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Discrimination of Diploid Crucifer Species Using PCR-RFLP of Chloroplast DNA

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Abstract. **Chloroplast DNA (cpDNA) was used to identify polymorphisms between crucifer species using the polymerase chain reaction-random fragment-length polymorphism (PCR-RFLP) technique. Ten primer pairs based on cpDNA gene sequences were used to amplify cpDNA fragments in *Brassica oleracea* L., *B. rapa* L., *B. nigra* (L.) Koch, *B. napus* L., *B. carinata* Braun, *B. juncea* (L.) Czern, and *Raphanus sativus* L. accessions. Amplified DNA sequences were then digested using 11 restriction enzymes to identify polymorphisms between the 7 species. Of the 110 combinations, 38 generated polymorphisms that discriminated one or more of the species. Genotyping of these polymorphisms in 10 accessions of each of the diploid species (*B. oleracea*, *B. nigra*, *B. rapa* and *R. sativus*) did not reveal segregating polymorphisms among accessions within species, indicating that they can be used to help determine species identity. Ten accessions of each of the amphidiploids *B. napus*, *B. carinata* and *B. juncea* were genotyped to infer their maternal ancestry. The diploid source of cpDNA in *B. carinata* was *B. nigra* in all accessions tested and *B. rapa* for nine of ten *B. juncea* accessions tested. Two *B. napus* accessions amplified polymorphisms shared with *B. rapa*, and eight accessions produced unique polymorphisms from neither *B. rapa, B. oleracea* or *B. nigra*. The polymorphisms identified in this study can be used to help confirm identity of the diploid crucifer species for taxonomic and conservation studies.**

The *Brassica* triangle or Triangle of U (U, 1935) consists of six cultivated crop species. Three of these species are diploid [*B. oleracea* (CC, 2n = 18), *B. nigra* (BB, 2n = 16) and *B. rapa* (AA, 2n = 20)] which through hybridization events have given rise to three amphidiploid species [*B. carinata* (BBCC, 2n = 34), *B. juncea* (AABB, 2n = 36) and *B. napus* (AACC, 2n = 38)]. The cultivated crop species *R. sativus* is closely related to the six *Brassica* species.

Crucifer species have a common evolutionary ancestry and it is possible to generate interspecific crosses among the six *Brassica* species, *Raphanus sativus* and other crucifer species with the aid of techniques such as embryo rescue and protoplast fusion (Ayotte et al., 1987; Chiang et al., 1977; Hansen and Earle, 1995; Metz et al., 1995). Accessions of these species can be difficult to discriminate morphologically and are frequently misidentified in germplasm collections (Guo et al., 1991, Taylor et al., 2002).

Comparison of nuclear DNA among *Brassica* species can help in determining species and genetic similarities among the species; however, polymorphisms identified typically segregate within the species. Chloroplast DNA (cpDNA) is highly conserved throughout the plant kingdom, as changes in the structure and sequence of cpDNA are infrequent in the origin and evolution of crop plants (Palmer and Stein, 1986). Primers based on cpDNA sequences can be used to amplify DNA in many plant species. The infrequency of base changes can be used to identify differences between species, while minimizing segregation within species.

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Evaluation of chloroplast genes can enable not only the discrimination of crop species type, but also the direction of the cross of the ancestral hybrid. The aim of this study was to use cpDNA sequences to discriminate the diploid *Brassica* species within the *Brassica* triangle and *R. sativus*, and determine the maternal ancestry of amphidiploid *Brassica* species.

Materials and Methods

Plant material. Ten accessions of each of seven cultivated *Brassica* species (*B. oleracea*, *B. rapa*, *B. nigra*, *B. napus*, *B. carinata*, *B. juncea* and *R. sativus*) were used to discriminate polymorphisms and passport information from respective sources were used to identify species of the accessions (Table 1). All seeds were planted in Cornell Mix (Boodley and Sheldrake, 1972) in 32-cell (125-cm^3) Styrofoam trays (Speedling, Sun City, Fla.) with 1 seed/cell, and 10 seeds/accession.

DNA extraction. Plants were grown to the second true leaf stage and 1 cm^2 of leaf tissue from each of the ten plants for each accession was harvested for extraction of DNA. Total genomic DNA was extracted with a standard procedure (Doyle and Doyle, 1990) and stored in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) at –20 °C. DNA was quantified with a spectrophotometer (ThermoSpectronic; BioMate, Pittsford, N.Y.) and final concentration was adjusted to 40 ng·μL^{-1}.

Amplification of cpDNA and restriction digest. Thirteen primer pairs were used in the present study. Twelve primer pairs were based on chloroplast DNA sequences from previous studies, and one primer pair (*BnpsbA* F-*BnpsbA* R) was developed based on available cpDNA sequence information from *B. napus* (Table 2). All primers were synthesized by Genosys Biotechnologies (The Woodlands, Texas). Optimum annealing temperatures were established for each primer pair and 10 of these primer pairs were used to amplify cpDNA from 70 accessions. DNA was amplified in a gradient thermal-cycler (Brinkman Inc., Westbury, N.Y.) with 0.2 μM of each of the primer pairs, in a modified 5× PCR buffer (0.5 M Tris, pH 8.3, 10 mM MgCl2, 14% Ficoll) with 0.5 unit *Taq* polymerase and 0.13 mM each of the dNTPs. DNA was added to each sample at a rate of 40 ng and total volume was adjusted to 18 μL with 8 μL distilled sterile water. PCR products (6 μL) were digested according to manufacturer's instructions (Promega, Madison, Wis.) with 11 restriction enzymes (*Eco*R I, *Eco*R V, *Hae* III, *Rsa* I, *Sac* I, *Hinf* I, *Taq* I, *Bam*H I, *Dra* I, *Hind* III and *Pst* I). One accession from each species was used initially to identify polymorphisms among species (HRI 6178, PI 169074, PI 169057, PI 458949, PI 271455, PI 193959, PI 29302). Digested fragments were separated by electrophoresis on a 1.5% agarose gel, at 80 V, for 1 h in 1× TAE buffer and visualized under UV light with Gel Doc 2000 and pictures were digitally recorded with Quantity One software (Bio-Rad Labs., Hercules, Calif.) after staining with ethidium bromide (6 ng·mL^{-1}). Primer pair/restriction enzyme combinations that identified polymorphisms were used to evaluate all 70 accessions of the 7 species.

Results

Thirteen primer pairs were used to amplify cpDNA from seven different species, of which ten amplified clear single bands. Primer pair *rbcL* 1F-*rbcL* 1460R produced a single band of expected size; however, the band stained very weakly under several different annealing temperatures. Primer pairs *trnH* F-*trnK* R and *matK* 16F-*matK* 1495R produced multiple bands even under increased annealing temperatures. The remaining 10 primer pairs evaluated in this study produced clear single bands within the specified size range with no size variation among the seven species evaluated.

Of the 110 enzyme/primer pair combinations evaluated, 38 identified fragments that were unique to at least one of the 7 species (Table 3). The restriction enzymes *Bam*H I, *Hae* III, *Pst* I and *Sac* I were the least effective in discriminating species revealing only 1 polymorphism each, and the enzyme *Taq* I was the most effective in discriminating the species producing 8 polymorphisms among the 10 amplified cpDNA regions. The most polymorphic fragments were *trnK* P1-*trnK* P2, *trnM-rbcL* R, *trnC-trnD* and *psaA-trnS* R, which generated 7, 7, 5, and 6 amplified variable regions respectively when cut with the 11 restriction enzymes. The combination of *psaA-trnS* R with *Taq*I discriminated all diploid species, and was the only combination to do so (Fig. 1).

The amphidiploid species of the *Brassica* triangle were also evaluated to find out usefulness of primer/enzyme combinations to discriminate those species. *B. carinata* had the same restriction profile as *B. nigra* in all accessions tested, *B. juncea* had the same profile as *B. rapa* in 9 of

Table 1. Accessions of *Brassica* species and *Raphanus sativus* evaluated for polymorphisms in cpDNA.

Species	Accession no.	Source	Origin	Species	Accession no.	Source	Origin
B. oleracea	6178	HRI[z]	China	*B. napus*	PI 470004	NC7	Poland
	5577	HRI	Holland		PI 470038	NC7	Taiwan
	5887	HRI	Holland		PI 470049	NC7	S. Korea
	8667	HRI	U.S.		PI 502303	NC7	Russia
	12700	HRI	Japan		PI 531274	NC7	Hungary
	8680	HRI	Portugal	*B. juncea*	PI 271455	NC7	India
	06990	CGN	France		PI 280637	NC7	Ethiopia
	06993	CGN	Holland		PI 379103	NC7	Yugoslavia
	PI 183214	NE9	Egypt		PI 426178	NC7	Afghanistan
	PI 462217	NE9	Italy		PI 426347	NC7	Pakistan
B. rapa	PI 169074	NC7	Turkey		PI 42648	NC7	Pakistan
	PI 254541	NC7	Afghanistan		PI 432377	NC7	Bangladesh
	PI 271447	NC7	India		PI 458928	NC7	Canada
	PI 352795	NC7	India		PI 458993	NC7	India
	PI 352815	NC7	India		PI 478325	NC7	China
	PI 426175	NC7	Afghanistan	*B.carinata*	PI 193959	NC7	Ethiopia
	PI 426244	NC7	Pakistan		PI 193459	NC7	Ethiopia
	PI 426254	NC7	Pakistan		PI 194904	NC7	Ethiopia
	PI 432366	NC7	Bangladesh		PI 197402	NC7	Ethiopia
	PI 537015	NC7	Pakistan		PI 273640	NC7	Ethiopia
B. nigra	PI 169057	NC7	Turkey		PI 360879	NC7	Sweden
	PI 175073	NC7	India		PI 360884	NC7	Sweden
	PI 195922	NC7	Ethiopia		PI 360887	NC7	Sweden
	PI 220282	NC7	Afghanistan		PI 390133	NC7	Pakistan
	PI 253722	NC7	Iraq		PI 597822	NC7	Sweden
	PI 367904	NC7	Mozambique	*R. sativus*	G29302	NE9	Nepal
	PI 368377	NC7	Yugoslavia		G30006	NE9	Germany
	PI 426410	NC7	Pakistan		G32362	NE9	USA
	PI 458981	NC7	U.S.		PI 140432	NE9	Iran
	PI 459011	NC7	India		PI 183242	NE9	Egypt
B. napus	PI 458949	NC7	Germany		PI 262955	NE9	Russia
	PI 458958	NC7	Germany		PI 269589	NE9	Pakistan
	PI 469786	NC7	S. Korea		PI 288609	NE9	India
	PI 469797	NC7	France		PI 506452	NE9	Moldova
	PI 469985	NC7	Japan		PI 518857	NE9	China

[z]NC 7: USDA, North Central Reg. Plant Intro. Sta., Ames, Iowa., NE 9: USDA, North Eastern Reg. Plant Intro. Sta., Geneva, N.Y., CGN: Center for Genetic Resources, Wageningen, Netherlands., HRI: Hort. Research Inst., Wellesburne, U.K.

Table 2. Chloroplast-derived primer pair sequences used for amplifying cpDNA.

Primer name	Primer sequence (forward–reverse 5'–3')	Annealing temp (^{0}C)	References
trnH F	ACGGGAATTGAACCCGCGCA		
trnK R	CCGACTAGTTCCGGGTTCGA	59.0	Demesure et al., 1995
trnK P1	GGGTTGCCCGGGACTCGAAC		
trnK P2	CAACGGTAGAGTACTCGGCTTTTTA	65.5	Demesure et al., 1995
trnC	CCAGTTCAAATCTGGGTGTC		
trnD	GGGATTGTAGTTCAATTGGT	61.0	Demesure et al., 1995
trnS	GAGAGAGAGGGATTCGAACC		
trnfM	CATAACCTTGAGGTCACGGG	65.5	Demesure et al., 1995
psaA	ACTTATGGTTCCGGCGAACGAA		
trnS R	AACCACTCGGCCATCTCTCCTA	61.0	Demesure et al., 1995
trnM	TGCTTTCATACGCCGGGAGT		
rbcL R	GCTTTAGTCTCTGTTTGTGG	59.0	Demesure et al., 1995
ndhF 972F	GTCTCAACTCGGTTATATGATG		
ndhF 2110R	CCACCTATATATTTTGTGACTTCTCC	63.0	Hall et al., 2002
matK 16F	GGATATTTAGAGTTCGATGGGGCTC		
matK 1495R	CTTAATGATTTGGTCAATCATTGAATAA	50.0	Marcus et al., 2001
trnS	GGTTCGAATCCCTCTCTCTC		
psbC	GGTCGTGACCAAGAAACCAC	64.0	Parani et al., 2001
trnT Pa	CATTACAAATGCGATGCTCT		
trnF Pf	ATTTGAACTGGTGACACGAG	64.0	Taberlet et al., 1991
rbcL 1F	ATGTCACCACAAACAGAAAC		
rbcL 1460R	TCCTTTTAGTAAAAGATTGGGCCGAG	51.0	Olmstead et al., 1992
atpB 2F	TATGAGAATCAATCCTACTACTTCT		
atpB 1494R	TCAGTACACAAAGATTTAAGGTCAT	50.0	Hoot et al., 1995
BnpsbA F	AGAGACGCGAAAGCGAAAG		
BnpsbA R	CAAATACCTACTACCGGCCAAG	50.0	In this study

the 10 accessions tested. Accession PI 280637 exhibited the banding pattern of *B. nigra* instead of *B. rapa*. There was no variation between accessions of *B. juncea* and *B. carinata* for the restriction patterns. Two *B. napus* accessions (PI 458949 and PI 502303) exhibited *B. rapa* restriction profile. The rest of the *B. napus* accessions (PI 458958, PI 469786, PI 469797, PI 469985, PI 470004, PI 470038, PI 470049, and PI 531274) did not show *B. oleracea* or *B. rapa* restriction profiles for the primer/enzyme combinations tested.

Discussion

In the present study, diploid species of *Brassica* triangle and *R. sativus* were discriminated from each other with the PCR-RFLP of cpDNA. However, amphidiploid species could not be

Table 3. Number of polymorphisms discriminating diploid crucifer species between the 10 cpDNA primer pairs and 11 restriction enzyme combinations.

Restriction enzyme	Primer pairs									
	ndhF 972F-*ndhF* 2110R	*trnS*-*psbC*	*trnT* Pa-*trnF* Pf	*atpB* 2F-*atpB* 1494R	*BnpsbA* F-*BnpsbA* R	*trnK* P1-*trnK* P2	*trnC*-*trnD*	*trnS*-*trnfM*	*psaA*-*trnS* R	*trnM*-*rbcL* R
*Eco*R I	1	0	1	0	0	1	0	0	0	1
*Eco*R V	0	0	0	0	0	0	1	1	0	1
Hae III	0	0	0	0	0	0	1	0	0	0
Rsa I	0	0	0	0	1	1	1	1	1	1
Sac I	0	0	0	0	0	0	0	0	0	1
Hinf I	1	0	1	0	1	1	0	1	0	0
Taq I	0	0	0	0	0	3	1	1	3	0
*Bam*H I	0	0	0	0	0	1	0	0	0	0
Dra I	0	0	1	1	0	0	1	0	1	1
Hind III	0	1	0	0	0	0	0	0	1	1
Pst I	0	0	0	0	0	0	0	0	0	1

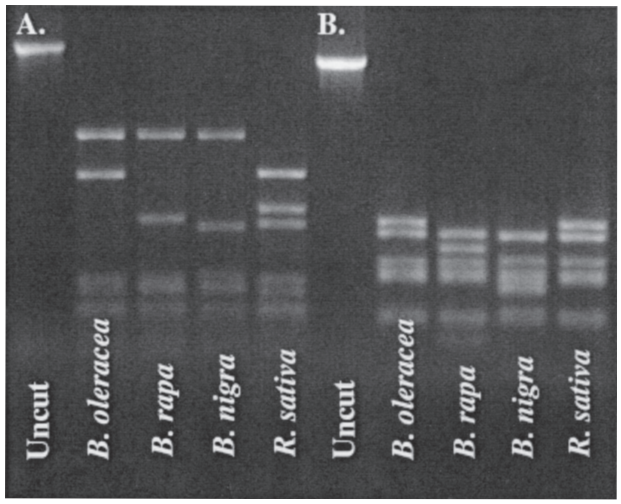

Fig. 1. (**A**) *Taq* I-digested *cp*DNA following amplification with the primer pair *psaA-trnS* R and (**B**) *Taq* I-digested *cp*DNA following amplification with the primer pair *trnK* P1 and *trnK* P2.

discriminated effectively in this study indicating that cpDNA has not shown accumulated fixed mutational differences observed in diploid crucifer species. In the present study, *B. carinata* accessions had the banding pattern of *B. nigra*. Based on these results *B. nigra* appears to be the maternal parent of all *B. carinata* accessions studied. All *B. juncea* accessions with the exception of PI 280637 (which had a *B. nigra* type profile) had the banding pattern of *B. rapa*. PI 280637 was collected in Ethiopia, and is currently under review as a misidentified *B. carinata* (Ethiopian mustard) accession, indicating that all *B. juncea* accessions tested have *B. rapa* maternal origin. *Brassica napus* accessions, with two exceptions, did not follow either *B. rapa* or *B. oleracea* banding patterns. In previous studies some *B. napus* accessions showed contradictory relations with other *Brassica* species (Palmer et al., 1983; Song et al., 1988) having four different cytoplasms with the majority of the accessions showing the banding pattern of *B. montana*, (Song and Osborn, 1992). Our study showed the same phenomena where most *B. napus* accessions did not show either the *B. rapa* or *B. oleracea* cytoplasm type. Increasing the number of primers and restriction enzymes may not solve this problem due to the low mutation rate of cpDNA. It is possible to use the PCR-RFLP method to assay polymorphisms in nuclear and mitochondrial genes (Ge et al., 2001), which could produce additional polymormphisms to use discriminating among *Brassica* amphidiploid species.

The type of cytoplasm may influence the success of interspecific hybridizations in *Brassica* (Ayotte et al., 1987; Chiang et al., 1977; Song et al., 1988). It may also influence the direction of cross and the genotype used. Evaluation of potential genotypes using PCR-RFLP may contribute to the success rate of interspecific hybridization in crop improvement. Use of wild relatives in *Brassica* breeding is important to transfer various traits into cultivated germplasm. The passport data of accessions from gene banks may not always be reliable (Wang et al., 1992). Use of cpDNA could also help the identification of other crucifer species for taxonomic, genetic and conservation studies.

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