Assessment of genetic variation within *Brassica campestris* cultivars using amplified fragment length polymorphism and random amplification of polymorphic DNA markers

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Genetic relationships were evaluated among nine cultivars of *Brassica campestris* by employing random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers. RAPDs generated a total of 125 bands using 13 decamer primers (an average of 9·6 bands per assay) of which nearly 80% were polymorphic. The per cent polymorphism ranged from 60–100%. AFLP, on the other hand generated a total of 319 markers, an average of 64 bands per assay. Of these, 213 were polymorphic in nature (66·8%). AFLP methodology detected polymorphism more efficiently than RAPD approach due to a greater number of loci assayed per reaction. Cultivar-specific bands were identified, for some cultivars using RAPD, and for most cultivars with AFLP. Genetic similarity matrix, based on Jaccard's index detected coefficients ranging from 0·42 to 0·73 for RAPD, and from 0·48 to 0·925 for AFLPs indicating a wide genetic base. Cluster analyses using data generated by both RAPD and AFLP markers, clearly separated the yellow seeded, self-compatible cultivars from the brown seeded, self-incompatible cultivars although AFLP markers were able to group the cultivars more accurately. The higher genetic variation detected by AFLP in comparison to RAPD was also reflected in the topography of the phenetic dendrograms obtained. These results have been discussed in light of other studies and the relative efficiency of the marker systems for germplasm evaluation.

1. Introduction

Estimates of genetic relatedness are important in designing crop improvement programmes. Information on genetic diversity is also valued for the management of germplasm and for evolving conservation strategies. Molecular markers are the best tools for determining genetic relationships. Different types of marker systems have been used for biodiversity analysis. These include restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), random amplification of polymorphic DNA (RAPD) (Williams *et al* 1990; Karp *et al* 1997) and the recently developed amplified fragment length polymorphism (AFLP) (Vos *et al* 1995). These techniques differ in their principles and generate varying amounts of data. RFLPs are well suited for the construction of linkage maps because of their high specificity (Chyi *et al* 1992) and their codominant nature although they have also been used for the analysis of genetic diversity (Song *et al* 1988a, b). However, RFLP analysis is labour intensive, time consuming and expensive. Later, with the development of polymerase chain reaction (PCR) based RAPD and SSR, most of the problems associated with RFLP were overcome. SSRs, which are based on microsatellite sequences, have been shown to detect very

Keywords. *Brassica*; RAPD; AFLP; germplasm evaluation

Abbreviations used: RFLP, restriction fragment length polymorphism; SSR, simple sequence repeats; RAPD, random amplification of polymorphic DNA; AFLP, amplified fragment length polymorphism; PCR, polymerase chain reaction.

high levels of polymorphism. However, prior information about genome is necessary before SSR markers can be exploited to their fullest potential. On the other hand, the technique of RAPD gained importance due to its simplicity, efficiency, relative ease to perform and non-requirement of sequence information (Karp *et al* 1997). However, RAPD technique has been shown to be non-reproducible as it is highly influenced by experimental conditions (Devos and Gale 1992; Staub *et al* 1996). With the advent of AFLP technique the problems encountered with both RFLP and RAPD have been overcome. AFLP is currently the method of choice for analysis of germplasm, genetic diversity and phylogeny, gene tagging and molecular map construction (Breyne *et al* 1997).

A variety of molecular markers have been used to study the extent of genetic variation among the diverse group of important crops in the genus *Brassica*. Among the different techniques, RFLP was used to examine the taxonomic status of a number of Brassicaceae members based on nuclear and chloroplast data (Song *et al* 1988a, b; Pradhan *et al* 1992; Lanner *et al* 1997). RAPD analysis has also been extensively used to document the genetic variation in *Brassicas* (Demeke *et al* 1992; Jain *et al* 1994; Bhatia *et al* 1995; Thormann *et al* 1994). However, most of the earlier studies were carried out with *B. napus* and *B. juncea* germplasms, and not much information is available on the extent of genetic variation present in *B. campestris* using DNA based marker systems. The present study was undertaken to assess the genetic diversity present within the *B. campestris* cultivars using RAPD and AFLP, and to compare the results obtained with the two marker systems for their relative efficiencies for germplasm analysis.

2. Materials and methods

The plant material used in the present study included *B. campestris* cultivars YsPb, Tobin, Debra, DYS-I, DYS-III, D-3 toria, Y1-D, Pant toria and Pusa kalyani. DNA was isolated from lyophilized leaf material following the modified CTAB method (Doyle and Doyle 1990).

2.1 *RAPD analysis*

The PCR reaction was performed in a final volume of 25 µl containing 1X *Taq* polymerase buffer (Gibco, BRL), 0·5 units of *Taq* polymerase (Gibco, BRL), 200 µM of each dNTPs (Promega), 15 ng of random primer (Operon technologies), 2·5 mM $MgCl_2$ and 30 ng of total genomic DNA. The reaction mixture was overlaid with 25 µl of mineral oil and PCR was performed using the following cycling parameters: 1 cycle of 1 min at 94°C, 30 s at 37°C, 1 min at 72°C; 35 cycles of 5 s at 94°C, 15 s at 37°C, 1 min at 72°C; and at 72°C for 7 min (Yu and Pauls 1992). The PCR products were resolved on 1·5% agarose gels and visualized under UV light after ethidium bromide staining (Sambrook *et al* 1989).

2.2 *AFLP analysis*

AFLP fingerprints were generated based on the protocol of Vos *et al* (1995) with minor modification. Total genomic DNA (300–500 ng) was restricted to completion with *Eco*RI + *Mse*I (1·25 U/µl) enzymes in a total reaction volume of 20 µl at 37°C. The restriction enzymes were heat inactivated at 70°C for 10 min. An aliquot of the restricted DNA samples (10 µl) was ligated to adapter ligation solution containing *Eco*RI and *Mse*I adapters using T4 DNA ligase in a reaction volume of 20 µl, at 20°C, for 4–6 h. The adapter ligated DNA was diluted 10-fold with sterile distilled water and used as a template for pre-amplification. Pre-amplification was carried out with primers complimentary to the *Eco*RI and *Mse*I adapters, with one selective nucleotide at the 3′ end, using the following parameters: 20 cycles of 30 s at 94°C, 60 s at 56°C and 60 s at 72°C. The pre-amplified DNA was diluted in a ratio of 1 : 50 using sterile distilled water and used as template for selective amplification with *Eco*RI and *Mse*I primers, each having three selective nucleotides at their 3′ end. Prior to selective amplification, the *Eco*RI selective primer was end-labelled with [γ-^{32}P]dATP. The cycling parameters were: 1 cycle of 30 s at 94°C, 30 s at 65°C and 60 s at 72°C. For the next 12 cycles, the annealing temperature was lowered by 0.7°C in each cycle, the other parameters remaining the same. The last 23 cycles were performed at 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. After the selective amplification, the reaction was stopped by the addition of equal amount of formamide dye and the products were separated on a 6% polyacrylamide gel followed by autoradiography (Sambrook *et al* 1989).

2.3 *Data analysis*

Bands were scored as 1 for their presence or 0 for their absence across the cultivars for both RAPD and AFLP to generate a binary matrix. A genetic similarity (GS) was computed based on Jaccard's coefficient of similarity [(Jaccard 1908; $GS(ij) = 2a/(2a + b + c)$, where GS (ij) is the measure of genetic similarity between individuals i and j, a is the number of polymorphic bands that are shared by i and j, b is the number of bands present in i and absent in j, and c is the number of bands present in j and absent in i]. The data was subsequently used to construct a dendrogram using the unweighted pair group method of arithmetical averages (UPGMA) algorithm. All the computations were carried out using the NTSYS-pc software (ver. 1·5, Rohlf 1989).

3. Results

3.1 *Genetic variation*

Nine cultivars of *B. campestris* were subjected to RAPD and AFLP analysis. RAPD assay of the total genomic DNA from *B. campestris* was performed using 13 random primers (table 1). The assay revealed a large amount of polymorphism, and the size of amplification products ranged between 300–2000 bp. Figure 1 shows the amplification profile obtained with two primers OPN-01 and OPN-02. The bands were scored as either monomorphic or polymorphic. Primer OPN-01 generated a total of 11 products of which 8 (72·7%) were scored as polymorphic. The monomorphic products were present at 0·6, 1·3 and 1·5 kb. One cultivar-specific band for Pant toria was observed at 0·5 kb (figure 1, marked with arrow). With primer OPN-02, 5 out of 7 bands (71·42%) were polymorphic. Bands specific to YsPb, Tobin and Debra (marked with arrows in the respective lanes) were identified (figure 1). Using 13 decamer primers, a total of 125 bands were scored (an average of 10 bands per primer) and about 80% were found to be polymorphic between the cultivars (table 1). The number of scorable bands ranged between 4 (OPK-03) to 18 (OPF-01). The percentage polymorphism varied from 60% (OPF-06) to 100% (OPM-02). Cultivar-specific bands were generated by a number of primers although their distribution was limited among four cultivars namely, YsPb, Tobin, Debra and Pant toria (table 1).

The genetic diversity among the *B. campestris* cultivars was also assayed with AFLP technique using five *Eco*RI + *Mse*I primer combinations. Amplification products were generated in the size range of 50–350 bp. Figure 2 represents the AFLP fingerprint pattern for the different cultivars of *B. campestris* using primer combination E-ACC + M-CTA and E-ACC + M-CTG. A total of 81 bands were scored with primer combination E-ACC + M-CTA. Of these 76·54% bands were polymorphic (such as those marked as 1,4,8 in figure 2) whereas bands marked as 6,7, and 9 were monomorphic (figure 2). Unique bands specific to a particular cultivar were also obtained, for e.g., bands marked as 2, 3, and 5 were specific to Tobin (figure 2). Such bands could be used for identification of a cultivar. Apart from unique bands, products specific to cultivars with a particular phenotype such as seed coat colour were identified. Bands marked as 1 and 4, were present in all the brown seeded cultivars namely, Tobin, Debra, D-3 toria, Pant toria and Pusa kalyani.

Using primer combination E-ACC + M-CTG (figure 2), a total of 70 bands were scored of which 48 were polymorphic (C and G; figure 2) whereas others were monomorphic (A and B; figure 2). Using this primer combination also, cultivar-specific bands were observed such as those marked as D (for Debra; figure 2), E and F (for Tobin; figure 2). In addition, bands present only in brown seeded cultivars (C and H; figure 2) were also identified.

Using five primer combinations, a total of 319 AFLP bands were scored (an average of 63·8 bands per primer). Of these, 213 bands were polymorphic (an average of 42·6 bands per primer or 66·8%; table 2). The number of bands scored with each primer ranged between 31

Table 1. Analysis of the polymorphism obtained with random primers among various cultivars of *B. campestris*.

Primer	Total number of bands (a)	Number of polymorphic bands (b)	Polymorphism = b/a × 100 (%)	Unique products
OPE-01	11	9	81·8	1 (Tobin, at 0·5 kb)
OPE-04	9	7	77·8	–
OPF-01	18	13	72·2	–
OPF-06	5	3	60·0	–
OPF-07	7	6	85·7	2 (Tobin, at 1·8 kb; Debra, at 1·3 kb)
OPK-01	17	15	88·2	2 (Pant toria, at 0·55 and 1·7 kb)
OPK-02	7	6	85·7	3 (Tobin, at 1·0 kb; Debra, at 0·70 and 1·6 kb)
OPK-03	4	3	75·0	–
OPK-04	11	10	90·9	1 (YsPb, at 0·55 kb)
OPM-01	13	9	69·2	1 (YsPb, at 1·4 kb)
OPM-02	5	5	100·0	–
OPN-01	11	8	72·7	1 (Pant toria, at 0·5 kb)
OPN-02	7	5	71·4	3 (YsPb, at 0·6 kb; Tobin, at 1·0 kb; Debra, at 0·8 kb)
Total	125	99	79·2 (average)	14

(E-ACC + M-CAG) to 81 (E-ACC + M-CTA). The per cent polymorphism ranged between 58·7% with primer E-ACC + M-CAT to 76·54% with E-ACC + M-CTA (table 2). Of the 213 polymorphic bands scored, 57 were specific to individual cultivars. All the five primer combinations generated cultivar-specific product which ranged between 6 bands for E-ACC + M-CAG to 17 bands for E-ACC + M-CTG (table 2). Specific amplification profiles distinguishing most cultivars except Y1D and Pant toria were obtained (table 2). Apart from cultivar-specific products, a number of products that could distinguish between yellow seeded and brown seeded cultivars were also observed. Sixteen products specific for brown seeded cultivars and 4 products specific to yellow seeded cultivars were scored.

3.2 *Genetic similarity matrix and cluster analyses*

The bands obtained with both RAPD and AFLP analyses were used to compute the Jaccard's similarity coefficient using the NTSYS-pc programme (Rohlf 1989). For RAPD, the data set with 125 bands and for AFLP, the data set with 319 bands were employed.

With RAPD data matrix, the highest genetic similarity value of 0·728 was obtained between DYS-I and DYS-III. The lowest value obtained was 0·419, between YsPb and Pant toria. With AFLP data matrix, the genetic similarity values ranged from 0·925 between DYS-I and DYS-III, to 0·476 between YsPb and Tobin. The genetic similarity matrices thus obtained with the two different data sets, RAPD and AFLP, were used to cluster the cultivars using the un-weighted pair group method of arithmetic averages (UPGMA) algorithm (NTSYS-pc, Rohlf 1989). The resultant dendrograms are shown in figure 3.

The cultivars were grouped into clusters A and B with both RAPD and AFLP data (figure 3). Cluster A consisted of YsPb, DYS-I, DYS-III and Y1D, all yellow seeded, self-compatible and within this cluster, DYS-I and DYS-III were grouped together with both RAPD and AFLP data (figure 3) at a similarity value of 0·728 (RAPD) and 0·925 (AFLP), respectively. Cluster B comprised of cultivars Tobin, Pusa kalyani, D-3 toria, Debra and Pant toria, all brown seeded and self-incompatible cultivars. However, the arrangement of these cultivars within the cluster was different for RAPD and AFLP data sets. The RAPD data showed close linkage between Tobin and Pusa kalyani, and between Debra and Pant toria. On the other hand, the AFLP data clustered all the Indian brown seeded cultivars namely D-3 toria, Pant toria and Pusa kalyani together whereas the exotic Tobin and Debra remained distinct.

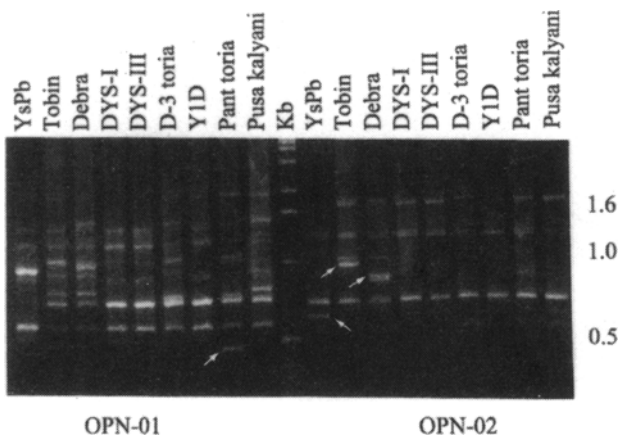

Figure 1. RAPD profile of the nine *B. campestris* cultivars obtained with random primers OPN-01 and OPN-02. Unique or cultivar-specific bands are indicated with arrows. Lane marked as 'Kb' displays 1 kbp ladder as DNA molecular size marker.

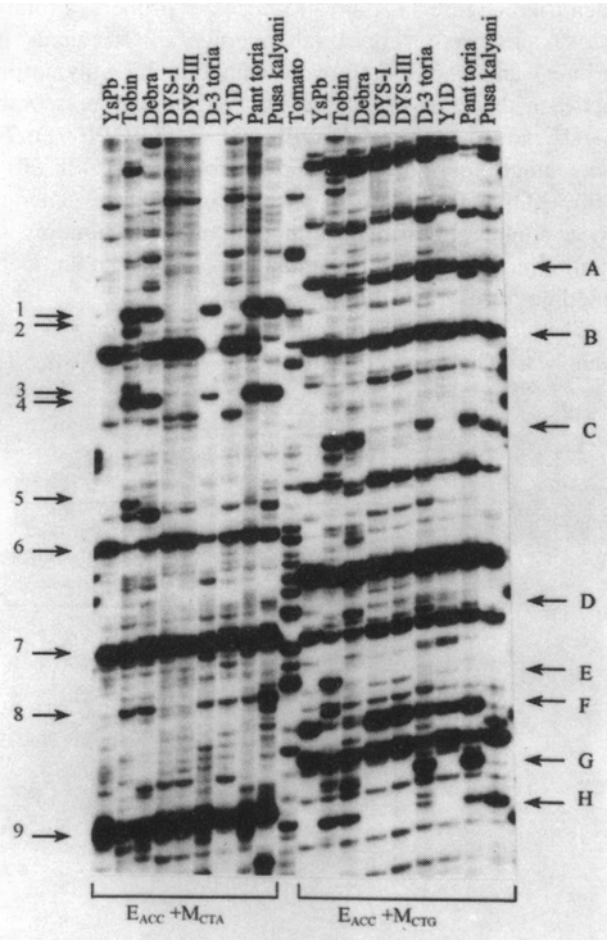

Figure 2. AFLP between nine *B. campestris* cultivars amplified with primer combinations E-ACC + M-CTA and E-ACC + M-CTG. Some of the monomorphic, polymorphic and unique bands are indicated with arrows and have been assigned either numbers (in E-ACC +M-CTA) or alphabets (in E-ACC + M-CTG). Tomato DNA was used as control.

The minimum number of amplification products required for accurate assessment of genetic relationship was determined by calculating the Jaccard's similarity value and clustering of cultivars using subsets of both the RAPD and AFLP data matrix. The RAPD data was split into matrices consisting of 33, 67 and 96 products and analysed (data not shown). The lowest and highest genetic similarity values obtained with the different subsets of RAPD data matrix remained near-identical to the values obtained with the complete matrix. However, the cultivars linked by lowest values were different in each case whereas the highest similarity value were obtained between DYS-I and DYS-III with all the data matrices. The dendrogram based on 33 RAPD products arranged the cultivars in an entirely different manner. The clustering obtained with 67, 96 and 125 products were near identical with minor changes.

The AFLP data matrix of 319 products was split into data matrices consisting of 63 and 137 products, equivalent to data generated with one, and two primers respectively. The genetic similarity values obtained with 63 AFLP products and 137 AFLP products were close

Table 2. Analysis of the polymorphism obtained with AFLP primer combinations among various cultivars of *B. campestris*.

Primer	Total number of bands (a)	Number of polymorphic bands (b)	Polymorphism = b/a × 100 (%)	Unique products
E-ACC + M-CAT	63	37	58·7	6 for Tobin; 2 for Pusa kalyani; 1 each for YsPb and DYS-III
E-ACC + M-CTC	75	47	62·7	8 for Tobin; 2 each for Debra and D-3 toria; 1 for Pusa kalyani
E-ACC + M-CAG	31	19	61·3	4 for Tobin; 2 for Debra
E-ACC + M-CTA	81	62	76·5	5 for Pusa kalyani; 4 for Tobin; 1 each for YsPb and Debra
E-ACC + M-CTG	70	48	68·5	10 for Tobin; 3 for Debra; 1 each for DYS-1, DYS-III, D-3 toria and Pusa kalyani
Total	319	213	66·8 (average)	57

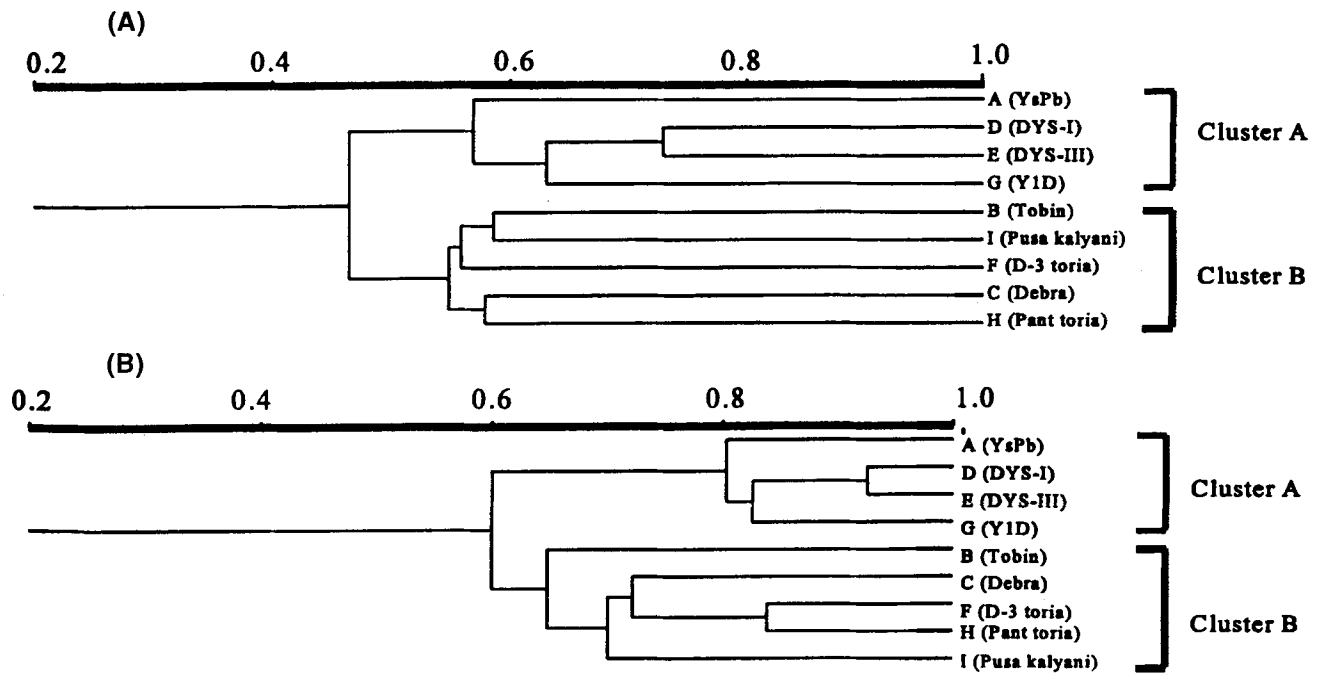

Figure 3. Phenetic dendrograms generated by UPGMA analysis based on RAPD (**A**) and AFLP (**B**) data showing the relationships among different *B. campestris* cultivars.

to values obtained with the 319 AFLP data set (0·476 to 0·925). The highest similarity values were always obtained between DYS-I and DYS-III. The two dendrograms with 63 and 137 products were identical and grouped the cultivars in similar fashion (data not shown). The clustering and arrangement of cultivars was similar to those obtained with 319 AFLP products. Thus about 60–70 RAPD or AFLP products are sufficient for assessment of genetic similarity and clustering of *B. campestris* cultivars.

4. Discussion

In the present study we undertook a comparative analysis of the RAPD and AFLP marker technologies for diversity analysis. RAPD markers have been earlier used to study taxonomic relationships (Demeke *et al* 1992) and shown to detect higher polymorphism than RFLP markers (Thormann *et al* 1994). In this study, AFLP generated an average of 63·8 bands per reaction as compared to only 9·6 with each RAPD reaction. However, the level of polymorphism was higher with RAPD (80·0%) than with AFLP (66·8%). Although AFLPs do not reveal high percentage of polymorphism but are more efficient as they produce more number of bands per reaction as compared to RAPD. Thus, AFLP has a higher marker index, an overall measure of marker efficiency (Powell *et al* 1996; Nakajima *et al* 1998). AFLP has also been reported to be highly reproducible with low error rates (Jones *et al* 1997), which provides a definite advantage over RAPD. In our study, both RAPD and AFLP generated unique or cultivar-specific bands. With RAPD, cultivar-specific bands for YsPb, Tobin, Debra and Pant toria were obtained. In comparison, AFLP could generate unique bands for most of the cultivars except Y1D and Pant toria, and could easily distinguish between such closely related cultivars as DYS-I and DYS-III. These results are in agreement with the studies carried out in *Vitis vinifera* which allowed for the identification of species-specific bands (Cervera *et al* 1998). A valid reason for AFLP being able to generate higher number of cultivar-specific bands and group-specific bands is its wide genome coverage. It has been shown in rice and in *A. thaliana* that the AFLP markers were not confined to particular regions of the chromosome but were widely distributed (Alonso-Blanco *et al* 1998; Zhu *et al* 1998).

The cluster analyses of RAPD and AFLP data revealed nearly similar groupings of the *B. campestris* cultivars. It is known that *B. campestris* ssp. *oleifera* consists of three ecotypes i.e., brown sarson, yellow sarson and toria. Both the marker systems clearly grouped the self compatible yellow sarson as one cluster (A). Whereas cluster B in both cases consisted of toria and brown sarson. This was expected since toria is known to closely resemble brown sarson. It has been reported by Hinata and Prakash (1984) that toria evolved as a mutant of brown sarson. Within cluster B the groupings based on AFLP were in confirmation with the evolutionary data. With AFLP the two toria ecotypes showed highest similarity whereas with RAPD, these were grouped separately. Moreover, with AFLP, the Indian varieties (D-3 toria, Pant toria and Pusa kalyani) formed part of the same cluster, which clustered separately from the exotic Tobin. On the other hand, the RAPD clustering not only separated the two toria ecotypes, but also showed close linkage between the Indian and exotic types which was in contradiction to the evolutionary data. Therefore, analysis based on AFLP was found to be more reliable. Clustering of cultivars based on various attributes have been reported earlier. Grouping has been observed based on crossability and breeding habits in tomato and coconut (Miller and Tanksley 1990; Perera *et al* 1998), seed size, as in *Lens*-var. microsperma and macrosperma (Sharma *et al* 1996), and geographical distribution, as in *Camellia sinensis* (Paul *et al* 1997).

Several previous studies have compared the relative advantages of using RAPD and RFLP to examine genetic relationships (Hallden *et al* 1994; Thormann *et al* 1994). In this study, AFLP detected a higher genetic similarity value, which is due to higher band sharing displayed by AFLP. The level of band sharing as displayed by AFLP renders it useful to assess both genetic similarity and variation at inter- as well as intra-specific level. Cluster analysis in *B. campestris* grouped the cultivars into two clusters A and B. Cultivars of cluster A were self-compatible and displayed higher band sharing and thus higher genetic similarity values. Members of cluster B are self-incompatible and displayed lower band sharing or lower genetic similarity values. The two clusters shared only 42·5% (RAPD) to 48·5% (AFLP) genetic similarity indicating a wide genetic base. It has been observed that hop cultivars had genetic similarity values ranging between 89 to 100% thus indicating a narrow genetic base (Hartl and Seefelder 1998) which is not the case in *B. campestris*. Cultivars belonging to cluster B displayed/exhibited lower genetic similarity values which correlates well with their outbreeding nature and their place of origin. Among the five cultivars (cluster B), Tobin and Debra are exotics obtained originally from Canada, whereas the remaining three are Indian types. Therefore, the wide genetic base observed is on account of the wide range of geographical distribution. Outbreeding nature which has evolved as preventive mechanism for selfing increases genetic exchange and diversity. Similar correlation between outbreeding nature and higher genetic diversity has also been observed in *Cocos nucifera* L. (Pererra *et al* 1998). It was observed that tall, outbreeding coconut varieties exhibited higher genetic diversity in comparison to dwarf, inbreeding varieties (Pererra *et al* 1998). Similar observations has been made in *Camellia sinensis* (Paul *et al* 1997) and *Azadirachta indica* (Singh *et al* 1999) which are outbreeding/outcrossing in nature.

Such information can be very useful in plant system where *a priori* knowledge on breeding habits are not available.

It was found that at least 60–70 products, derived from either RAPD or AFLP were required to achieve a reliable clustering of the *B. campestris* cultivars. This minimum number of products could be obtained by using 6–8 RAPD primers or from a single AFLP primer combination. It has been demonstrated that the data generated by one to two AFLP primer combinations was sufficient to obtain consistent grouping of the rice germplasm and to identify barley lines (Schut *et al* 1997; Zhu *et al* 1998). Thus very few experiments were required with AFLP markers for a reliable estimation of diversity in the germplasm. Even with RAPD it has been shown that the genetic relationship among *B. oleracea* cultivars varied minimally after 50–60 products were analysed and for better resolution of genetic relationship, analysis of 60–75 RAPD products were sufficient (Kresovich *et al* 1992).

Acknowledgements

This work was supported by financial assistance from the Indo-French Centre for the Promotion of Advanced Research (IFCPAR–CEFIPERA). We would like to thank Dr R K Pachauri (TERI), for his encouragement at all stages of this work. We also take the opportunity to thank Dr Shyam Prakash, Indian Agricultural Research Institute, New Delhi for providing the germplasm. The Senior Research fellowship from the Council of Scientific and Industrial Research, New Delhi to SD is gratefully acknowledged.

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MS received 15 May 1999; accepted 23 July 1999

Corresponding editor: RAKESH TULI