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AFLP-based genetic diversity assessment amongst agronomically important natural and some newly synthesized lines of *Brassica juncea*

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Abstract AFLP markers were employed to assess the genetic diversity amongst 21 established natural and nine synthetic varietes and lines of *Brassica juncea* originating from Asia, Australia, Canada, Eastern Europe and Russia. Six of the synthetics used for diversity studies have been developed recently. Twenty one *Eco*RI/*Mse*I-based AFLP primer pairs generated a total of 1251 scorable fragments among the 30 genotypes studied, of which 778 bands were polymorphic with an average of 37 polymorphic bands per primer pair. On the basis of the similarity coefficients (*F* value), cluster analysis was performed using the UPGMA method. The 30 *B. juncea* lines could be grouped into three distinct clusters. All the Indian, Chinese and previously developed synthetics formed one cluster (cluster A), the recently developed synthetics formed a separate cluster (cluster B) and the lines from Australia, Canada, Eastern Europe and Russia formed the third cluster (cluster C). A majority of the lines were uniquely identified by one or more primer pairs due to the presence or absence of variety specific band(s). Four primer pairs were found to be most informative, since these uniquely identified all the genotypes assayed. These four primer pairs, could therefore be used as fingerprinting primers for varietal identification.

Keyword AFLP markers · *Brassica juncea* · Genetic diversity

Introduction

Evaluation of genetic divergence and relatedness among breeding materials has significant implications for the improvement of crop plants. Besides providing predictive estimates of genetic variation within a species these studies also facilitate the planning of new breeding approaches for cultivar development. Diverse parental combinations could be either used to create segregating progenies with maximum genetic variability for selection or for heterosis breeding.

Brassica juncea (Indian mustard) is a major oilseed crop of India and is grown in about six million hectares of land, particularly in the rainfed areas of Northern India during the winter growing season. It is also a potential crop in the western prairies of Canada and to some extent is grown in Australia, China and Russia. *B. juncea* is predominantly a self-pollinated crop with 5–15% cross-pollination (Asthana and Singh 1973). Cultivar improvement programmes in *B. juncea* have generally followed breeding methodologies for self-pollinating grain crops. Over the past two decades, breeding programmes, particularly in India, have endeavoured to make selections from segregating populations of crosses amongst varieties from regional programmes. Crossing within such germplasm, has resulted in only marginal improvements in the productivity of *B. juncea*. To realize further gains in productivity, it would be important to utilize new sources of variation which would lead to broadening the genetic base of the existing varieties. Productivity can also be substantially increased by heterosis breeding. In *Brassica* species, it has been shown that F_1 hybrids between genetically diverse genotypes and/or genotypes of different geographical regions are more productive than those involving genotypes which are more regional and have a narrow genetic base (Banga and Labana 1984; Lefert-Buson et al. 1987; Pradhan et al. 1993).

DNA markers are increasingly being recognised as useful tools for assessing genetic diversity amongst germplasm since these are least influenced by the environment (Lee 1995). The marker systems include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence re-

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peat polymorphism (SSR). RFLP has been used to study genome organization, varietal identification and phylogeny in the genus *Brassica* (Song et al. 1988; Diers and Osborn 1994; Hallden et al. 1994; Santos et al. 1994). However, the procedure is laborious, expensive and few loci are detected per assay. The random amplified polymorphic DNA (RAPD) technique (Welsh and Mc-Clelland 1990; Williams et al. 1990) is considered to be more straight forward compared to RFLP analysis and requires only nanograms of genomic DNA. It uses single oligonucleotide primers of arbitrary sequence in a polymerase chain reaction and allows a large number of markers to be assayed inexpensively. RAPD has been employed in different *Brassica* species for varietal identification and genetic diversity studies (Demeke et al. 1992; Kresovich et al. 1992; Jain et al. 1994). However, this technique is sensitive to subtle changes in reaction conditions and hence difficult to reproduce (Jones et al. 1997). Simple sequence repeats (SSRs) are locus-specific and co-dominant PCR-based markers. However, the development of oligonucleotide primers to be used as SSR markers is time-consuming, expensive and few loci are identified per reaction.

The recently developed AFLP technique (Vos et al. 1995) has generated considerable interest and appears to be most promising for varietal fingerprinting, mapping and genetic diversity studies. One of the main advantages of the AFLP technique is its multiplex ratio, which means that a large number of amplified products are generated in a single reaction (Powell et al. 1996). Furthermore, the method is generic and does not depend on the availability of sequence information. The AFLP technique has been applied to analyze intraspecific diversity in rice (Mackill et al. 1996), soybean (Maughan et al. 1996), tea (Paul et al. 1997), grape (Sensi et al. 1996), barley (Ellis et al. 1997), wheat (Barret and Kidwell 1998) and *Vigna* (Yee et al. 1999) but has not been used in any of the *Brassica* species. In this paper, we report the use of the AFLP technique to: (1) study the genetic variability amongst 30 *B. juncea* accessions comprising Indian, exotic and synthetic lines, some of which were recently synthesized in our laboratory, (2) identify the AFLP primer combinations which would be highly informative in varietal identification, and (3) to develop a number of polymorphic markers with AFLP primers for eventual linkage-based tagging of important agronomic traits available in the germplasm studied here.

Materials and methods

Plant materials

The plant materials used in this study comprised 30 *B. juncea* lines (see Table 1) representing established varieties and lines from India, Russia, China, Australia, Yugoslavia, Poland, Turkey and Canada. It also included nine synthetic lines of which six were synthesized recently in our laboratory (see Table 1). All the materials used in the study were maintained by selfing for at least three generations except for the newly developed synthetics which were used after one generation of selfing.

AFLP analysis

DNA was isolated from leaves of field-grown plants following the protocol of Rogers and Bendich (1994). AFLPs were generated using the protocol of Keygene Inc. (Vos et al. 1995). AFLP Analysis System I kit (Life Technologies, Gaithersburg, USA) based on *Eco*RI/*Mse*I primer pairs was used for generating AFLP fragments. Restriction endonuclease digestion of the DNA and ligation of adapters, pre- and selective-amplification of the restriction fragments, and gel analysis of the amplified fragments were performed according to the manufacturer's instructions supplied with the kit. Selective amplification was carried out with 21 *Eco*R1/*Mse*I (see Table 2) primer pairs. *Eco*RI primers were end-labelled with γ^{32}P ATP (4000 ci/mmol). PCR reactions for pre- and selective-amplification were performed using a Perkin Elmer 9600 thermocycler.

Data analysis

AFLP markers were generated using 2 pairs of primers (see Table 2). Each locus was treated as a separate character and scored as either present (1) or absent (0) across all genotypes. Bands within genotypes were scored amissing data if poorly resolved on the gel or if template DNA did not amplify properly. Clearly distinguishable bands were used in the genetic analysis. The *F* value (an estimate of the similarity coefficient, $F=2a_{ij}/b_i+c_j$) was computed according to the method of Nei and Li (1979) where a is the number of common bands between any two lanes i and j, b is the number of bands present in lane i, and c is the number of bands present in lane j. Cluster analyses were performed using the unweighted pair group method with arithmetical averages (UPGMA). All computations were done using NTSYS-pc 2.0 software for multivariate analysis (Rohlf 1998). The goodness of fit of the dendrograms and correlations between similarity matrices were assayed by the Mantel test statistic (Mantel 1967) of the NTSYS MXCOMP function (Rohlf 1998).

Results

Level of polymorphism

The twenty one pairs of primers generated a total of 1251 scorable fragments among the 30 genotypes studied, of which 778 bands were polymorphic with an average of 37 polymorphic bands per primer combination. All bands that could be reliably read on the autoradiograms were treated as individual dominant loci and scored as either present (1) or absent (0) across all genotypes. Part of a typical gel is shown in Fig. 1. The number of fragments detected by individual primer pair ranged from 39 (primer pair E-ACC/M-CAG) to 88 (primer pair E-AAG/M-CAT (see Table 2). The percentage of polymorphism for each primer combination varied from 42% (primer pair E-AAG/M-CAA) to 85% (primer pair E-ACC/M-CAG).

Genetic relatedness and cluster analysis

The AFLP data generated from 1251 fragments over 21 primer pairs was used to calculate pair-wise *F* values according to the formula of Nei and Li (1979) and cluster analysis was performed using the UPGMA method. The

Table 1 List of *B. juncea* lines included in the present study

Line. no.	Name	Origin	Primer pairs[a] exhibiting bi-allelic[b] variety specific bands
	Indian		
1	Varuna	India	(18)
2	Pusa Bold	India	(11), 18
3	Pusa Jai Kisan	India	14
4	Kranti	India	(11), 3
5	RH 30	India	8, 14
6	RLM 198	India	4, 16
7	TM 4	India	(2)
	Exotic		
8	Donskaja IV	Russia	19
9	Skorospieka II	Russia	3
10	Hanzahang Gaoyou Cai	China	(2, 8, 10, 16, 17, 18, 20), 3, 7, 14
11	Shi Yian Kuyou Cai	China	(2, 4), 3
12	Acc. No. 409	Tibet	(3, 6, 10, 15), 3, 4, 9, 10, 13, 14, 16, 17
13	Zem 1	Australia	(9)
14	ATC 94395	Australia	4, 13, 14
15	Acc. No. 404	Yugoslavia	(5)
16	Malopoloska	Poland	–
17	Secus	Poland	(2, 5, 6), 15, 18
18	Acc. No. 2548	Turkey	–
19	Cutlass	Canada	–
20	Domo IV	Canada	(15)
21	Heera	Canada/India	(2, 5), 1, 12, 19
	Synthetics		
	Existing		
22	Sej II (R.N. Raut, personal communication)		1
23	Chinensis-nigra (Prakash 1973)		(7, 10, 19)
24	PNMB (Prakash 1973)		(8, 16, 17), 7, 14, 21
	Newly synthesized		
25	AS 1 (*B. campestries*, Acc. No. 94211×*B. nigra* var. Sangam)		(2, 15, 16)
26	PS 6 (*B. campestries* var. Pant toria×*B. nigra* var. Sangam)		–
27	DIS 1.3 (*B. campestries* var. DYS 1×*B. nigra* var. Sangam)		(2, 7, 9, 12, 19, 21)
28	D3A2 (*B. campestries* var. DYS III×*B. nigra* Acc. no. 94029)		(1, 3, 6, 7, 14, 16, 17)
29	D3S33 (*B. campestries* var. DYS III×*B. nigra* var. Sangam)		(17, 19, 15)
30	TS 2 (*B. campestries* var. TLC 1×*B. nigra* var. Sangam)		–

[a] Number indicates the primer no. mentioned in Table 2. Primer pairs exhibiting variety specific band(s) for more than one line is due to the presence or absence of band(s) of different molecular weight. Numbers in parenthesis are due to the presence of band(s)

[b] Bi-allelic means either presence or absence of band(s)

F value ranged between 0.80 and 0.97 with a mean of 0.86. The goodness of fit of the dendrogram was high (r=0.87). At the 0.86 level, 30 genotypes analysed were grouped into three distinct clusters (Fig. 2). Twelve lines comprising all the Indian genotypes, previously developed synthetics and Chinese types, formed one cluster (cluster A) in which RH 30 and Pusa Jai Kisan exhibited the highest *F* value of 0.97. The two Chinese lines, Hanzahang Gaoyou Cai and Shi Yian Kuyou Cai formed a separate subgroup at an *F* value of 0.88 within cluster A. Cluster B comprised all the six newly developed synthetics. All the exotic lines formed the third cluster (cluster C). In this cluster ATC 94395 and Acc. No. 404 formed a separate subgroup at an *F* value of 0.89 where-

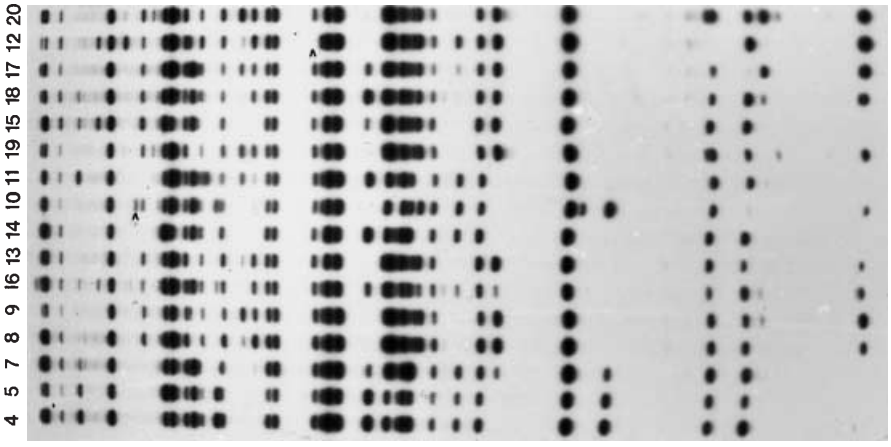

Fig. 1 AFLPs generated from genomic DNA of some lines of *B. juncea* using primer combination E-AAC/M-CTG. The *number* in the lane corresponds to the line no. of Table 1. *Arrows* indicate bi-allelic variety specific bands generated by this primer pair

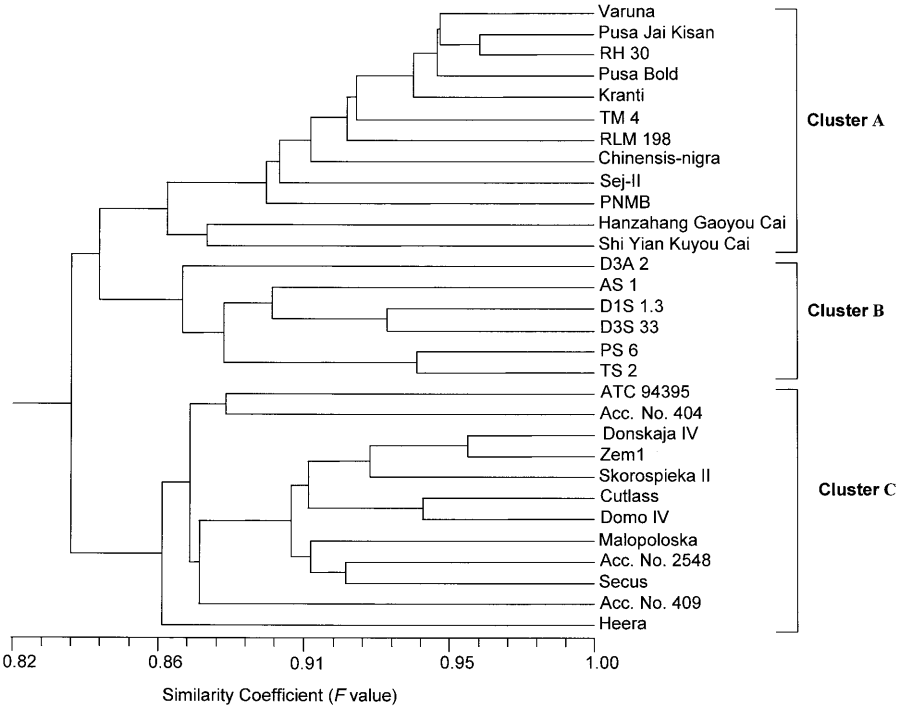

Fig. 2 UPGMA-based dendrogram of *B. juncea* lines generated from 1251 AFLP markers

as Heera was found to be the most divergent line and merged in cluster C at an *F* value of 0.86.

Identification of informative primer pairs

No single primer pair could distinguish all 30 genotypes on the basis of the presence or absence of variety specific band(s) in a qualitative manner. However, all the lines except Malopoloska, Acc. No. 2548, Cutlass, PS6, and TS2 were uniquely identified by one or more primer pairs due to the presence or absence of variety specific band(s). For example, Acc. No. 409 from Tibet was uniquely distinguished by 12 primer pairs followed by Hanzahang Gaoyou Cai (by ten primer pairs). All the Indian genotypes were also uniquely distinguished by one or more primer pairs (Table 1).

Clustering of AFLP data from individual primer pairs indicated that all the primer combinations except E-AAG/M-CAC uniquely identified in excess of 90% of the genotypes they assayed. Only four primer pairs, namely, E-ACT/M-CTC, E-AAC/M-CTA, E-AGC/M-CAT and E-AAG/M-CTT, uniquely identified all the genotypes they assayed (Table 2) and therefore can be regarded as the most informative out of the 21 primer combinations that were tested. In order to determine whether these informative primer pairs could be useful in determining the genetic diversity in comparison to the diversity discerned with 21 primer pairs (Fig. 2), a dendrogram was constructed from the AFLP data based on these four informative primer pairs. Subsequently this dendrogram was compared with the dendrogram obtained with 21 primer pairs and the correlation between the two types of similarity matrices were assayed by

Table 2 Number of AFLP fragments, the level of polymorphism and their clustering behaviour detected over 30 genotypes by using 21 primer combinations

Serial no.	Primer combination	Total no. of Bands	No. of polymorphic bands	Percent polymorphic band	No. of genotypes assayed	Distinct genotype patterns	Percent genotypes identified
1	E-ACT/M-CTC	48	32	67	28	28	100
2	E-AAC/M-CTA	76	57	75	27	27	100
3	E-AAC/M-CAA	73	43	59	28	26	93
4	E-ACT/M-CTG	51	32	63	28	27	96
5	E-AAG/M-CAC	50	32	64	28	25	89
6	E-AAG/M-CAA	78	33	42	29	26	90
7	E-ACG/M-CAA	45	28	62	29	27	93
8	E-ACT/M-CAT	58	40	69	27	26	96
9	E-AAG/M-CAT	88	40	45	30	29	97
10	E-ACT/M-CTT	54	38	70	25	24	96
11	E-ACA/M-CTG	49	34	69	30	28	93
12	E-AAG/M-CAG	52	27	52	27	25	93
13	E-AAC/M-CAT	68	36	53	26	25	96
14	E-AAG/M-CTA	64	43	67	26	25	96
15	E-AGC/M-CAT	51	40	78	29	29	100
16	E-AAC/M-CTG	58	42	72	29	28	97
17	E-AAC/M-CTC	57	36	63	30	29	97
18	E-AAG/M-CTC	59	34	58	29	27	93
19	E-AAG/M-CTT	85	51	60	30	30	100
20	E-ACC/M-CAG	39	33	85	30	29	97
21	E-ACC/M-CTA	48	27	56	28	27	96

Mantel test statistic of the NTSYS MXCOMP function. The correlation between the two similarity matrices was very high (r=0.93). The F values of these similarity matrices ranged from 0.75 to 0.98 with a mean of 0.84. The goodness of fit of the dendrogram was also high (r=0.83). The clustering pattern of the two dendrograms was comparable. The only exception was the shifting of the Chinese line Hanzahang Gaoyou Cai in the dendrogram constructed on the basis of the four informative primers. It formed a separate cluster by itself and joined clusters A and B at an F value 0.83 (dendrogram not shown).

Discussion

The primary objective of this study was to characterize the levels and patterns of genetic diversity among the 30 representative lines of *B juncea* using AFLP markers. The genetic diversity in *B. juncea* has been previously studied using isozyme markers (Kumar and Gupta 1985) and morphological traits (Gupta et al. 1991; Pradhan et al. 1993). Jain et al. (1994) first studied the genetic diversity in *B. juncea* lines by using RAPD markers. Since there are some accessions common between the study undertaken by Jain et al. (1994) and the present study, a direct comparison between the RAPD and AFLP markers for diversity analysis can be made. By using 32 RAPD primers, Jain et al. (1994) observed 378 polymorphic fragments out of a total of 500 fragments scored, with an average of 11.8 polymorphic loci per primer. In our study, using 21 AFLP primer pairs, we surveyed 1251 fragments with an average of 37 polymorphic loci per primer pair. This indicated that AFLP primers on an average detected more than 3-times polymorphism per primer pair than the RAPD primers even though the proportion of polymorphic bands per total number of bands detected was lower for AFLPs in *B. juncea* as compared to RAPDs. Similar observations were reported in lentil (Sharma et al. 1996) and *Vigna* (Yee et al. 1999). AFLP has been reported to be highly reproducible as compared to the RAPD technique and the number of polymorphic markers detected by AFLPs far exceeds the number of polymorphic bands detected by RAPDs (Jones et al. 1997). Vogel et al. (1994) observed that compared to RAPDs, AFLP analysis detected about 12-times the number of polymorphic loci per assay in soybean varieties.

In the present study, estimates of F values based on 21 primer pairs showed the highest similarity between Pusa Jai Kisan and RH 30 (0.97) and between Donskaja IV and Zem 1 (0.96). From the survey of individual autoradiograms and the clustering patterns of individual primer pairs, it was observed that Pusa Jai Kisan and RH 30 were discriminated by 18 primer pairs (except primer nos. 4, 5, and 6) and Donskaja IV and Zem 1 were discriminated by 15 primer pairs (except primer nos, 3, 5, 8, 10, 12, and 13; data not shown). Jain et al. (1994) reported that Varuna and Pusa Bold showed the greatest similarity among *B. juncea* lines with RAPD markers and could be differentiated by only 2 out of 32 primer that were used. In comparison, our study with AFLP markers indicated that these two lines could be differentiated by 19 out of 21 primer pairs (except primers nos. 9 and 17). Thus, the results obtained in the present study indicate the robustness of the AFLP technique in providing a higher degree of resolution for discriminating closely related germplasm than is possible with RAPDs.

Cluster analysis in the present study is broadly in accordance with earlier clustering studies based on morphological traits (Pradhan et al. 1993) and RAPD data (Jain et al. 1994). However, our study has included germplasm from regions that were not previously represented. All the Indian lines were grouped in cluster A and were found to be very close, indicating that these have been bred from a narrow genetic base. The closeness of Pusa Jai Kisan with RH 30 in our study is intriguing. Pusa Jai Kisan is a released Indian variety reported to have evolved through somaclonal variation from the variety Varuna (Katiyar 1995). The presence of many RH 30-specific AFLP bands in Pusa Jai Kisan indicates that the original somaclone might have outcrossed with RH 30 prior to its stabilization or else strictly selfed material of Varuna was not taken for the production of somaclones.

The newly synthesized *B juncea* lines formed an entirely separate cluster (cluster B). The diversity exhibited by these synthetic *B. juncea* lines might act as a new source of variation since these were developed using diverse *Brassica campestris* and *Brassica nigra* lines (Table 1). Pradhan et al. (1993) have shown that hybrids involving Indian and synthetic lines exhibit higher heterosis than those involving only Indian lines. This new class of synthetics has to be carefully checked for the component traits contributing to yield; for example, the number of primary and secondary branches, the number of pods per plant, the number of seeds per pod and their yield performance. Selected lines with desirable traits from amongst these new synthetic lines could then be used for heterosis breeding.

All the Australian, Canadian, Eastern European and Russian lines formed the third cluster (cluster C) and were found to be the most-divergent group from the Indian types. Amongst these exotic lines, Heera was the most divergent one. It was developed through pure-line breeding from the cross between ZYR 4 (a Canadian yellow-seeded variety) and BJ 1058 (a derivative of a three-way cross) (A.S. Khalatkar, personal communication). It is an important line in the *B. juncea* collection. It has many desirable attributes such as low erucic acid, low glucosinolates, yellow seed-coat colour and many other contrasting traits with the Indian types such as seed size, time of flowering and plant height. Being the most diverse genotype, a molecular linkage map between Heera and the predominant Indian variety Varuna would allow tagging of some of the most important agronomic traits in *B. juncea.*

Another objective of the present study was to identify the primer combinations that could be more informative for varietal identification of *B. juncea.* In a previous study in barley, Ellis et al. (1997) have shown that AFLP primer pairs that have greatest discriminatory power also have the most comprehensive genome coverage and were considered to be the most-informative primer pairs. We observed that four primer pairs, namely, E-ACT/M-CTC, E-AAC/M-CTA, E-AGC/M-CAT and E-AAG/M-CTT, were most informative for *B. juncea* germplasm because they had 100% discriminatory power. Their informativeness was further confirmed by comparing the dendrograms constructed on the basis of these four primer pairs with the dendrogram constructed on the basis of 21 primer pairs. We conclude that these primer pairs, being the most informative and having the highest discriminatory power, could be effectively used as fingerprinting primers for varietal identification in *B. juncea*. In addition, the AFLP primer pairs generating variety specific products could be of immense use in the establishment of proprietary rights and in the determination of cultivar purity.

In conclusion, the present AFLP analysis has provided better resolution for discerning genetic diversity amongst *B. juncea* lines and has the potential of complementing both conventional and other molecular markers in studying the genetic diversity in *B. juncea* germplasm. The identification of four informative primer pairs could be very useful for DNA fingerprinting for varietal identification. The increased resolution associated with the large number of markers available with the AFLP approach would provide sufficient markers to construct genetic linkage maps between carefully chosen cultivars, and many agronomically important traits could be tagged. The information generated in this study could have considerable relevance to *B. juncea* breeding.

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