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Potential taxonomic use of random amplified polymorphic DNA (RAPD): a case study in *Brassica*

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Summary. The potential use of RAPDs for taxonomic studies were investigated using *Brassica*, *Sinapis* and *Raphanus* taxa. Principal coordinate analysis of 284 RAPD bands revealed the classical U triangle relationship between diploid and amphidiploid *Brassica* taxa. *Raphanus sativus* and *S. alba* were distinct from the *Brassica* taxa. It appears that at least ten primers with approximately 100 total bands are needed to adequately portray these relationships. Cultivars of cabbage and cauliflower were separated by RAPDs. Analysis of RAPDs from individual plants of *B. carinata* cv. *dodola* resulted in 69 RAPDs, with 91.7% monomorphic and 8.3% polymorphic bands. RAPDs appear to be useful for taxonomic studies at levels ranging from populations to species and perhaps genera.

Key words: RAPDs – PCR – Primers – *Brassica* – Taxonomy

Introduction

The genomic relationships of *Brassica* and related taxa have been extensively studied. The different techniques used include restriction fragment length polymorphism (RFLP) (Hosaka et al. 1990; Song et al. 1990), chloroplast DNA analysis (Palmer et al. 1983), taxonomy and cytogenetics (Prakash and Hinata 1980; Vaughan 1977), isozymes and γDNA genes (Quiros et al. 1987). These studies have established the relationships of diploid and amphidiploid *Brassica* taxa and support a classical U triangle (see Fig. 1 A) relationship (U 1935). Recently, DNA polymorphisms have been detected by RAPDs (Welsh and McClelland 1990; Williams et al. 1990; Caetano-Anolles et al. 1991; Hu and Quiros 1991; Klein-Lankhorst et al. 1991; Martin et al. 1991; Michelmore et al. 1991; Rafalski et al. 1991; Carlson et al. 1991). RAPDS are generated by the amplification of genomic DNA with a single primer of arbitrary nucleotide sequence. The polymorphisms generated function as genetic markers and can be used to construct genetic maps (Williams et al. 1990). Quiros et al. (1991) have identified genome-specific markers in *Brassica* using RAPDs.

This report investigates the potential taxonomic use of RAPDs with *Brassica*. Because *Brassica* relationships have been well documented, comparisons can be readily made between the classical classification and that produced using RAPDs. We have also included in this study two more distantly related taxa (*Raphanus* and *Sinapis*), as well as analyses of cabbage and cauliflower cultivars and individual plants of the same species.

Materials and methods

DNA was extracted from seedlings of the plant materials shown in Table 1. Leaves from 4–6 plants per taxon were used for DNA extraction by the CTAB protocol (Doyle and Doyle 1987). The polymerase chain reaction (PCR) was performed in a volume of 25 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36 µM primer, 3.0 ng genomic DNA, and 1.0 unit of *Taq* DNA polymerase (Promega). A control PCR tube containing all components, but no genomic DNA, was run with each primer to check for contamination. Two hundred random 10-mer primers were obtained from Dr. John Carlson (University of British Columbia). One hundred and forty seven of these were screened using spinach DNA. Primers that gave several bright bands and did not have any false bands (in the controls) were then considered for further use. Thus, 19 10-mer primers were selected for various analyses (Table 2). In addition, six 9-mer primers that had proved useful in preliminary studies of other species were synthesized for use in this study (Table 2).

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Table 1. Sources of plant materials used for DNA extraction

B. napus	Jet neuf	Ag. Canada, Saskatoon
B. carinata	Dodola	Ethiopia
B. campestris	Parkland	Ag. Canada, Saskatoon
B. juncea	Mustard green	Locally grown
B. nigra	Black mustard (Type 2)	Ag. Canada, Saskatoon
S. alba	White mustard (Ochre)	Ag. Canada, Saskatoon
R. sativus	Radish (Rauola R-1368)	Ag. Canada, Saskatoon
B. oleracea	Cauliflower (Early snowball)	McKenzie Co.
	Broccoli (Green sprouting)	McKenzie Co.
	Cabbage	
	(Early copenhagen)	McKenzie Co.
	(Grand prize)	Peto Co.
	(Discovery hybrid)	Peto Co.
	(Regalia hybrid)	Stokes Co.
	(Red rookie hybrid)	Midwest Seed Co.
	(Grenadier hybrid)	Northrup King Co.
	Cauliflower	
	(Montano hybrid)	Northrup King Co.
	(Snowball impatiens)	Harris Moran Co.
	(Snowball 123)	Harris Moran Co.
	(Crystal)	Peto Co.

Table 2. List of primers used in this study

Code[a]	Sequence (5′–3′)	Code	Sequence (5′–3′)
9-mers:			
RC2[b,d]	TAC ACC CGT	RC3[b,d]	CAG TCG CTT
RC5[b,d]	CTC AGT CAC	RC13[b,d]	CCA AGC AGT
RC16[b]	GAG CGT TGT	RC20[b,d]	ACC CGG ACA
10-mers:			
RC42[b,d]	GCA AGT AGC T	116[b,c,d]	TAC GAT GAC G
122[b]	GTA GAC GAG C	125[b]	GCG GTT GAG G
143[d]	TCG CAG AAC G	147[b]	GTG CGT CCT C
150[b,c]	GAA GGC TCT G	153[b,c,d]	GAG TCA CGA G
184[b,c,d]	CAA ACG GCA C	204[d]	TTC GGG CCG T
212[b,c,d]	GCT GCG TGA C	218[c,d]	CTC AGC CCA G
220[c]	GTC GAT GTC G	223[b,c]	GAT CCA TTG C
227[b,c]	CTA GAG GTC C	232[c]	CGG TGA CAT C
234[c]	TCC ACG GAC G	237[c]	CGA CCA GAG C
244[c]	CAG CCA ACC G		

[a] The RC group primers were synthesized at the Plant Biotechnology Institute, NRC, and the numbered primers (116–244) were purchased from the University of British Colombia

[b] Primers used in the study of *Brassica* taxa plus *R. sativus* and *S. alba*

[c] Primers used in the study of cabbage and cauliflower cultivars

[d] Primers used in the study of variants among individuals of *B. carinata* cv. *dodola*

DNA amplification was performed in an MJ Programmable Thermal Cycler (MJ Research, Inc.). The thermal cycle used was: 94 °C (1.5 min) for initial strand separation, then 40 cycles of 37 °C (2 min) 72 °C (2 min), 94 °C (1 min). Two additional steps were used, 37 °C (2 min) and 72 °C (5 min), for final extension. Amplification products were analyzed by electrophoresis in 1.5% agarose gels and detected by staining with ethidium bromide. The gels were photographed under UV light with Polaroid film 667. pGEM DNA (Promega) was used as a marker. The size of the bands and their intensity was scored as: 0 = no band; 1 = very, very faint; 2 = very faint; 3 = faint; 4 = medium; 5 = bright; 6 = very bright band. In addition, analysis using presence/absence (0 = no band; 1 = band present) was tried but found to be less informative than using the character states. Similarity measures were computed using absolute character state differences (Manhattan metric) divided by the maximum value for that character over all taxa (= Gower metric; Gower 1971; Adams 1975). Division by the observed character state range was tried and found to be less informative than using the maximum character value (i.e., including zero in the range). Principal coordinate analysis (PCO) follows Gower (1966).

Results and discussion

PCR amplification of genomic DNA from eight *Brassica* taxa, plus *Raphanus sativus* and *Sinapis alba* yielded a total of 284 RAPDs, ranging in size from 190 to 2,600 bp. PCO of the similarity matrix of eight *Brassica* taxa plus *R. sativus* and *S. alba* gave seven eigenroots that accounted for 91.37% of the variance among the ten OTUs. Eigenroots 5, 6, and 7 appeared to asymptote in value and were not further examined. Thus, the first four eigenroots contained 71.59% of the variance among the ten taxa. These eigenroots contained 26.88, 16.88, 15.63, and 12.20%, respectively, of the variance.

A plot of the ten taxa onto the first three principal coordinates is shown in Fig. 1 B, with a minimum spanning network superimposed. *R. sativus* and *S. alba* are well separated from *Brassica*. *R. sativus* is added to the *Brassica* group via *B. juncea* at a similarity of 0.62, whereas *S. alba* is added to the rest of the group via *R. sativus* at a similarity of 0.60. Although a close relationship is indicated, it would be difficult to support the thesis that *Raphanus* and *Sinapis* should be merged with *Brassica* as suggested by Palmer et al. (1983), who grouped *R. sativus* and *S. alba* with *Brassica* based on chloroplast DNA relationships.

The diploid and amphidiploid *Brassica* taxa show a U triangle relationship (Figs. 1, 2). Even though the amphidiploids are not exactly at the center of the side of the triangle, their positions are generally intermediate to the putative parents. Hosaka et al. (1990) showed the presence of shared fragments between A, B, and C genomes which is indicative of the partial homology of the three genomes. A close relationship among the three genomes has also been proposed based on the number of allosyndetic pairs (Mizushima 1950). The RAPDs exhibited several bands that are shared among the C (*B. oleracea*)-containing genotypes (AC, BC, and C), and a few bands were shared among A, AB, AC, B, BC, and C genotypes. However, the B (*B. nigra*) genotype shared only a limited number of bands with the other taxa, showing its more distant relationship to other *Brassica* species. Hosaka et al. (1990) and Quiros et al. (1991) have reported that the B genotype (*B. nigra*) is less related to the A [*B.*

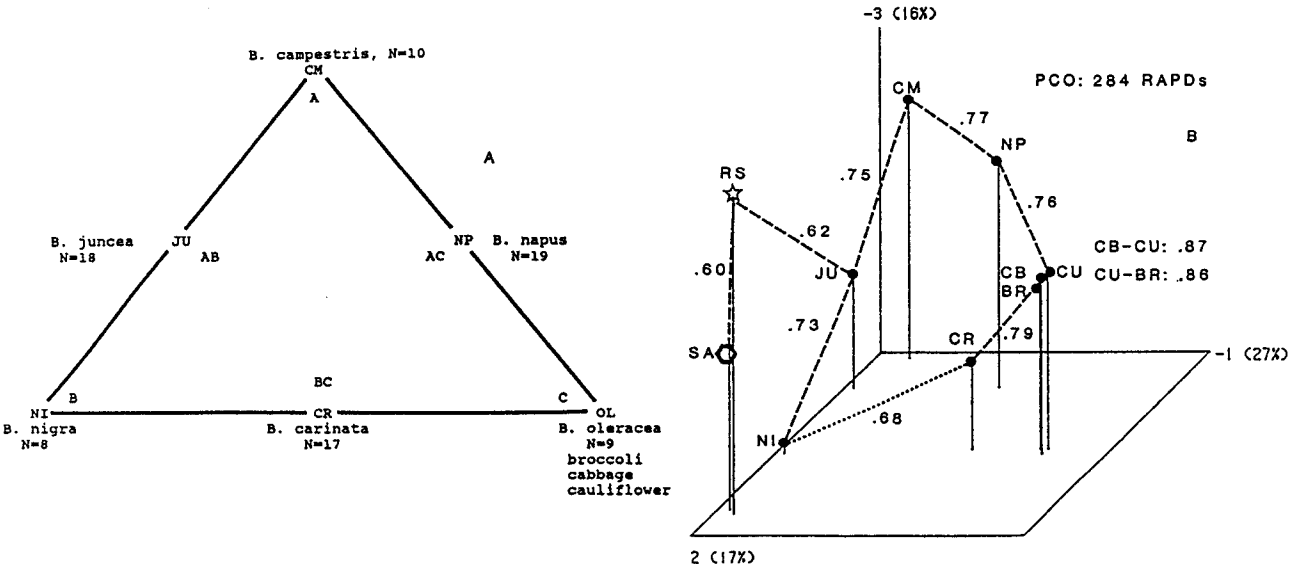


Fig. 1. **A** A classical U triangle depicting the phylogeny of diploid and amphidiploid *Brassica* taxa based on cytogenetic analysis (U 1935). **B** Principal coordinate analysis of the similarity matrix of eight *Brassica* taxa plus *R. sativus* and *S. alba*, where: *SA* = *S. alba*; *RS* = *R. sativus*; *NI* = *B. nigra*; *JU* = *B. juncea*; *CM* = *B. campestris*; *CR* = *B. carinata*; *NP* = *B. napus*; and *B. oleracea* (*CU* = cauliflower; *BR* = broccoli; *CA* = cabbage). *Dashed lines* are the minimum spanning network (with similarities noted); the *dotted line* shows the connecting link between NI and CR

campestris (= *B. rapa*)] and C (*b. oleracea*) genotypes. This is in agreement with the present RAPD data. The sharing of some RAPD bands among the A, B and C genomes (e.g., primers 125, 153, 184, 212, and 227) indicates the partial homology of the three genomes.

The fourth principal coordinate (12.2%) largely discriminated between *S. alba* and *R. sativus* and will not be discussed further.

The effects of using only 9-mers, only 10-mers, and using all 9- and 10-mer data in combination, for the computation of similarities and subsequent PCO are shown in Fig. 2. PCR using the six 9-mer primers on genomic DNA from ten taxa gave a total of 93 bands. Using these RAPDs does not give a clear representation of the taxa (Fig. 2A). *Brassica carinata* (CR) is only partially intermediate between *B. nigra* (NI) and *B. oleracea* (CB, CU, BR) and is only slightly more similar to *B. nigra* (NI, 0.68) than to *S. alba* (SA, 0.65). In addition, *B. juncea* (JU) has a slightly higher similarity to *B. carinata* (CR, 0.674) than to its putative parent *B. campestris* (CM, 0.673). *R. sativus* (RS) is placed in the middle of the *Brassica* triangle and the classical U triangle is quite distorted (Fig. 2A).

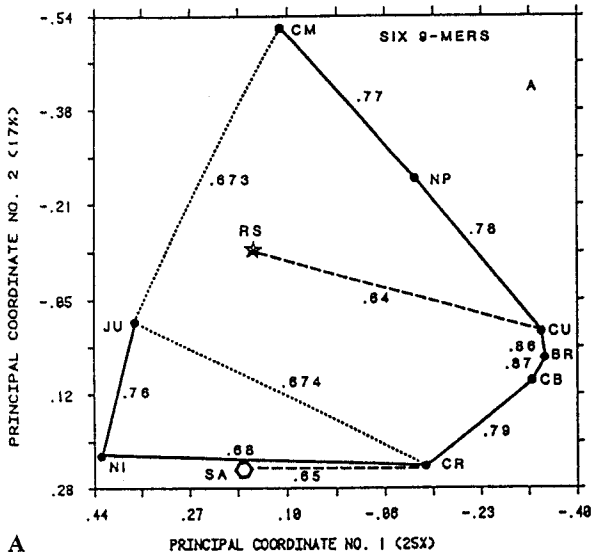
PCR using the eleven 10-mer primers gave a total of 191 bands among the ten taxa. PCO of the resulting similarity matrix clearly separates *S. alba* (SA) and *R. sativus* (RS) from *Brassica* and the U triangle is obvious (Fig. 2B).

Computing similarities using all 284 RAPDs from the 9- and 10-mers resulted in a very good U triangle in addition to separating *S. alba* (SA) and *R. sativus* (RS) (Fig. 2C). Note also that *B. juncea* (JU) is much more intermediate between *B. campestris* (CM) and *B. nigra* (NI) (Fig. 2C) than when only the 191 RAPDs were used (Fig. 2B).

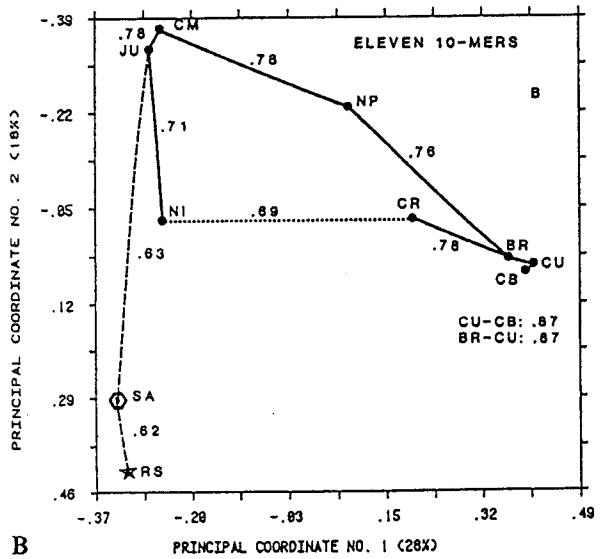
The poorer resolving power of the 9-mers appears to be due to the lack of data (fewer total RAPDs due to using only six primers), rather than any intrinsic characteristic of 9-mer primers. Thus, it appears, in this data set, that the classification stabilized with 11 primers (191 RAPDs) and only small changes in the species relationships were obtained by utilizing additional RAPDs (17 primers, 284 RAPDs).

A total of 122 RAPDs were obtained from five cabbage and four cauliflower cultivars using 13 primers (Table 2). PCO of the similarity matrix gave four eigenroots before the roots began to asymptote in value. The first four eigenroots removed 64.33, 12.92, 8.55, and 5.22% of the variance among the nine OTUs (total of 91.02%). The primary trend in the first three coordinates (85.5% of the variance) is to separate the cabbage from the cauliflower cultivars (Fig. 3). The cauliflower cultivars are tightly clustered together, whereas the cabbage cultivars are somewhat separated (Fig. 3). It appears that the cabbage cultivars are more genetically diverse than the cauliflower cultivars. The fourth eigenvector (5.22%) primarily separated the two cabbage cultivars, Grand prize (GP) and Grenadier (GR). Hu and Quiros (1991) also found RAPDs to be useful for the analysis of cultivars of broccoli and cauliflower.

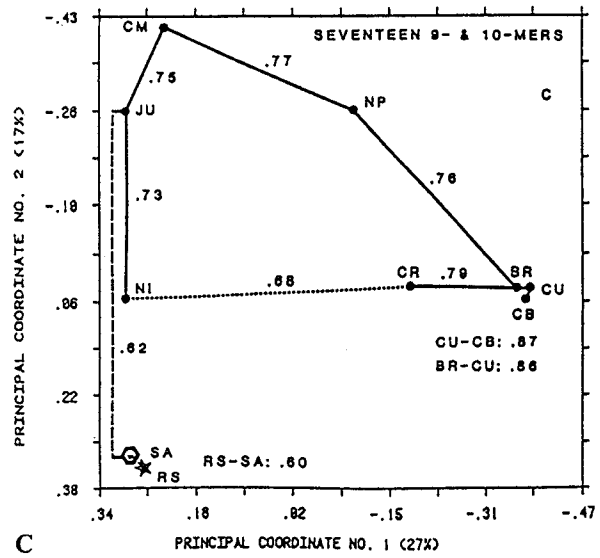
The separation of cabbage and cauliflower cultivars and the similarities within the varieties is further shown



A



B



C

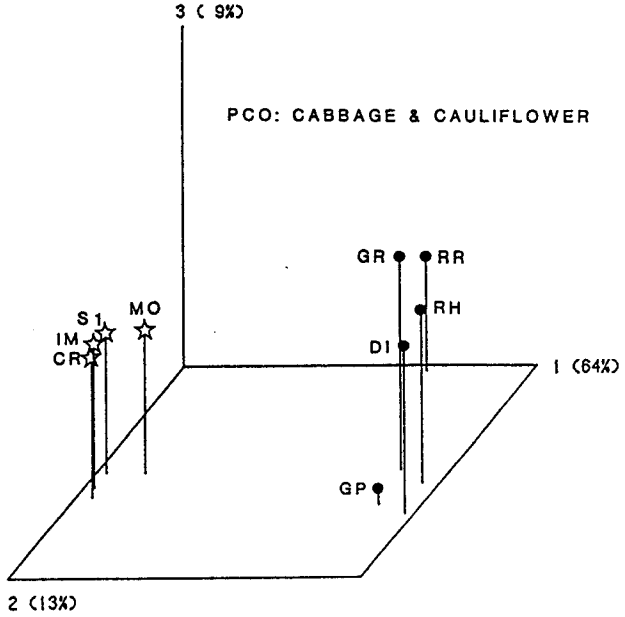


Fig. 3. Principal coordinate analysis of five cabbage (*solid circles*) and four cauliflower (*open stars*) cultivars (122 RAPD bands). Cabbage cultivars are: *GR*=Grand prize; *RR*=Red rookie; *RH*=Regalia hybrid; *DI*=Discovery hybrid; *GR*=Grenadier; and Cauliflower cultivars are: *MO*=Montano hybrid; *SI*=Snowball 123; *IM*=Snowball impatiens; *CR*=Crystal. Notice cabbage and cauliflower are well separated on the first principal coordinate axis. The cabbage cultivars show more diversity than the cauliflower cultivars

in Fig. 4. For this primer (#116) there are five bands that distinguish the cabbage and cauliflower cultivars. For many of the other primers most of the bands are in common for cabbage and cauliflower cultivars, indicating their close relationship. Thus, it appears that RAPDs will be useful for separating cultivars, if one runs enough RAPDs.

Fig. 2A–C. Principal coordinate analysis of the similarity matrix of eight *Brassica* taxa plus *R. sativus* and *S. alba*. In each case, the OTUs are plotted onto the first two principal coordinates and the *solid lines* are the minimum spanning network for *Brassica* taxa. The *dashed lines* indicate the similarities of SA and RS to *Brassica* taxa. A Ordination using only the six 9-mer primers (93 RAPD bands). The *dotted lines* indicate the similarities to the next nearest neighbors to JU (CR and CM). B Ordination using the eleven 10-mer primers only (191 RAPD bands). The *dotted line* indicates the connection to the second (next) nearest neighbor of NI (i.e., CR) and this completes a crude triangle between NI, CU, and CM but notice that JU is not intermediate between CM and NI as in the classical U triangle (Fig. 1a, b). C Ordination combining the six 9-mer primers and the 11 10-mer primers (=17 primers, 284 RAPD bands). The U triangle is now well defined and the amphitetraploids (JU, NP, CR) are more intermediate to the putative parents

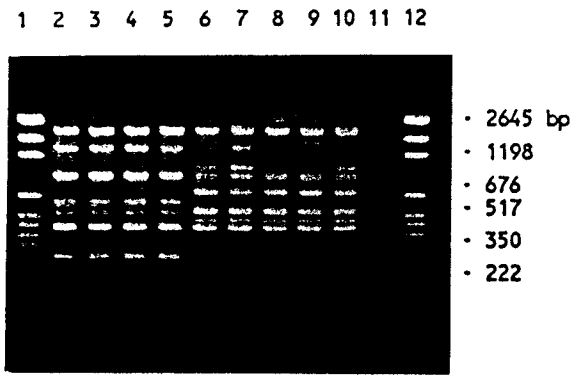


Fig. 4. Gel-electrophoresis of cabbage and cauliflower RAPD DNA bands produced by primer 116. Where: *lane 1 = pGEM* DNA marker; *2* = Crystal peto; *3* = Snowball 123; *4* = Snowball impatiens; *5* = Montano hybrid; *6* = Grenadier; *7* = Red rookie; *8* = Regalia hybrid; *9* = Discovery hybrid; *10* = Grand prize; *11* = Negative control; *12* = pGEM DNA marker

In order to examine variation in RAPDs among individuals, DNA was extracted from six individual seedlings of *B. carinata* cv. *dodola*. A total of 69 RAPDs were produced from 13 primers (data not shown). Sixty three (91.7%) were monomorphic, whereas six (8.7%) were polymorphic. These polymorphic bands could in future be used as genetic markers (Quiros et al. 1991; Williams et al. 1990).

In summary, we have found that RAPDs can be used for classification at taxonomic levels ranging from individuals, to cultivars, and species. Due to the speed and low cost of producing RAPDs, they will surely be widely used for taxonomy in the future.

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