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Identification of Different Species and Dultivars of *Brassica* by SDS-PAGE, Isozyme and Molecular Marker

Rahman Md. Mukhlesur[1,2*], Yutaka Hirata[1]

[1]Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Fuchu-shi, Tokyo 183-8509, Japan; [2]Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh-2002, Bangladesh

Abstract

Eighty-five different cultivars of *Brassica rapa, B. juncea, B. napus, B. carinata, B. oleracea* and hexaploid *Brassica* collected from Bangladesh, Japan, China and Denmark were analyzed by SDS-PAGE for seed and leaf protein variations, using esterase, acid phosphatase and peroxidase isozyme analysis. Ten polymorphic bands were identified from seed protein however no identifiable polymorphic band was found in the leaf protein. Polymorphic markers clearly distinguished the different *Brassica* species as well as yellow sarson (YS) and brown seeded (BS) cultivars of *B. rapa*. The F_1 cross between YS and brown seeded cultivars showed the existance of all polymorphic bands of the respective parents. The Bangladeshi and Japanese cultivars of *B. rapa* differed in the amount of seed protein. In the case of isozyme analysis, esterase showed the highest number of polymorphic bands (13) followed by acid phosphatase (9) and peroxidase (5). These polymorphic markers were very effective for classification of all the species studied in this experiment. In parentage tests using isozymes, the hybridity of intra-and-interspecific crosses of almost all the seedlings could be identified from their respective cross combinations. Esterase polymorphism showed a clear differentiation between YS and BS types of *B. rapa*. In addition, two esterase polymorphic markers were iden ified to differentiate some cultivars of *B. juncea*. Segregation patterns in these two esterase bands showed a simple Mendelian monohybrid ratio of 3:1 in F_2, 1:1 in test cross and 1:0 in back cross progenies. No polymorphic band was identified to distinguish different cultivars of the same species by acid phosphatase or peroxidase. Polymerase Chain Reaction (PCR) was carried out with seed coat color specific marker of *B. juncea*. The yellow seeded cultivars produced a strong band at 0.5 kb and weak band 1.2 kb. In the addition of these two specific bands, Japanese yellow-seeded cultivars expressed two more weak bands at 1.0 kb and 1.1 kb. Where the brown seeded cultivars generated a single strong band at 1.1 kb. In segregating population, the yellow seed coat color marker segregated at a ratio 15 (brown) : 1 (yellow), indicating the digenic inheritance pattern of the trait.

Key words: *Brassica*, SDS-PAGE, isozymes, PCR

Introduction

Brassica species are major oilseed crops as well as vegetables like broccoli, cabbages, Chinese cabbage. Leafy mustard is an increasingly important part of the human diet worldwide. In general, genetic improvement of crops can be accelerated when broad genetic diversity and the information of these genetic resources are available. The collection of these genetic resources and the assessment of genetic diversity within and among species should have priority for varietal improvement. At the same time it is necessary to develop better methods of characterization and identification of germplasm collections to improve strategies for conservation and collection of species or cultivars, and to increase the utilization of plant genetic resources.

* Corresponding author, E-mail: umrahm04@cc.umanitoba.ca
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Electrophoresis of proteins is one method to investigate genetic variation and to classify plant varieties (Takehisa et al. 2001). Seed protein is not sensitive to environmental fluctuations; its banding pattern is very stable which is useful for species identification in crops. This protein is useful tool for studying genetic diversity of wild and cultivated rice (Thanh and Hirata 2002). However, the information on the SDS-PAGE on different species or cultivars of *Brassica* for genetic diversity is still limited.

Despite the large numbers of molecular markers available for genetic analysis of *Brassica* species, isozyme polymorphism has still been used as complementary marker for its ease in detection of polymorphisms. This technique is an efficient tool for genetic, systematic and breeding studies, particularly in *Brassica* species because of their high level of polymorphism. Isozyme analysis has been widely used to screen the variability present in populations and to select desirable genotypes in plant populations (Tanksley and Jones 1986). Isozyme loci have also been used as markers in a number of genetic studies, such as genetic diversity in *Brassica juncea* (Kumar and Gupta 1985), genetic diversity in *B. rapa* (Person et al. 2001), testing genome construction of different species of *Brassica* (Chen et al. 1989); isozyme loci and their linkage in *B. campestris* (Nozaki et al. 1995); and isozyme markers as seed coat color marker in *Brassica* (Rahman 2001).

B. juncea is an important oilseed crop in Bangladesh, India and of growing importance in Canada and USA as an alternative to rapeseed. Quality and quantity of oil can be improved by the development of yellow-seeded cultivars in this species. In *B. juncea* two independent dominant genes with duplication effect (Anand et al. 1985; Chauhan and Kumar 1987) govern the seed coat trait. Here, we used the suggested primer combinations of SCAR marker developed by Negi et al. (2000) in simple PCR analysis. In this paper we report the variations of protein, isozymes and molecular markers for identification of different species and cultivars of *Brassica*.

Materials and Methods

Plant materials

Eighty-five different cultivars of *B. rapa, B. juncea, B. napus, B. carinata, B. oleracea* and hexaploid *Brassica*, collected from Bangladesh, Japan, China and Denmark were used for this experiment (Table 1).

SDS-PAGE

Leaf protein and seed proteins were extracted from young leaf and cotyledons of a single seed, and the extraction buffer was of 0.0625M Tris-HCl (pH 6.8), 8M Urea, 2% Sodium dodecylsulfate (SDS) and 5% 2-Mercaptoethanol. The crude extract was analyzed by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) using 12% (w/v) mini-slab gel. The bands were detected with 0.5% Coomassie Brilliant

Table 1. Seed protein subunits diversity in the cultivars of several *Brassica* species.

Species	Origin	Culti-vars	A[1]	B	C	D	E			F	G	H	I	J		
							E_1	E_2	E_3					J_1	J_2	J_3
YS *B. rapa*	Bangladesh	6	0[2]	0	6	6	6	0	0	6	0	0	6	0	6	0
BS *B. rapa*	Bangladesh	6	0	0	1	0	6	0	0	6	6	0	6	6	0	0
	Japan	12	0	12	0	7	12	0	0	12	12	0	12	12	0	0
	China	8	0	2	1	0	8	0	0	8	8	0	8	8	0	0
B. juncea	Bangladesh	10	0	10	0	10	0	10	0	0	10	10	10	0	10	0
	Japan	18	0	12	0	18	0	18	0	0	18	18	18	0	18	0
	Denmark	2	0	2	0	2	0	2	0	0	2	2	2	0	2	0
B. napus	Bangladesh	16	16	6	0	10	0	0	16	12	16	0	16	0	0	16
	Japan	2	2	2	0	2	0	0	2	2	2	0	2	0	0	2
B. carinata	Denmark	1	1	1	0	1	0	1	0	0	1	1	0	0	0	1
	Japan	1	1	1	0	1	0	1	0	0	1	1	0	0	0	1
B. oleracea	Japan	2	2	2	0	2	0	2	0	0	2	0	0	0	0	2
Hexaploid *Brassica*	Denmark	1	1	1	0	1	0	0	1	1	1	1	1	0	0	1
Total		85														

N.B.: YS= Yellow sarson, BS= Brown seeded.

1 - Designation of protein subunit based on polymorphic band.

2 - Number of cultivars polymorphic for protein subunit.

Blue (CBB) G-250 in acetic acid-ethanol-water (2:5:5 volume ratio). Banding patterns were scored from at least two electrophoregrams for each cultivar. When ambiguous band patterns were obtained, electrophoresis was further carried out by changing the gel concentration and/or electrophoresis time to determine the protein type.

Isozyme analysis

Peroxidase isozyme was extracted by 0.2 M phosphate buffer (pH 7.0). The enzyme was subjected to polyacrylamide gel electrophoresis (7.5% separation gel, 3.75% stacking gel, 0.02 M Tris-glycine running buffer) and detected by 0.044M phosphate-0.028M citric acid buffer (pH 4.4-4.6) containing 0.02% O-dianisidine & 1% H_2O_2. Acid phosphatase and esterase isozymes extraction, electrophoresis and detections were followed using the methods of Nozaki et al. (1995).

Rf values for isozyme

In order to compare the different allozymes, the Rf values were calculated as the ratio between the migration distance from the cathedral origin to the position of each allozyme and the distance from the origin to the most anodal band of the banding pattern.

DNA extraction & amplification

DNA was extracted from young leaves by SDS methods according to Dellaporta et al. (1983) with some modifications and was used as template in amplification. PCR was carried out in a final reaction volume of 25 :l in reaction buffer containing 20ng total genomic DNA, 0.1:l *Taq* polymerase (Takara), 2.5:l of 10x buffer, 2.5:l $MgCl_2$, 2.0:l dNTPs, 1.0:l of each primers (SCM08 & Walk8F) and 15.9:l autoclaved IEW (Ion Exchange Water). PCR conditions for amplification were 94℃ for 3 min, followed by 40 cycles of 94℃ for 45s, 69℃ for 45s, 71℃ for 1.5 min. Staining was performed with Ethidium Bromide (5:l EtBr/200ml 1x TAE buffer) for 20 min, and photographed under UV light.

Primers

The seed coat color specific primers of *B. juncea*, which was developed by Negi et al. (2000) were used in this experiment. The primers combinations were as follows:

Forward: SCM08 5'-GAGCATCTAAACCGTCGTGCTTCC-3' (24 mer)

Reverse: Walk8F 5'-GCGCGTCCCCTTCCAGAAGTGAAC-3' (24 mer)

Results

Leaf protein

The cultivars of *B. rapa, B. juncea, B. napus, B. carinata, B. oleracea* and hexaploid *Brassica* were tested for leaf protein analysis by SDS-PAGE. Results do not distinguish these different species of *Brassica* well.

Seed protein

In total, 33-35 bands per cultivars were detected in SDS-PAGE electrophoregrams of seed proteins. Of these, polymorphic bands appear in ten positions designated as 'A', 'B', 'C', 'D', 'E', 'F', 'G', 'H', 'I' and 'J', respectively (Table 1, Figure 1). Bands in the position 'A', 'G', 'H' and 'I', showed presence-or-absence type polymorphisms. In position 'E', some genotypes expressed a single band and other showed a pair bands, and two levels of mobility of the bands were detected. These banding patterns were recognized in the position as 'E_1', 'E_2' and 'E_3', respectively. Bands in the positions 'B', 'C', 'D' and 'F' differed in the intensity among genotypes. Bands in position 'J' were also divided into three patterns ('J_1', 'J_2' and 'J_3'), based on size and number of bands in cluster; 'J_1' represented two medium thick bands, 'J_2' as three relatively thin bands and 'J_3' as one very thick band.

The six Bangladeshi cultivars of yellow sarson, *B. rapa*, showed four uniquely identifiable bands at 'C', 'D', 'G' and 'J', respectively. Highly dense band 'B' was specifically expressed in Japanese cultivars as well as few cul-

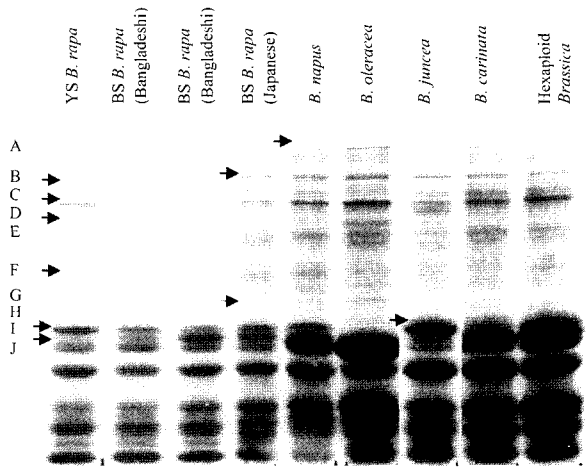

Figure 1. Electrophoregram types identified by SDS-PAGE of seed protein of different species of *Brassica*. Arrow heads indicate polymorphic bands at different positions.

tivars of Chinese brown seeded *B. rapa*. The cultivars of *B. rapa* exhibited a band at 'I' and failed to produce any band at 'A' and 'H' and also were rich in 'F' and 'E_1' types.

The cultivars of *B. juncea* exhibited a band at 'G', 'H' and 'I', and were 'C'-less, 'D'-rich, 'F'-less with 'E_2' and 'J_2' types. Polymorphism of different cultivars of *B. juncea* was obtained at band 'B'.

In *B. napus*, bands existed at band 'A', 'G' and 'I'; and were 'C'-less, 'E_3' and 'J_3' types of banding pattern. Polymorphisms of different cultivars of *B. napus* were obtained at 'D' and 'F' bands.

In this study two cultivars of each of *B. carinata* and *B. oleracea* were used to compare the bands with other species. However, *B. carinata* exhibited a band at 'A', 'G' and 'H' and was 'B'-rich, 'F'-less, 'E_2' and 'J_3' types. *B. oleracea* was specific to band 'A', 'G', and was 'B'-rich, 'C'-less, 'F'-less, 'E_2' and 'J_3' types.

The trigenomic hexaploid, which contained all three genomes of *Brassica*, exhibited the highest number of bands per assay. It produced band 'A', 'G', 'H' and 'I'. This cultivar was 'B'-rich, 'D'-rich, 'F'-rich, 'C'-less, 'E_3' and 'J_3' type.

Isozyme analysis

Esterase analysis

Thirteen polymorphic bands were identified from esterase analysis (Figure 2). The polymorphic band and Rf value were marked as 'A' (Rf=0.16), 'B' (Rf=0.30), 'C' (Rf=0.34), 'D' (Rf=0.42), 'E' (Rf=0.44), 'F' (Rf=0.46), 'G' (Rf=0.53), 'H' (Rf=0.58), 'I' (Rf=0.70), 'J' (Rf=0.77), 'K' (Rf=0.84), 'L' (Rf=0.89) and 'M' (Rf=0.95), respectively. The yellow sarson and brown seeded types of *B. rapa* commonly shared a band at position 'A', 'C', 'H' and 'J' and showed polymorphism at position 'I', 'L' and 'M'. The cultivars of *B. juncea* were distinguishable to band 'D', 'E', 'F', 'J', 'K' and 'M'. Polymorphisms were observed among different cultivars of *B. juncea* at 'H' and 'L'. Cultivars of *B. napus* was specific to bands 'A', 'B', 'D', 'G', 'H', 'I', 'J', 'L' and 'M'. Similarly the cultivars of *B. carinata* and *B. oleracea* were specific to band 'A', 'B', 'G', 'H', 'L' and 'M', except 'K', where cultivars of *B. oleracea* failed to have a band at 'K'. The hexaploid *Brassica* exhibited all the bands except two *B. juncea* specific bands, 'E' and 'F'.

Acid phosphatase analysis

In acid phosphatase isozyme analysis, nine polymorphic bands were identified at different positions. The polymorphic bands with Rf value were designated as 'A' (Rf=0.15), 'B' (Rf=0.30), 'C' (Rf=0.38), 'D' (Rf=0.43), 'E' (Rf=0.46), 'F' (Rf=0.53), 'G' (Rf=0.76), 'H' (Rf=0.81) and 'I' (Rf=0.87), respectively (Figure 3). Using this isozyme it was not possible to differentiate cultivars of the same species by polymorphic bands. However, different species were clearly identified through their polymorphisms. The cultivars of *B. rapa* exhibited a band at 'A', 'B', 'C' and 'G'. The band at 'D' and 'E' were uniquely specific to *B. juncea*. The cul tivars of *B. juncea* also showed a band at 'C', 'G', 'H'

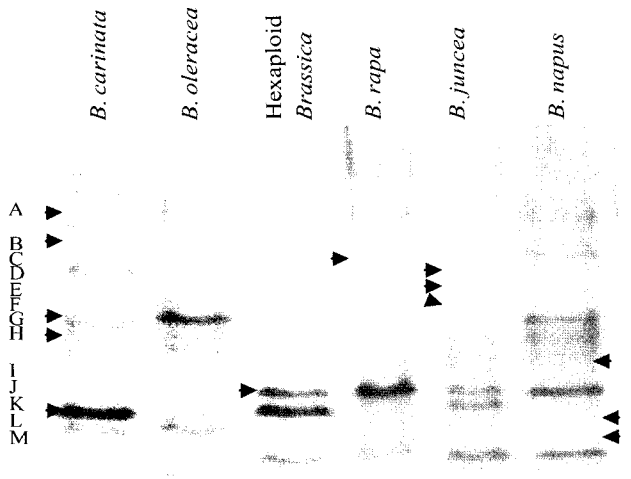

Figure 2. Esterase banding pattern of different species of *Brassica*. Arrow heads indicate polymorphic bands at different positions.

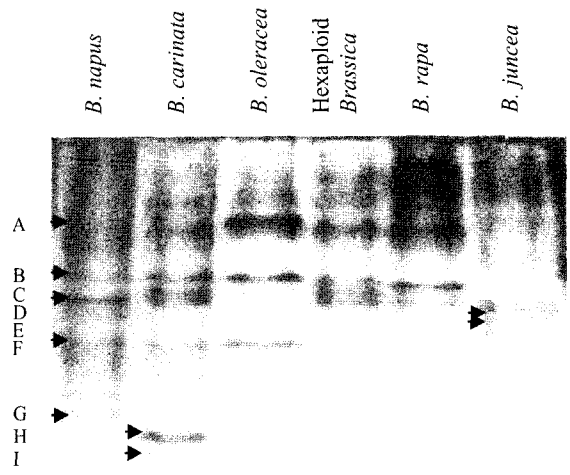

Figure 3. Acid phjosphate banding pattern of different species of *Brassica*. Arrow heads indicate polymorphic bands at different positions.

and 'I'. Cultivars of *B. napus* showed band at 'A', 'B', 'C', 'F' and 'G'. The cultivars of *B. carinata* were specific to band 'A', 'B', 'C', 'F' 'H' & 'I' and *B. oleracea* were at 'A', 'B' and 'F'. Hexaploid *Brassica* exhibited all bands except 'D', 'E' and 'I'.

Peroxidase analysis

Five different polymorphic bands viz 'A' (Rf=0.47), 'B' (Rf=0.59), 'C' (Rf=0.87), 'D' (Rf=0.93) and 'E' (Rf=1.00), were identified from peroxidase analysis (Figure. 4). The cultivars of *B rapa* showed only one band at position 'E'. *B. juncea* was specific to band 'B', 'C' and 'E', where *B. napus* showed a band at position 'A', and 'D'. The cultivars of *B. carinata* were specific to band 'B', 'C' and 'D', where *B. oleracea* were specific to band 'B' and 'D'. Hexaploid *Brassica* showed two bands at 'B' and 'D'.

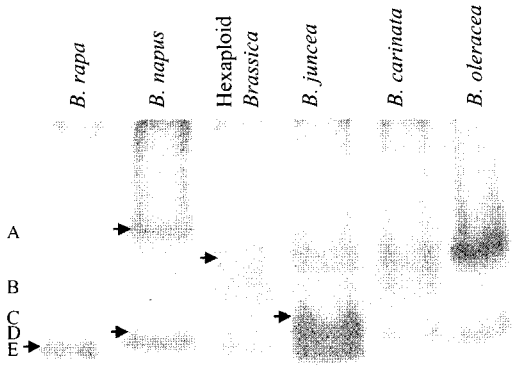

Figure 4. Peroxidase banding pattern of different species of *Brassica*. Arrow heads indicate polymorphic bands at different positions.

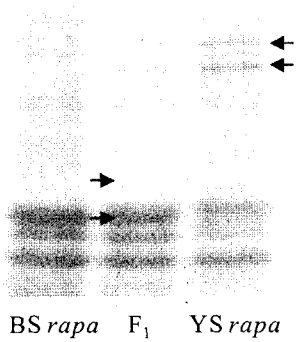

Figure 5. SDS-PAGE of F_1 cross of yellow sarson (Agrani) and brown seeded (R-307) cultivar of *B. rapa*.

Genetic studies of some polymorphic bands

SDS-PAGE for seed protein

The Bangladeshi yellow sarson cultivar (Agrani) and Japanese brown seeded cultivar (JR-310) were polymorphic at four bands. The F_1 between two cultivars showed the sum of all polymorphic banding types of the respective position of parents (Figure 5).

Esterase isozymes

Esterase polymorphism was observed in different cultivars of *B. juncea* at band 'H' and 'L'. One Japanese cultivar (Jun-26134) expressed 'H'-null type of band, which was crossed with 'H'-rich cultivar (Jun-26133). The progeny of cross and reciprocal cross showed 'H'-rich band (Figure 6a). Similarly, One Danish cultivar (Jun-83) was 'L'-null type, which was crossed with two Japanese 'L'-rich cul-

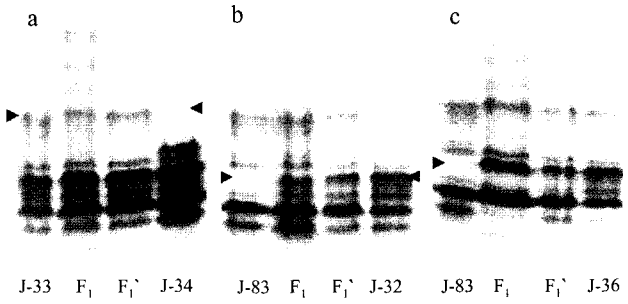

Figure 6. Genetic analysis of intra-specific esterase banding of *B. juncea*. a, *B. juncea* (*var. Jun- 33*) X *B. juncea* (var. Jun 34); b, *B. juncea* (*var. Jun- 83*) X *B. juncea* (var. Jun 32); c, B. *juncea* (*var. Jun- 83*) X *B. juncea* (var. Jun 36)

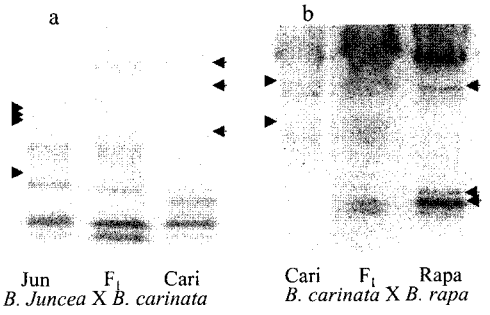

Figure 7. Crossing analysis of inter-specific esterase banding pattern. Arrow head indicate polymorphic bands at different positions.

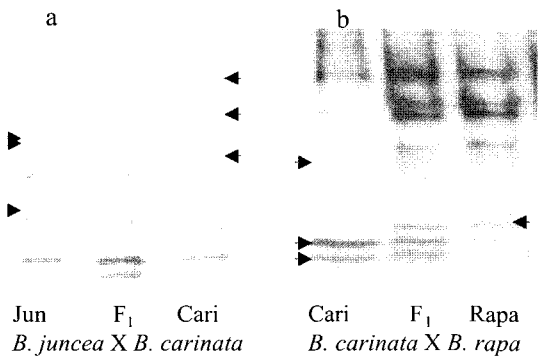

Figure 8. Crossing analysis of inter-specific acid phosphate banding pattern. Arrow head indicate polymorphic bands of the two species.

tivars (Jun-26132 and Jun-26136), where F_1 of the cross and reciprocal cross of the parents showed the dominant nature of the band (Figure 6b, c). In case of interspecific analysis, cultivars of *B. juncea* and *B. carinata* were polymorphic at band 'A', 'B', 'D', 'E', 'F', 'G', and 'J'. The F_1 of the cross between *B. juncea* and *B. carinata* showed the presence of all mentioned polymorphic bands (Figure 7a). Similarly, *B. rapa* and *B. carinata* were polymorphic at 'B', 'C', 'G', 'J' and 'K' bands and the F_1 of the cross exhibited the presence of all polymorphic bands (Figure 7b).

Acid phosphatase isozyme

B. juncea and *B. carinata* were polymorphic at band 'A', 'B', 'D', 'E', 'F' and 'G'. The F_1 of the cross between *B. juncea* and *B. carinata* showed the presence of all polymorphic bands (Figure 8a). Similarly, *B. rapa* and *B. carinata* were polymorphic at band 'F', 'G', 'H' and 'I' and F_1 exhibited the presence of all bands (Figure 8b).

Isozyme segregation

Two polymorphic esterase bands were identified in cultivars of *B. juncea*. Jun-83 was L-null type and where Jun32 and Jun-36 were L-rich types, were crossed to study the nature of L-band-rich band cultivars. Similarly, Jun-34 was H-band-null type which was crossed with H-band-rich Jun-33 cultivar. Segregation of L-rich and H-rich banding types were recorded. The segregation pattern of the cross Jun-83 X Jun-36 of L-allele fitted to the monogenic segregation ratio of 3:1 (χ^2=0.108, P=0.74). The test cross and back cross also fit the expected ratio i.e. 1:1 (χ^2=0.286, P=0.62) and 1:0 (χ^2=0, P=1.00), respectively (Table 2). Similarly, segregation pattern of cross of Jun-83 X Jun-32 of L-allele followed the same Mendelian 3:1 (χ^2=0.236, P=0.66), 1:1 (χ^2=0.066, P=0.81) and 1:0 (χ^2=0, P=1.00) for F_2, test-cross and back cross, respectively (Table 2). Segregation of H-rich allele in the cross of Jun-33 X Jun-34, appeared as monogenic segregation of 3:1 (χ^2=2.38, P=0.14) in F_2; where test-cross and back cross also followed the Mendelian monohybrid ratio i.e. 1:1 (χ^2=2.082, P=0.17) and 1:0 (χ^2=0, P=1.00) (Table 2).

PCR assay

In this experiment, yellow seed and brown seed coat color specific SCAR marker (SCM08 and Walk8F) were used. Two Danish yellow seeded cultivars (Jun-83, Jun-20), one yellow seeded (Jun-33) and two brown seeded (Jun-32, Jun-34) Japanese cultivars, and one Bangladeshi brown seeded (Rai-5) cultivar were used in this experiment. F_1 and 30 F_2 progenies of a cross of Jun-83 x Jun-32 were also studied. All three yellow seeded cultivars produced a strong band at 0.5 kb and a weak band at 1.2 kb. In addition of these two specific bands, the Japanese yellow seeded cultivar had tw more weak bands at 1.0 kb and 1.1 kb. The

Table 2. Segregation of esterase isozymes polymorphic bands in *B. juncea*.

Cross combination	Band position	Observed frequency		Expected ratio	χ^2-test	Probability
		+	-			
(Jun-83 X Jun-36)	L	59	18	3:1	0.108	0.74
(Jun-83XJun-36) X Jun-83	L	30	26	1:1	0.286	0.62
(Jun-83XJun-36) X Jun-36	L	24	0	1:0	0.000	1.00
(Jun-83 X Jun-32)	L	50	19	3:1	0.236	0.66
(Jun-83XJun-32) X Jun-83	L	31	29	1:1	0.066	0.81
(Jun-83XJun-32) X Jun-32	L	24	0	1:0	0.000	1.00
(Jun-33 X Jun-34)	H	47	9	3:1	2.380	0.14
(Jun-33XJun-34) X Jun-34	H	29	19	1:1	2.082	0.17
(Jun-33XJun-34) X Jun-33	H	24	0	1:0	0.000	1.00

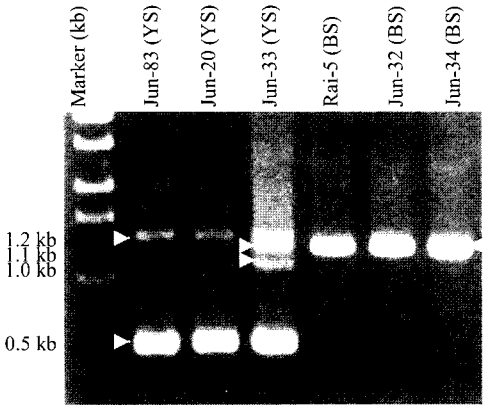

Figure 9. Gel amplification using specific marker for the classification between yellow-seeded (Jun-83, Jun-20, Jun-33) and brown-seeded (Jun-31, Jun-32, Jun-34) cultivars.

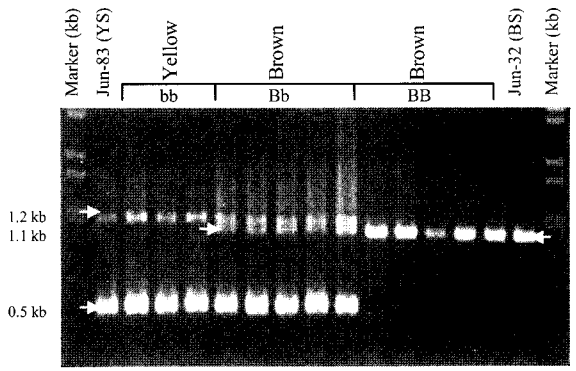

Figure 10. Analysis of PCR products obtained using the SCAR primer on individual plants. Jun-83 is the yellow-seeded parents and Jun-32 is the brown seeded parent. F2 showed 15:1 segregation ratio between brown-seeded and yellow-seeded specific markers.

brown seeded cultivars produced a single intense band at 1.1 kb (Figure 9).

The yellow seeded individuals in the segregating populations showed an amplification pattern similar to that of yellow parents (Figure 10), whereas differences were observed in the banding pattern among the brown seeded individuals (Figure 10). This is due to the fact that the brown seeded lines might be either homozygous (BB) or heterozygous (Bb). The homozygous brown seeded individuals showed a pattern similar to that of the brown parents, whereas the heterozygous (Bb) brown individuals produced 3 bands corresponding to the banding pattern of both the yellow and brown parents. A total of 30 segregating (F_2) individuals were screened with the primers and only 3 plants showed the yellow seeded specific marker. The Chi-square (χ^2= 0.72, p= 0.42) test showed that the seed coat color marker segregated in the ratio 15 (brown) : 1 (yellow) ratio, confirming the digenic inheritance pattern with duplicate gene interaction of the traits.

Discussion

Intra-and- interspecific variation and geographical distribution of leaf protein and seed storage protein of eighty-five cultivars of *Brassica* were analyzed. Banding pattern of SDS-PAGE of leaf protein of different species of *Brassica* cultivars did not show any significant difference. No reports on SDS-PAGE for leaf protein of *Brassica* are available, so it seems to justify that more diverged cultivars and species are necessary to find polymorphism in leaf protein.

Seed protein types and their variation differed among different species, and this information will be helpful for identification of the species at seed level as well as providing information on purity of *Brassica* species accessions or seed lots. The genotypes of *B. rapa* from Bangladesh, Japan and China were found to differ in protein types. This information may help us to establish the origin of the various Asian germplasm. Thanh and Hirata (2002), observed seed storage protein diversity in wild and cultivated rice. The greatest genetic diversity was found in the cultivars of Bangladeshi yellow sarson and other brown seeded *B. rapa*, where yellow sarson showed four unique protein types. The yellow sarson are special ecotypes from India, belonging to ssp. *trilocularis* and are self-compatible, whereas other brown seeded *B. rapa* belong to ssp. *oleifera* and are self-incompatible (Röbbelen 1960). The result of differentiation of yellow sarson and brown seeded types of *B. rapa*, shows a similar agreement with the report of Das et al. (2000), where the result clearly separated the yellow seeded, self-compatible cultivars from the brown seeded, self-incompatible cultivars by using RAPD and AFLP analysis.

Polymorphism as a total of 27 isozyme loci were detected by three isozyme systems viz. esterase, acid phosphatase and peroxidase. The result of this study reveals a high level of interspecific variation as indicated by high proportion (87% - 100 %) of polymorphic bands among different species. All the species were fully characterized based on isozymes polymorphisms. The polymorphic markers will help to identify different species at the seedling stage. The variability has provided an efficient tool for studies of genetics and evolution of crucifers (Chevre et al. 1994). In this study esterase polymorphism at three bands showed a clear difference with yellow sarson and brown seeded *B. rapa*. This information will be helpful to identify the cultivars and useful markers in a breeding program of *B. rapa*. The result showed an agreement with Das et al. (2000). Nozaki et al. (1995) observed polymorphism between two cultivars of *B. campestris*

by using the seven enzyme systems. Variation of esterase was also observed in a few cultivars of *B. juncea*.

Isozymes are co-dominant markers that enable us to distinguish between homozygous from heterozygous plants. However, since a number of isozyme bands showed different intensities, homozygous plants could not be distinguised. Therefore, zymograms showing presence or absence of bands only were analyzed. In this experiment isozyme banding pattern shows that F_1 hybrids represented the sum of the bands from their respective parents, indicating that some genes are controlling the bands. Isozyme electrophoresis is a very useful method as a purity test of the F_1 hybrid variety, because the data can be obtained within a short time under stable environmental conditions. Isozymes have been used to distinguish F_1 interspecific hybrids of *Brassica* by many scientists (Quiros et al. 1988; Ripley et al. 1992; Chevre et al. 1994; Eber et al. 1994). Segregation tests of two esterase loci from the F_2, test-cross and back-cross progenies were found to fit a monogenic segregation. The results were in agreement with Moriyuki et al. (1999) where esterase and peroxidase markers exhibited a monogenic segregation ratio of 3:1.

Isozyme analysis can be easily performed with a large number of samples and requires only a small amount of tissue. This analysis can also be applied for cultivar identification and parentage test in *Brassica* breeding programs. This could be a useful marker to investigate the inheritance of traits in *Brassica* species.

Yellow seeded and brown seeded cultivars of *B. juncea* and their progenies were tested with a seed coat color specific SCAR marker (Negi et al. 2000). The three yellow-seeded cultivars produced two common bands and the Japanese yellow-seeded cultivar generated two additional bands, where the brown seeded cultivars produced a single band. The results showed almost full agreement with Negi et al. (2000), except for two additional bands in the Japanese yellow-seeded cultivar. Negi et al. (2000) used a brown seeded cultivar of Indian origin and yellow seeded cultivar from Poland. This study used Japanese and Danish yellow seeded cultivars, and Japanese and Bangladeshi brown seeded cultivars. Therefore, perhaps due to genetic diversity of yellow seeded *B. juncea*, the Japanese cultivar produced two additional bands. The current results give a further authentication of seed coat color specific markers in diversified origin of cultivars. These results also confirmed the tight linkage of the SCAR marker to seed coat color and indicated that this marker could be useful for marker-assisted breeding. The markers were extended to analyze a segregating population of *B. juncea*. The PCR assay using the SCAR primers were useful as all the individuals could be classified as bb, Bb or BB. Thus, the SCAR markers have the advantage of being co-dominant and useful for identifying heterozygous individuals.

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