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Research Article

SSR marker based DNA fingerprinting and diversity studies in mustard *(Brassica juncea)*

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Abstract
Seventeen mustard cultivars were subjected to SSR assay to identify the efficient primers for developing DNA fingerprinting. Two SSR primers of mustard Ra1H11 and Ra1F03 revealed a considerable DNA polymorphism of 41.17% and 35.29% respectively. The SSR primer Ra1H11 (0.772) of mustard had showed highest discriminating power followed by Ra1F09 (0.617) then Ra1F03 (0.558), Ra2B02 (0.382) and Ra1G07 (0.228) respectively. The highest PIC value had showed by Ra1F03 (0.525), then followed by), Ra1H11 (0.363), Ra2B02 (0.359), Ra1F09 (0.290) respectively. The primer Ra1H11 had showed highest MI value (0.423) then followed by Ra1F03 (0.262), Ra1F09 (0.193), Ra2B02 (0.119) and Ra1G07 (0.071) respectively. Among all the mustard SSR primers Ra1H11, Ra1F09, Ra1F03 followed a descending order of their efficiency to discriminate the mustard cultivars so that they can be used for developing DNA finger printing patterns for these cultivars. A combination of SSR primer Ra1F03 with Ra1F09 had distinguished the cultivar Bhagirathi from Panchali and Sanjukta, Ra1F09 with Ra1H11 had distinguished the cultivar kranti from Agrani and PT-303, Ra1F03 with Ra1F09 had distinguished the cultivar JD-6 from Pusa Bold, and Ra1F03 with Ra1H11 had distinguished the cultivar YST-151 from Jhumuka by producing a unique banding pattern.

Key Words
Mustard, DNA fingerprinting, genetic diversity, SSR markers.

Introduction

Brassica juncea, a well-known plant of family brassicaceae is grown as an oil crop. Mustard oil is one of the major edible oils in India. Mustard oil has also got medicinal importance (Yousuf *et al.* 2013) it is the third largest contributor of the world supply of vegetable oil. Diversity at marker loci is currently the most feasible strategy for characterizing diversity in mustard (Iqbal *et. al.*, 2015), molecular marker is a sequence of DNA, which are located with a known position on the chromosome (Kumar 1999), or a gene whose phenotypic expression is frequently easily discerned and used to detect an individual, or as a probe to mark a chromosomes, nucleus, or locus (King and Stansfield 1990; Schulmann 2007). Despite the fact that the mechanism of microsatellite evolution and function remains unclear, SSRs were being widely employed in many fields soon after their first description (Litt and Luty 1989; Tautz 1989; Weber and May 1989) because of the high variability which makes them very powerful genetic markers. Microsatellites have proven to be an extremely valuable tool for genome mapping in many organisms (Knapik *et al.* 1998), but their applications span over different areas ranging from kinship analysis, to population genetics and conservation / management of biological resources (Jarne and Lagoda 1996). Identification and successful deployment of efficient molecular markers can minimize ambiguity in varietal identification. DNA markers such as microsatellites or simple sequence repeats (SSRs) and inter-SSR (ISSR) portray genetic variation at the DNA level thereby overcoming the influence of environments, independent of tissue effects, and providing more precise characterization of genotypes and measurement of genetic relationships than other markers (Autrique *et al.*; 1996, Ajibade *et al.*; 2000, Souframanien and Gopalakrishna, 2004). But such studies do not focus on the important problem that is the way to optimally apply these new techniques for elite variety identification purposes. In particular, the greatest challenges are to reduce the cost of analysis, (i.e. the number of amplifications, and thus the number of primers) as well as the risk of confusing one of these elite genotypes with a randomly chosen genotype taken from a larger sample. Amongst the various types of molecular markers used, Simple Sequence Repeat (SSR) markers are the most preferred one because of their higher reproducibility, co-dominance nature, abundance, wide distribution throughout the genome, easy scorability, and multi-allelic variation (Powell *et al*., 1996). Simple sequence repeat markers have been used for genetic diversity analysis in a number of crops including Indian

bread wheat (Mir *et al*., 2011), rice (Rahman *et al*., 2012), and maize (Sivaranjini *et al*., 2014). The objectives of this study was to develop keys to identify varietal purity of mustard using PCR based techniques and to develop DNA fingerprinting pattern of cultivars of mustard crops.

Materials and Methods

As a part of the development of a molecular tool kit for the study of diversity within the collected crop cultivars, SSR technology has been applied to the selected cultivars. The following cultivars of Mustard crop were used for the cultivars identification study (Table 1.).

DNA was extracted by the modified CTAB method, (Rajendra *et al*., 2006) for isolation of high quality and quantity of DNA from single rice grain. The procedure involves soaking of dehusked rice seed (or) grain and whole seed of green gram, black gram and mustard in 600 µl extraction buffer (100mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 1.25 M NaCl, 2% CTAB and 3% PVP) for 30-45 minutes at 37°C water bath (TB-85 Thermo bath) in a sterile 1.5 ml micro centrifuge tube and grinding the sample using a sterile mortar pestle till the tissue disintegrates. Then, 600 µl of chloroform is added; the contents are mixed gently for 2-3 minutes and centrifuged at 12000 x g for 10 minutes at room temperature in high speed cooling centrifuge (HERMLE). The supernatant is carefully transferred to a fresh sterile 1.5 ml micro centrifuge tube; and 2 µl RNase A was added into it and kept at 37º C water bath (TB-85 Thermo bath, Shimadzu) for 30 minutes to degrade the RNA present in the sample. The DNA is precipitated using an equal volume of ice-cold isopropanol. The DNA is pelleted by centrifugation at 12000 rpm for 10 minutes at room temperature. After centrifugation, the supernatant is discarded and the DNA pellet is washed twice with 70% ethanol. The pellet is air dried for 1 hr and dissolved in 50 µl, 100 µl, 100 µl and 50 µl of sterile TE buffer (10 mM Tris HCl, pH 8.0 and 1mM EDTA, pH 8.0) for rice, green gram, black gram and mustard respectively and centrifuged at 12000 x g for 10 minutes at room temperature in high speed cooling centrifuge (HERMLE). The supernatant is carefully transferred to a fresh sterile 1.5 ml micro centrifuge tube. Then the transferred supernatant is kept for PCR analysis and the pellet precipitated is discarded. Extracted DNA samples were quantified through electrophoresis in 1 % agarose gel at constant voltage (65 V) for 90 minutes and further diluted accordingly to a uniform concentration of 50 ng / µl. The extracted DNA samples, along with the diluted samples were stored in –20^{0}C freezer (VESTFROST) with proper labeling.

About 6 pre-validated primers of those having high PIC value in another study were selected for SSR marker based characterization (Chattopadhyay *et al*., 2008) and are distributed throughout the genome of mustard. Primers were synthesized from Metabion (Germany). Details of the primers are given in the following table 2.

The reaction volume (25 µl) contained diluted DNA sample 2 µl (40 ng) with 1 µl (100 ng) forward primer, 1 µl (100 ng) reverse primer, along with 2.5 µl 10X buffer, 1 µl 2.5 mM dNTPs, 17.5 µl HPLC grade sterile water and 5 U Taq DNA polymerase enzyme. Amplification was performed in 200 µl PCR tube (TARSONS) in a thermal cycler (Gene Amp PCR System 9700) where the reaction condition was set as follows: 94ºC for 5 minutes of initial denaturation followed by 35 cycles each of denaturation at 94ºC temperature for 45 seconds, annealing of primer pairs at appropriate temperature (55ºC) for 45 seconds, and polymerization at 72º C for 1 minute. Further extension was followed at 72º C for 7 minutes, followed by holding the samples at 4º C for 5 minutes. On completion of PCR, the amplification products were stored in –20º C freezer.

Result and Discussion

Seventeen (17) mustard cultivars of West Bengal were subjected to SSR assay to identify the efficient primers for developing DNA fingerprinting. Two SSR primers of mustard (Ra1H11: 41.17% and Ra1F03: 35.29%) revealed a considerable DNA polymorphism among the selected cultivars of West Bengal for the study (Fig. 2). Six SSR primers of mustard cultivars used in the study produced an average of 21.56 per cent polymorphism. The number of polymorphic bandings ranged up to seven (Table 3) with an average polymorphic banding patterns per SSR primer was four in mustard.

The appearance of multiple bands was found in the banding pattern of SSR markers of mustard cultivars (Fig. 2). The appearance of multiple bands has been reported earlier as the result of amplification of more than one locus by each SSR (Holton T. *et al*.; 2002). The occurrence of multiple bands from the same locus could be explained by the presence of cryptic site upstream, downstream and between the primer binding sites (Winter P. *et al*.; 1999).

The SSR primer Ra1H11 (0.772) of mustard had showed highest discriminating power followed by Ra1F09 (0.617) then Ra1F03 (0.558), Ra2B02 (0.382) and Ra1G07 (0.228) respectively. The primers Ra2C07 used in the study to discriminate

the mustard cultivars were produced monomorphic banding pattern (Table 4). The highest PIC value had showed by Ra1F03 (0.525), then followed by), Ra1H11 (0.363), Ra2B02 (0.359), Ra1F09 (0.290) respectively. The primer Ra1H11 had showed highest MI value (0.423) then followed by Ra1F03 (0.262), Ra1F09 (0.193), Ra2B02 (0.119) and Ra1G07 (0.071) respectively.

The discriminating power (D) parameter, which evaluates the efficiency of a primer for the purpose of identification of varieties; i.e. the probability that two randomly chosen individuals have different patterns. The analysis of power discrimination revealed that the efficiency of a given primer does not depend only on the number of patterns it generates. For example, even if two primers produce the same number of patterns (e.g. primers Ra1F03 and Ra1G07), they can have very different discriminating powers (0.558 and 0.228 respectively). The scale of banding pattern variation was ranging up to seven. On the contrary, two primers with quite a different number of banding patterns can have similar discrimination powers which were also judged in similar fashion while using RAPD profile in *Vitis vinifera* L. (Tessier C. *et al*; 1999).

It was also found that high polymorphic information content (PIC) value does not always generate a high polymorphic banding pattern as earlier reported by Turi *et. al*., 2012. For example, the highest PIC value of Ra1F03 was 0.525 (Table 4) whereas their polymorphic banding pattern % is 11.76 (Table 3). But, the D value of each primer relate proportionally with their polymorphic banding pattern %. But, higher PIC value with higher marker index (MI) for each primer relate linearly with their polymorphic banding pattern % (Table 5), for e.g. Ra1H11. The similar results also were found by Chattopadhyay K. *et al*. (2008) while studying the "PCR-based characterization of mung bean (*Vigna radiata*) genotypes from Indian subcontinent at intra and inter specific level". From this it was revealed that neither PIC value nor MI value alone could be used for the discriminant analysis, whereas considering with a combination to both the PIC and MI values, it could be used for the discriminant analysis. On the contrary, the D value alone could be used for the discriminant analysis and it need not require a second parameter for the same. Therefore the D parameter could be used more efficiently for the cultivar discrimination and could also be used for diversity analysis and similar result was also found by Tessier C *et al*; (1999) and Gupta *et. al*., 2014.

Ra1H11 alone had clearly distinguished Sanjukta by amplifying a unique banding pattern of 200 and 230 bp fragment (Table 6), from Panchali and Bhagirathi by producing a common banding pattern of 200 and 300 bp fragment (Fig. 2). This primer alone also distinguished to both of the cultivar Sarama and Seeta by amplifying a unique banding pattern of 220, 300 bp fragments and 230 bp fragment respectively (Parida *et. al.,* 2010). The grains/seeds of these cultivars were almost alike (Fig. 1). The cultivar Seeta also distinguished by primer Ra1F03 with amplifying 150 bp fragment from others. Primer Ra1F09 alone had clearly distinguished Pusa Bold by amplifying a unique banding pattern of 110, 130 and 170 bp fragments (Table 4.2.6) from JD-6. The grains/seeds of these two cultivars were almost alike (Fig. 1). Primer Ra1G07 alone also distinguished to both of the cultivar kalian by amplifying a unique banding pattern of 220, 470 bp fragments, from others and Rajendra sarsoon by amplifying a unique banding pattern of 470 bp fragment, from PYS-2005 respectively. The grains/seeds of these cultivars were almost alike (Fig. 1). So, employing these single SSR primers alone can be used for ascertaining the seed purity or admixture and can be used for ascertaining genetic purity at any stage of the growth period.

A combination of SSR primer Ra1F03 with Ra1F09 had distinguished the cultivar Bhagirathi from Panchali and Sanjukta, Ra1F09 with Ra1H11 had distinguished the cultivar kranti from Agrani and PT-303, Ra1F03 with Ra1F09 had distinguished the cultivar JD-6 from Pusa Bold, and Ra1F03 with Ra1H11 had distinguished the cultivar YST-151 from Jhumuka by producing a unique banding pattern (Table 6 and Fig. 2) whose grains/seeds of these cultivars were almost alike (Fig.1). So, employing a combination of these SSR primers, it can be used for ascertaining the seed purity or admixture and genetic purity at any stage of the growth period.

Here, the SSR primers used for the study was failed to discriminate the cultivar Agrani from Panchali, Jhumuka from PYS-2005 and B-9 from NDYS-2018. This appears to us that the cultivars are seems to be genetically very closely related. That's why, no polymorphism was found among these cultivars respectively.

It also be noted that the SSR primers used in maximum number of cultivar cases i.e. six was Ra1H11 then followed by four in Ra1F09, three in Ra1F03 and two in Ra1G07 (Table 7) respectively to discriminate among all. This could be concluded that primer Ra1H11 was the most efficient followed by Ra1F09, Ra1F03 respectively for the discrimination of the cultivars used in the present

study and also was supported to the earlier result obtained for the discriminating power (D) value alone and also with the % of polymorphic banding pattern (Table 5) whereas the PIC and MI value alone did not supported to the results and with the % of polymorphic banding pattern. Therefore the D parameter could be used more efficiently for the cultivar discrimination and could also be used for diversity analysis. Furthermore the binary scoring of resulted banding pattern of SSR marker of mustard is presented in Table 8. It is also appearing to me that, it may be required a more number of SSR primer for obtaining DNA polymorphism among the undistinguished cultivars of mustard as well as for discriminating to all in an efficient way. Lower number of alleles per locus, and lower PIC values may be attributed either to the use of less informative SSR markers, or the presence of lesser genetic diversity among the tested genotypes. Several other researchers have also used SSR markers for diversity analysis in Brassica species (Abbas *et al.*, 2009; Celucia *et al*., 2009). Among all the mustard SSR primers Ra1H11, Ra1F09, Ra1F03 followed a descending order of their efficiency to discriminate the mustard cultivars so that they can be used for developing DNA finger printing patterns for these cultivars. The informative molecular markers could be used alone or in combination with morphological descriptors for assessing the variability or differentiating cultivars of these crops in a more conclusive manner. The greater resolving power of the SSR assay can provide more informative data than other techniques.

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Table 1. Name of the cultivars and source of collection of mustard genotypes

Name of the Crops	Number of cultivars	Name of the cultivars	Source of collection
Mustard	1	Agrani (B-54)	Pulses and Oil seed Research Station, Berhampore, West Bengal.
	2	Sanjukta Asech.	
	3	Panchali (TWC-3)	
	4	Sarama	
	5	B-9	
	6	Kalyan (WBBN-1)	
	7	Seeta (B-85)	
	8	PT-303	
	9	Jhumuka (NC-1)	
	10	Bhagirathi (RW-351)	
	11	JD-6	
	12	Kranti	
	13	Pusa Bold	
	14	Rajendra sarsoon	
	15	YST-151	
	16	NDYS-2018	
	17	PYS-2005	

Table 2. Details of SSR primers used

S.No	Primer code	Orientation	Sequence of the primer (5/ - 3/)	Annealing temp. ^{0}C
1	Ra1F03	F	AACTCG CTT TTA CCGTCGTC	50
		R	CAAGACGTGGAGCTGAAGTG	
2	Ra1F09	F	AAAACGGATAAACGTCACCG	45
		R	GAACAGTCTTACACCCGATTTAG	
3	Ra1G07	F	ATCGACATCGAACGAAAAGC	50
		R	TCACCCTCTACCTCCACCAC	
4	Ra1H11	F	CGCTAATGTGTGGTGGATTG	48
		R	ACCGGAGCGGTTTACATAAC	
5	Ra2B02	F	GATGGTTTTTCGTTTTCACG	45
		R	TCAGCTGTCACGTCTTGTCG	
6	Ra2C07	F	ATTTCCGAATCGGGAGTTTC	50
		R	ACTTGCAAACGCACACACAC	

Table 3. Analysis of SSR banding pattern in mustard cultivars

S.No.	Name of the primer code	No. of polymorphic banding patterns	Total no. of banding patterns	Per cent(%) of polymorphic banding patterns
1	Ra1F03	6	17	35.29
2	Ra1F09	4	17	23.52
3	Ra1G07	3	17	17.64
4	Ra1H11	7	17	41.17
5	Ra2B02	2	17	11.76
6	Ra2C07	0	17	0
	Total	**22**	**102**	**21.56**

Table 4. D, PIC and MI calculation from resulted banding pattern of SSR marker in mustard cultivars

Primer code Name	Banding pattern in bp size	No. of i^{th} banding pattern	Total no. of cultivars	Proportion of i^{th} banding pattern (F)	Confusion probability of i^{th} banding pattern	Confusion probability of j^{th} primer	Discriminating power of j^{th} primer	2F(1-F)	No. of loci detected	PIC of j^{th} primer	MI of j^{th} primer
Ra1 F03	150	10	17	10/17	0.3308	0.441	**0.558**	0.4844	2	**0.525**	**0.262**
	160	1	17	1/17	0.0			0.1107			
	150,160	6	17	2/17	0.1103			0.4567			
Ra1 F09	130	4	17	4/17	0.0441	0.382	**0.617**	0.3598	4	**0.290**	**0.193**
	130,170	10	17	10/17	0.3308			0.4844			
	130,190	2	17	2/17	0.0073			0.2076			
	110,130 170	1	17	1/17	0.0			0.1107			
Ra1 G07	470	1	17	1/17	0.0	0.772	**0.228**	0.1107	3	**0.143**	**0.071**
	190,470	15	17	15/17	0.7720			0.2076			
	220,470	1	17	1/17	0.0			0.1107			
Ra1 H11	200	1	17	1/17	0.0	0.227	**0.772**	0.1107	4	**0.363**	**0.423**
	230	1	17	1/17	0.0			0.1107			
	300	5	17	5/17	0.0735			0.4152			
	200,300	7	17	7/17	0.1544			0.4844			
	220,300	1	17	1/17	0.0			0.1107			
	230,300	1	17	1/17	0.0			0.1107			
	200,230 300	1	17	1/17	0.0			0.1107			
Ra2 B02	350	13	17	13/17	0.5735	0.617	**0.382**	0.3598	2	**0.359**	**0.119**
	310,350	4	17	4/17	0.0441			0.3598			
Ra2 C07	130	17	17	17/17	1.0	1.0	**0.0**	0.4152	1	**0.0**	**0.0**

Table 5. Comparison of PIC, MI, D and polymorphic banding pattern (%) value of SSR primers of Mustard

S.No.	Name of the primer code	PIC value	Marker Index (MI)	Discriminating power (D)	Polymorphic (%) banding patterns
1	Ra1F03	0.3840	0.4291	0.8163	35.29
2	Ra1F09	0.2900	0.2160	0.6178	23.52
3	Ra1G07	0.1430	0.0799	0.2280	17.64
4	Ra1H11	0.3632	0.4735	0.7721	41.17
5	Ra2B02	0.3598	0.1340	0.3824	11.76
6	Ra2C07	0	0	0	0

Table 6. Distinctiveness of mustard cultivars through SSR marker analysis

Name of the distinctive variety from all others	Name of the distinctive primer code	Particulars of the use of primers (single or in combination with)	Unique distinctive feature (amplicon in bp size)
Sanjukta	Ra1H11	single	230,300
Sarama	Ra1H11	single	220,300
Kalyan	Ra1G07	single	220,470
Seeta	Ra1F03 and Ra1H11	single	150 and 230
Bhagirathi	Ra1F03+Ra1F09	combination	130,150 +130,170
JD-6	Ra1F03+Ra1F09	combination	130,150 +130
Kranti	Ra1F09+Ra1H11	combination	130,190 + 200,300
Pusa Bold	Ra1F09	single	110,130,170
Rajendra sarsoon	Ra1G07	single	470
YST-151	Ra1F03+ Ra1H11	combination	120+200,300

Table 7. Efficiency of SSR markers in mustard cultivars

Primer's code name	Polymorphic band (in bp size)										
	110	120	130	150	170	190	200	220	230	300	470
Ra1F03		YST-151	Bhagirathi, JD-6	Seeta, Bhagirathi, JD-6							
Ra1F09	Pusa bold		Bhagirathi, JD-6, Kranti, Pusa bold		Bhagirathi, Pusabold	Kranti					
Ra1G07								Kalyan			Kalyan, Rajendra sarsoon
Ra1H11							Kranti, YST-151	Sarama	Sanjukta, Seeta	Sanjukta, Sarama, Kranti, YST-151	

Table 8. Binary scoring pattern of SSR marker in mustard

	Name of the cultivars																
Banding Pattern (In bp size)	Agrani	Sanjukta	Panchali	Sarama	B-9	Kalyan	Seeta	PT-303	Jhumka	Bhagirat-hi	JD-6	Kranti	Pusa Bold	Rajendra	YST-151	NDYS-2018	PYS-2005
(Ra1 F03) 150	1	0	1	0	1	1	0	1	1	0	0	0	0	1	1	1	1
160	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
150,160	0	1	0	1	0	0	0	0	0	1	1	1	1	0	0	0	0
(Ra1 F09) 130	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0
130,170	1	0	1	0	1	0	0	1	1	1	0	0	0	1	1	1	1
130,190	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
110,130,170	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
(Ra1 G07) 470	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
190,470	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1
220,470	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
(Ra1 H11) 200	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
230	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	1	1
200,300	1	0	1	0	0	0	0	0	0	1	1	1	1	0	1	0	0
220,300	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
230,300	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200,230,300	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
(Ra2 B02) 350	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	1
310,350	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0
(Ra2 C07) 120	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

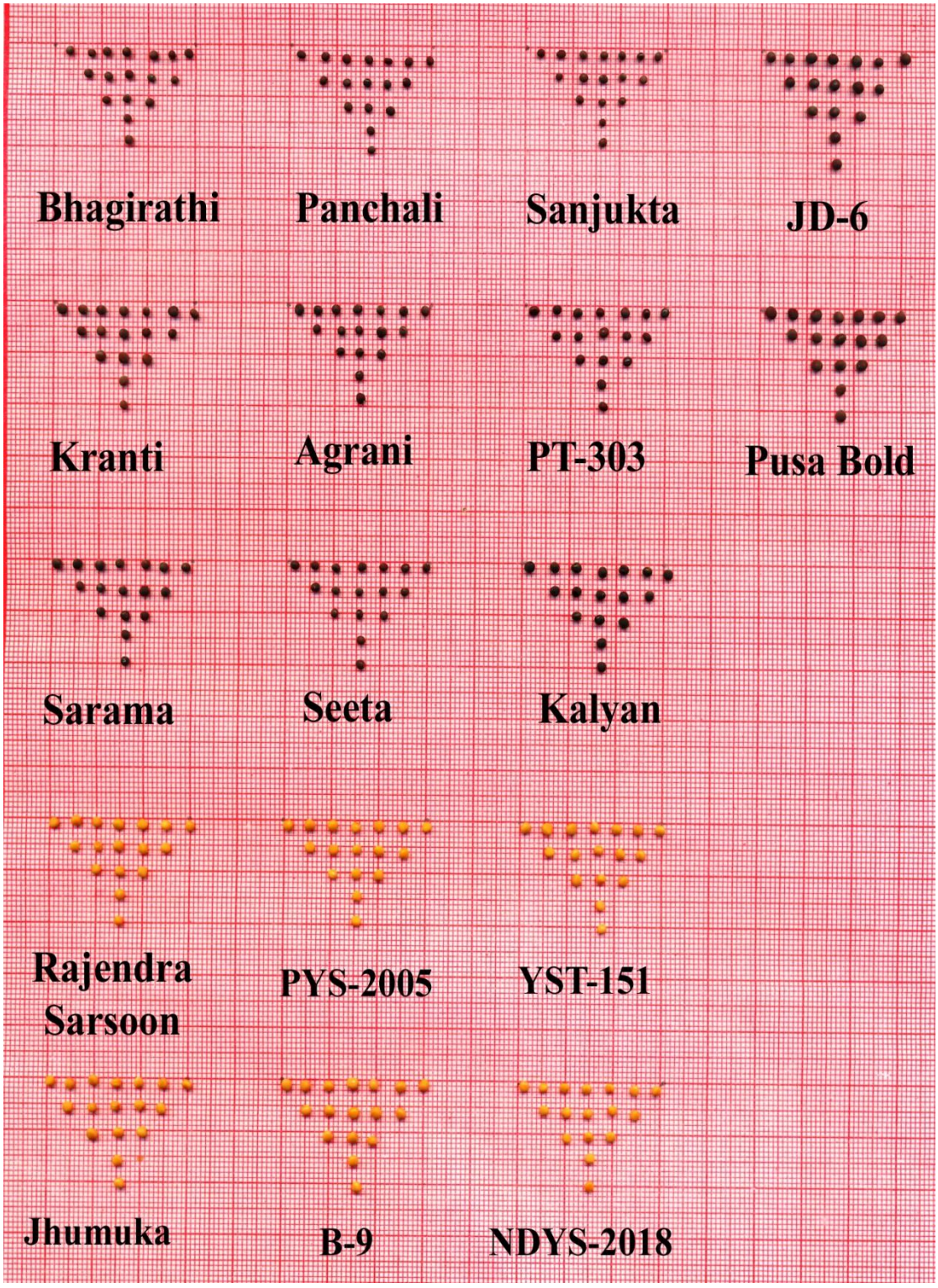

Fig. 1. Seed morphology of mustard cultivars

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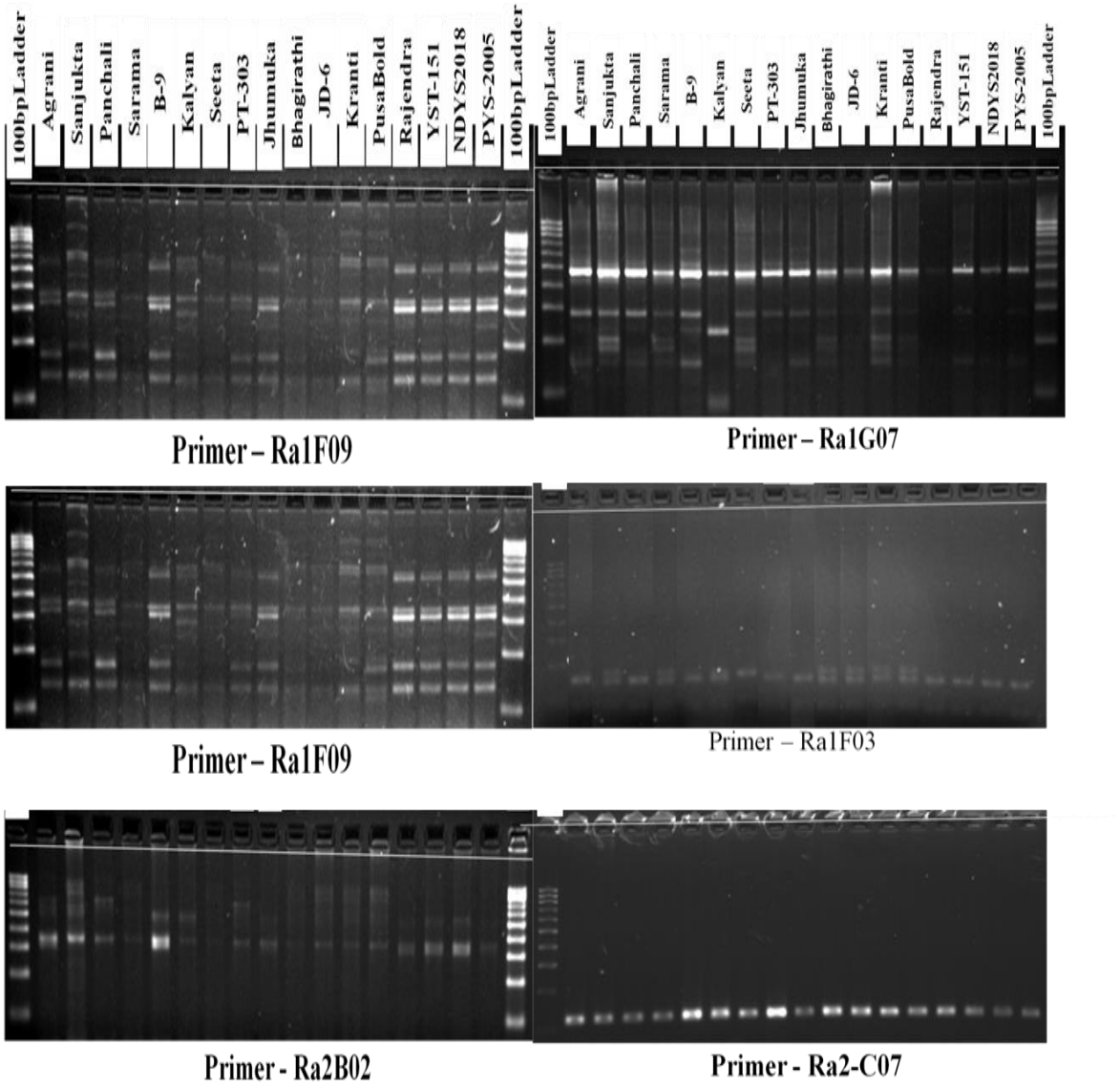

Fig. 2. Banding pattern of SSR markers in mustard cultivars