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GENETIC DIVERSITY OF *Brassica rapa* GERMPLASM OF AZAD JAMMU AND KASHMIR, PAKISTAN REVEALED BY MOLECULAR MARKERS

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ABSTRACT

Brassica species commonly known as rapeseed are the second largest oil yielding crop of Pakistan and belongs to the family *Brassicaceae*. This scientific investigation was carried out to explore the genetic diversity of *Brassica rapa* from Azad Jammu and Kashmir (AJK) Pakistan. Seeds of *Brassica rapa* L. genotypes of Azad Jammu and Kashmir were collected from Plant Genetic Research Institute (PGRI), National Agricultural Research Centre (NARC), Islamabad Pakistan. CTAB method was used for the extraction of DNA from leaf samples. SSR and ISSR markers were used to explore the genetic diversity and cluster analysis was used for data analysis. Out of 6 SSR markers, Ra2E11 and Na10-E02 showed maximum genetic diversity while Ra2-E07, Ra2-D04, and Na10-D03 showed moderate diversity while Ra2-E01 showed minimum genetic diversity. Among the selected genotypes 026503 (Bhimber), 026514 (Kotli), 026510 (Kotli) and 026539 (Bagh) showed maximum diversity with 6 SSR markers. From 10 ISSR markers ISSR 888 and ISSR 5 showed highest number of bands (7). ISSR 3, ISSR 5, ISSR 888 showed maximum genetic diversity among mustard genotypes, while ISSR 887 and ISSR 840 showed no results and ISSR 2, ISSR 4, ISSR 80, ISSR 811, ISSR 889 showed moderate diversity. Among the selected genotypes 026557 showed maximum diversity with ISSR markers. Based upon the present studies the genotypes 026503, 026514, 026510, 026539 and 026557 had been recognised as probable most significant genotypes which could be used in future plant breeding program.

Key words: *Brassica rapa*, Azad Kashmir, genetic diversty, SSR, ISSR, markers

INTRODUCTION

Brassica L., genus which belongs to family *Brassicaceae* consists of six important species which are economically important and cultivated worldwide [Saha et al. 2008]. *Brassica* species commonly known as rapeseed-mustard is important oil seed crop having third position in the world after soybean and palm. Higher quantity of seed oil and its quality raised the rapeseed to second largest oilseed crop. In the production of biodiesel *Brassica* species are also used [Burbulis et al. 2004, Hasan et al. 2006]. *Brassica* is grown on 307,000 ha in Pakistan and total oil seed production is 233,000 t and 17% is used as domestic purposes [PARC 2006].

The wide range of molecular and morphological diversity is present in *Brassica rapa* germplasm and due to this huge diversity this crop is used as vegetable and

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for oil production [Prakash and Hinata 1984]. The genetic variability present in any germplasm is the base for the utilization and exploitation of various genes for genetic advancement of open pollinated varieties and hybrids. For the development of the hybrids it is necessary to have the knowledge of genetic diversity [Shinwari et al. 2013, 2013a, Sultan et al. 2013, Ahmad et al. 2014]. In family *Brassicaceae*, gene flow is low in natural conditions so hybridization at interspecific level can be easily forced [Sandhu and Gupta 2000]. Molecular characterization as well morphological and biochemical assessment is vital for the identification of genetic association as high degree of polymorphism can be detected by molecular markers and these markers are independent of developmental and ecological variations [Ahmad et al. 2014, Rabbani et al. 2010, Zeb et al. 2011]. Molecular markers associated with genes of important characters are very useful in in selection of two or more genes in a single genotype [Ahmad et al. 2013]. Molecular markers are important tool for the assessment of genetic differences and relationship among the species of plants. The markers simple sequence repeats (SSRs), also known as microsatellite markers have been successively used to distinguish related *Brassica* genotypes and for the investigation of genetic variability present in genotypes [Louarn et al. 2007]. Due to the presence of co-dominance and ability to identify maximum number of alleles at each locus, it results in high variation and reproducibility [Marriete et al. 2000]. The microsatellite known as simple sequence repeats (SSRs) have been extensively used for different studies of plants which include plant breeding, genetics including mapping of genes and recognition of diversity at generic level [Gupta and Varshney 2000]. In earlier studies only SSR and RAPD markers were used to study the genetic diversity *Brassica* germplasm [Wang et al. 2008, Song et al. 2015]. SSRs are being preferred in breeding programs because of their multiallelic nature, abundance and wide genomic analysis [Ijaz and Khan 2009]. In previous studies Leroy et al. [2000] discriminated *B. oleraceae* accessions by using four different microsatellite markers. Of the total 136 reproducible fragments, 25 fragments were common for all accessions, 27 bands were distinctive and 84 were phylogenetically useful. The genetic diversity in *Brassica*, *Camelina, Raphanus*, *Arabidopsis* and *Sinapis* was investigated by Flannery et al. [2006] using 10 SSR primer sets. Different eight loci showed polymorphism and divided the individuals of *Brassica*ceae into taxon-specific groups. In European winter *Brassica rapa* cultivars, diversity was evaluated by Ofori et al. [2008] by SSR markers and found maximum (83%) genetic variation in the selected cultivars. In other studies Moghaddam et al. [2009], measured the genetic variability among the 32 rapeseed cultivars by using the SSRs markers and found polymorphism information content (PIC) of SSRs loci ranged from 0.60–0.91. From all previous studies it is confirmed that microsatellite markers are effective for measuring the genetic diversity and to determine the relation in *Brassica* species. Hence the present study was aimed to estimate the genetic diversity and phylogenetic relationships of the *Brassica* rapa genotypes used locally in the Azad Jammu and Kashmir Pakistan by using SSr and ISSr markers.

MATERIAL AND METHOD

The experiment was conducted at the National Institute of Genomics and Advanced Biotechnology (NIGAB) National Agricultural Research Centre (NARC) Islamabad Pakistan. Seeds of mustard genotypes were collected from different regions of Azad Jammu and Kashmir (Table 1) and provided by seed center NARC Islamabad and were sown in the pots. DNA was extracted from three weeks old seedlings, by using Cetyl Trimethyl Ammonium Bromide (CTAB) method as designated by Murray and Thompson [1980] with some modifications [Thakur et al. 2013]. After collection of DNA pellet it was dissolved in 100 lL TE buffer, quality was checked by using 1% agarose gel. The concentration of the DNA samples were intended by using Epoch Microplate Spectrophotometer (Epoch, USA) and then the final dilution was made 100 ng L^{-1} before use and was stored at –180°C.

To evaluate the genetic diversity among 30 *Brassica rapa* germplasm genomic SSR and ISSR markers were used. Optimization was made for each primer by using different temperature and best temperature for each primer was noted. Molecular analysis was performed by following the protocol of Hasan et al. [2006] with minor modifications. For the SSR markers analysis a solution of 20uL volume was prepared by using 1 uL template DNA, taq polymerase 0.2 uL

Table 1. List of *Brassica rapa* germplasm from Azad Jammu and Kashmir Pakistan

Sr. No.	Acc. No.	Genus	Species	Local name	Origin	Province	Location
1	026502	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bhimber
2	026503	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bhimber
3	026508	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Kotli
4	026509	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Kotli
5	026510	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Kotli
6	026512	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Kotli
7	026513	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Kotli
8	026514	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Kotli
9	026515	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Kotli
10	026517	*Brassica*	*rapa*	Ghobi Sarsoon	Pakistan	AJK	Kotli
11	026518	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Kotli
12	026527	*Brassica*	*rapa*	Ghobi Sarsoon	Pakistan	AJK	Rawalakot
13	026529	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Rawalakot
14	026532	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Rawalakot
15	026533	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Rawalakot
16	026538	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bagh
17	026539	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bagh
18	026540	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bagh
19	026542	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bagh
20	026545	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bagh
21	026548	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Muzaffarabad
22	026551	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Muzaffarabad
23	026557	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Muzaffarabad
24	026562	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Muzaffarabad
25	026563	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Muzaffarabad
26	026564	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Muzaffarabad
27	026496	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bhimber
28	026498	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bhimber
29	026499	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Bhimber
30	001321	*Brassica*	*rapa*	Sarsoon	Pakistan	AJK	Muzaffarabad

PCR buffer 2 uL, dntps mix 0.4 uL, $MgCl_2$ 2.4 uL, ddH_2O 12 uL, 1 uL forward and 1 uL of reverse primers were used. For ISSR primers 20 uL sample reaction was prepared by little changes as for ISSR only forward sequence of nucleotides is present. Its amount in reaction mixture was 1 uL and 13 uL ddH_2O was used to make it 20 uL. The reaction was carried out in the thermal cycler and amplification was performed at 94°C for five minutes, along with 35 cycles. Each cycle consists of three steps involving denaturation at 94°C for 40 seconds, second step is of annealing that was performed by applying different temperature depending upon the primer used for 5 seconds, while extension was done at 72°C for 40 seconds. At end final extension was done for 10 minutes at 72°C.

GEL electrophoresis to visualize amplified products. Next to amplification, 10 µl of the amplified samples were combined with 3 µl of a loading buffer and then amplified products were stained with ethidium bromide on 1.5-percent agarose gel. For comparison of size of amplified products 100 bp DNA ladder was used. At the end, bands were visualized by using a UVI Doc. Gel Documentation System and images were saved.

Scoring of alleles and data analysis. By using agarose gel, different bands of PCR products were amplified and size of bands was compared with 100 bp

ladder. The data was then put into the binary matrix as presence (1) and absence of the bands (0). Based on polymorphism most informative markers were selected. The genetic similarities (F) between genotypes was calculated according by Using pair-group method and dendrogram was constructed to get genetic relationships among accessions [Nei et al. 1979]. For ISSR markers polymorphic information content (PIC) value was calculated using formula PIC = 1-Σpi^2, where Pi is the frequency of the ith allele, exposed by the ith primer summed along all patterns showed by the primers [Botstein et al. 1980].

RESULTS

Examination of the results of ISSR-PCR based amplification. Significant variations were observed through ISSR markers among 30 genotypes of *Brassica rapa.* Out of 10 ISSR markers used in the study, 8 ISSR markers showed high polymorphism among *Brassica rapa* germplas and two ISSR markers did not amplify any fragment in mustard genotypes. After studying the presence of diverse bands it was observed that DNA amplification of 30 mustard genotypes with 10 ISSR markers produced 40 distinguish bands, from which 33 bands were polymorphic and the size of the bands ranged between 100 to 700 base pairs. On average 5 bands were amplified by each primer, from which 4.1 were polymorphic. ISSR 5 and ISSR 888 were observed to be best markers to evaluate the diversity among *Brassica* germplasm as they have shown maximum number of bands with maximum polymorphism (85.7%). ISSR 890 showed few bands while ISSR 840 and ISSR 887 showed no results (Tab. 2).

Examination of the results of SSR-PCR based amplification. To evaluate the *Brassica rapa* germplasm 6 SSR markers were used. From 6 SSR markers only two SSR markers showed polymorphic and reproducible bands whose polymorphic percentage was 50% for both while other showed monomorphic bands. It was observed that DNA amplification of 30 mustard genotypes with 6 SSR markers produce 8 distinguish bands, from which 2 were polymorphic and the size of the bands ranged between 50 to 200 base pairs. With 6 SSR markers 8 distinguishable bands were produced. SSR marker Na10E02 showed maximum bands whereas SSR Ra2E01 showed few bands with mustard germplasm (Tab. 3).

Table 2. ISSR marker, sequence, annealing temperature, number of amplified bands, number of polymorphic bands and proportion of polymorphic loci (%)

Primer	Sequence (5`–3`)	Annealing temperature (°C)	Number of amplified bands	Number of polymorphic bands	Proportion of polymorphic loci (%)
ISSR 2	5'-(AC)8T-3'	55°C	3	2	66.6
ISSR 3	5'-(AC)8C-3'	58°C	6	5	83.3
ISSR 4	5'-(AG)8C-3'	58°C	6	5	83.3
ISSR 5	5'-(AG)8T-3'	56°C	7	6	85.7
ISSR 811	GAGAGAGAGAGAGAGAC	56°C	6	5	83.3
ISSR 888	BDBCACACACACACACA	56°C	7	6	85.7
ISSR 890	VHVGTGTGTGTGTGTGT	56°C	2	1	50
ISSR 889	DBDACACACACACACA C	52°C	3	2	66.6
ISSR 887	DVDTCTCTCTCTCTCTC	55°C	–	–	–
ISSR 840	GAGAGAGAGAGAGAGAYT	55°C	–	–	–
Total	–	–	40	33	–
Average			5	4.1	–

Table 3. SSR markers, sequence, annealing temperature, number of amplified bands, number of polymorphic bands and proportion of polymorphic loci (%)

Markers	Sequence (5`–3`)	Annealing temperature (°C)	No. of amplified bands	No. of polymorphic bands	Proportion of polymorphic loci (%)
Ra2E01	F. TCTATATTAACGCGCGACGG R. GCACACACACACTCAAACCC	62°C	1	–	–
Ra2-E11	F. GGAGCCAGGAGAGAAGAAGG R. CCCAAAACTTCCAAGAAAAGC	60°C	2	1	50%
Ra2-D04	F. TGGATTCTCTTTACACACGCC R. CAAACCAAAATGTGTGAAGCC	56°C	1	–	–
Ra2-E07	F. ATTGCTGAGATTGGCTCAGG R. CCTACACTTGCGATCTTCACC	62°C	1	–	–
Na10-D03	F. ATGATTTGCCTTGAAATGCC R. GATGAAACAATAACCTGAGACACAC	60°C	1	–	–
Na10-E02	F. TCGCGCATGTAATCAAAATC R. TGTGACGCATCCGATCATAC	58°C	2	1	50%
Total	–	–	8	2	–

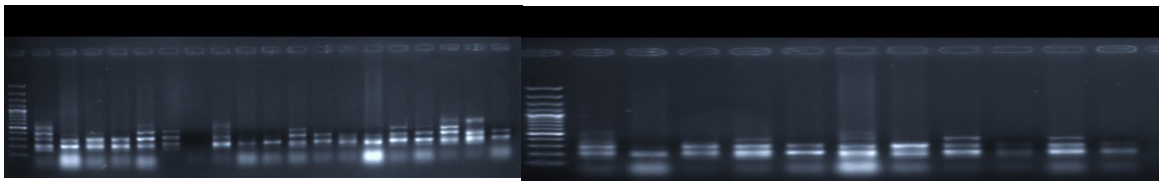

Fig. 1. ISSR markers banding pattern of 30 *Brassica rapa* genotypes

Table 4. SSR based main cluster, subgroups, total number of accessions in each cluster

Main Cluster	Subgroup	Accessions
Cluster-A	A1	8
	A2	7
Cluster-B	A3	1
	A4	14

The bivariate data of 30 *Brassica rapa* genotypes based upon SSR markers was analysed using unweighted pair group method with arithmetic mean (UPGMA) (Fig. 1). All accessions were grouped into two main clusters (Tab. 4), Cluster-A was with 15 genotypes which was divided into subgroup A1 with 8 genotypes 026548, 026563, 026498, 026499, 026564, 026562, 026540, 026518 and A-2 with 7 genotypes 026542, 026515, 001321, 026545, 026527, 026557, 026551.

Cluster-B was also with 15 genotypes with two subgroups A3 and A4. A3 was smallest group with only 1 genotype (026509) and A4 was largest group with 14 genotypes 026533, 026532, 026502, 026503, 026508,026510, 026539, 026517, 026529, 026538, 026514, 026513. All the genotypes grouped together were native to different geographical regions, as group A1, A2, A4 were with geographically diverse genotypes, showing that native area of genotypes does not have any effect on cluster formation (Fig. 2).

Table 5. ISSR based main cluster, groups, subgroups, total number of accessions in each cluster

Main Cluster	Group	Subgroup	Accsessions
Cluster-A			1
Cluster-B	B-1		1
	B-2	B-21	3
		B2-2	25

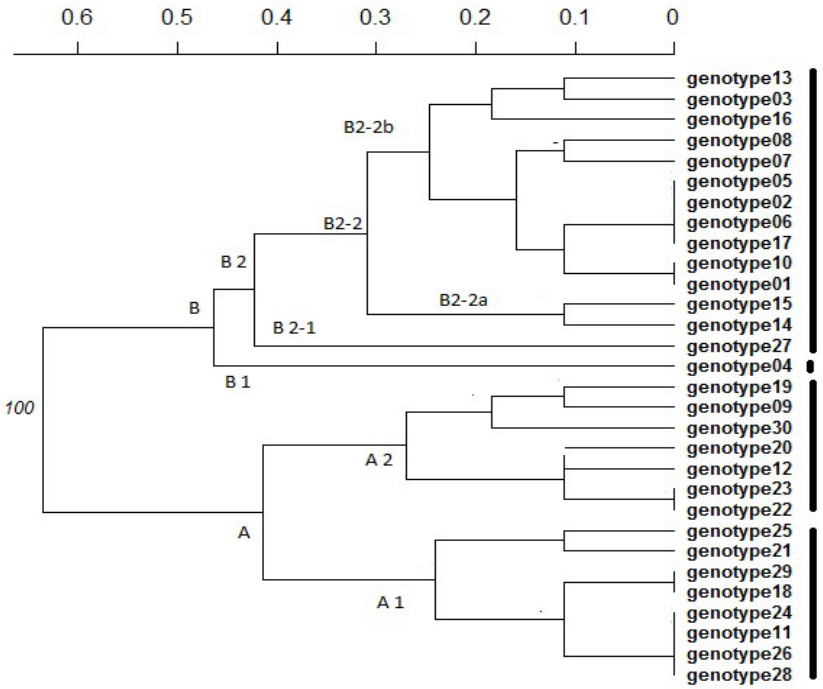

Fig. 2. Dendrogram of 30 mustard genotypes based on SSR markers

By using ISSR markers bivariate data was generated and was analysed by cluster formation where all genotypes were grouped into two main clusters (Tab. 5). Cluster-A was smallest cluster with only 1 genotype 026539, while cluster-B was with 29 genotypes. Cluster-B was further divided into two groups B-1 with 1 genotype (026502) and B-2 with 28 genotypes. Group B-2 was further divided into subgroups with geographically distinct genotypes. Analysis by ISSR markers showed maximum diversity among *Brassica rapa* germplasm as compare to SSR markers (Fig. 3).

DISCUSSION

Genetic diversity of the plants is necessary for the conservation programs which can be helpful for the germplasm users for the identification of the required accessions and propose the breeders to use the chosen genotypes for the crops improvement [Cruz et al. 2007]. PCR based ISSR makers are powerful tools which are polymorphic, reproducible and abundantly found in the plant genome [Bornet et al. 2002]. In this study, 6 SSR markers were used which produced re-

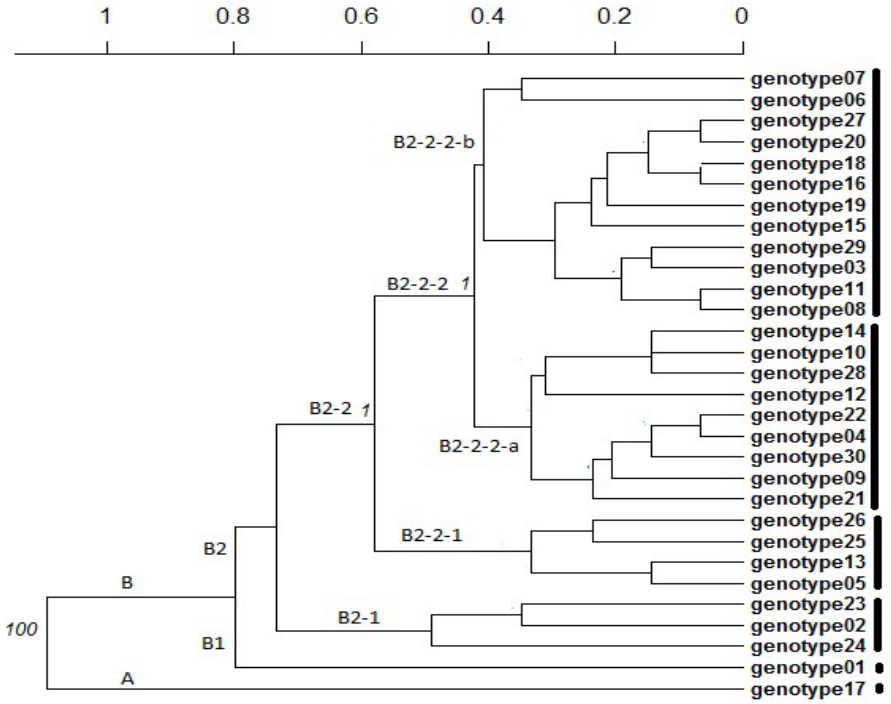

Fig. 3. Dendrogram of 30 mustard genotypes based on ISSR markers

producible products in 30 mustard genotypes. This is the first study which was carried out on the local germplasm of the Azad Jammu and Kashmir. SSR markers have high ratio of genetic diversity in *B. rapa* than in *B. juncea* and *B. napus*. It has been that *B. rapa* is the indigenous species of the Himalayan region of the Pakistan [Turi et al. 2012]. The six primers used in this study produced monomorphic and polymorphic results. Out of six only 1 primer showed polymorphic results. Similar results were shown by Thakur et al. [2017] who revealed both monomorphic and polymorphic nature of these primers. Our results showed that one primer Na10-E02 gave polymorphic bands. These findings are controversial to the findings of Turi et al. [2012] who revealed polymorphic results for the Na10-E02. The difference in results may due to lab conditions or due to handling. SSR based polymorphisim was also observed in Pakistani based *Brassica* genotypes by Sadia et al. [2009]. Cluster analysis revealed that genotypes from the same region were not grouped together while genotypes from different regions were grouped in the same cluster. These findings showed that geography of an area does not associate with the genetic diversity. Similar findings were also confirmed by the Jahan et al. [2013]. Westman and Stephen [1999] assessed the genetic diversity of *Brassica* germplasm collected from four different areas and their results showed that specific difference was observed among the genotypes belonging to similar region while in this there was no grouping observed for the geographical dispersal of the genotypes. This showed that a genetic base of Pakistani oilseed crops is narrow as suggested by Rabbani et al. [1998].

The ISSR markers provide multilocus patterns which are polymorphic, reproducible and abundantly found in the genomes of the plants [Bornet et al. 2002]. In this study 10 ISSR markers were used which gave polymorphic results. The findings of the research showed that primer ISSR840 produced highest number of bands than other primers used. Similar results were also obtained in previous research as shown by Shen et al. 2016. Our findings were also in confirmation of the results of Shen et al. [2016]. Talebi et al. [2010] also used ISSR markers to assess the genetic diversity

of international germplasm pool of 47 accessions of the *B. rapa* and revealed that an average number of 7.6 bands/primer. The current study has lower value than this study. Bornet and Branchard [2004] showed that ISSR markers have amazing power for identification of cultivars of *Brassica* taxa for registration of the plant. Such extraordinary power of identification was also confirmed by using ISSR markers for 30 *Brassica rapa* germplasm in this study. Consequently ISSR markers showed high level of capability for detecting DNA polymorphisim and may be used a valuable device for breeders. Cluster analysis of the ISSR markers showed that genotypes were grouped into two main clusters which were further subdivided into subgroups. In sub clusters the genotypes from same geographical regions were grouped into same clusters. Such results were also provided by Talebi et al. [2010] who reported that based on origin most of the Korean and Chinese *Brassica rapa* accessions were grouped together in same cluster. Polymorphic information content (PIC) is found to be significant features of molecular markers that can be used to evaluate the diversity ability of the markers. In the present study PIC value was calculated and the PIC value for SSR marker Na10E02 was 0.92. For ISSR markers PIC value was also calculated based upon polymorphic bands. PIC value for primer ISSR 2 was 0.95, for ISSR 3 PIC value was 0.7, and for ISSR 888 PIC value was 0.9. By using similar method PIC value was calculated for ISSR 5 that was 0.7, ISSR 890 and ISSR 889 were with 0.9 PIC value. While for ISSR 811 calculated PIC value was 0.8. ISSR 4 showed monomorphism, so its PIC value was not calculated and ISSR 840 and ISSR 887 did not amplify any allele in mustard germplasm.

CONCLUSIONS

In this study reasonable amount of diversity has been observed and frequently can be used in the breeding programs. Based upon molecular techniques ISSR markers showed greater diversity as compare to SSR markers. ISSR 888 and ISSR 5 produce maximum number of bands, 7 bands in a single genotype and evaluated highest level of diversity among genotypes, while ISSR 887 and ISSR 840 showed no results with *Brassica* germplasm. With ISSR markers the genotype 026557 showed maximum diversity. SSR markers showed mostly monomorphic results except Na10-E02. The maximum diversity was concluded with Ra2-Ell and Na10-E02. SSR markers Ra2-E07, Ra2-D04 and Na10-D03 showed moderate diversity among *Brassica rapa* germplasm.

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