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Brassica taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs)

1. Genome evolution of diploid and amphidiploid species

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Summary. Restriction fragment length polymorphisms (RFLPs) of nuclear DNAs have been used to explore the origin and evolution of the six cultivated *Brassica* species. Extensive RFLP variation was found at the species, subspecies and variety levels. Based on RFLP data from *Brassica* and related genera, a detailed phylogenetic tree was generated using the PAUP microcomputer program, which permits a quantitative analysis of the interrelationships among *Brassica* species. The results suggested that 1) *B. nigra* originated from one evolutionary pathway with *Sinapis arvensis* or a close relative as the likely progenitor, whereas *B. campestris* and *B. oleracea* came from another pathway with a possible common ancestor in wild *B. oleracea* or a closely related nine chromosome species; 2) the amphidiploid species *B. napus* and *B. juncea* have evolved through different combinations of the diploid morphotypes and thus polyphyletic origins may be a common mechanism for the natural occurrence of amphidiploids in *Brassica;* 3) the cytoplasm has played an important role in the nuclear genome evolution of amphidiploid species when the parental diploid species contain highly differentiated cytoplasms. A scheme for the origins of diploid and amphidiploid species is depicted based on evidence gathered from nuclear RFLP analysis, cpDNA RFLP analysis, cytogenetic studies and classical taxonomy.

Key words: *Brassica* – Genome evolution – Phylogenetic tree – Molecular taxonomy – Restriction fragment length polymorphisms

Introduction

Brassica is an agriculturally important genus containing species with highly diverse morphology and wide ranging utility. Over the past 60 years, there has been considerable interest in the taxonomy and evolution of economically important *Brassica* species. Based on interspecific hybridization and cytogenetic evidence, U (1935) proposed interrelationships among six *Brassica* species, which have become known as the U-triangle. According to the U-triangle, *B. napus* ($n=19$), *B. juncea* ($n=18$) and *B. carinata* ($n=17$) are amphidiploid species evolved from interspecific hybridizations between the pairs of diploid species *B. campestris* ($n=10$) × *B. oleracea* ($n=9$), *B. campestris* × *B. nigra* ($n=8$) and *B. nigra* × *B. oleracea,* respectively. Many studies have confirmed U's hypothesis using a number of taxonomic criteria including flavonoid composition (Dass and Nybom 1967), seed protein serology (Vaughan 1977), isozymes (Coulthart and Denford 1982; Quiros et al. 1985; Takahata and Hinata 1986) and ribosomal DNA (Quiros et al. 1985, 1986). Restriction pattern analysis of chloroplast DNA (Erickson et al. 1983; Palmer et al. 1983) has provided information on the maternal contributors of various amphidiploids, specifically that *B. juncea* has the cytoplasm of *B. campestris* and *B. carinata* has the cytoplasm of *B. nigra* whereas the cytoplasm of *B. napus* is of a more complex origin.

Although most studies on the species of U's triangle have confirmed the diploid origins of amphidiploid species, many questions remain concerning how the amphidiploid species evolved from their parental diploids (Prakash and Hinata 1980). Also, very little is known about the origins of the diploid species.

Table 1. Plant materials used in the experiment

Abbr.	Species	Subspecies	Cultivar	Source[a]
A1	*B. campestris*	chinensis	Flowering pakchoi	CrGC
A2	(syn. rapa)	pekinensis	Wong Bok	WGB
A3		pekinensis	WR 70 days	UCD
A4		pekinensis	Michihili	Olds
A5		pekinensis	Chi Hi Li	WGB
A6		japonica	Mizuna	CrGC
A7		narinosa		CrGC
A8		perviridis	Tendergreen	Olds
A9			Broccoletto	WGB
A10			Broccoletto	WGB
A11			Spring broccoli raab	CrGC
A12			Broccoletto	WGB
A13		utilis	Hong Tsai Tai	CrGC
A14		parachinensis	Choi sum	WGB
A15		rapifera	Presto	Sakata
A16		rapifera	Purble Top white Globe	CrGC
A17		oleifera?	R500, High GS	CrGC
A18		oleifera	Trunip rape	UCD
A19		trilocularis	Sarson	UCD
B1	*B. nigra*		WPBS	CrGC
B2	*B. nigra*		Black mustard	CrGC
C1	*B. oleracea*	gongylodes	Kohl rabi	Olds
C2		sabauda	Savoy cabbage	WGB
C3		botrytis	Cauliflower (All Year Round)	WGB
C4		botrytis	Cauliflower (White Rock)	CrGC
C5		gemmifera	Brussels sprout (Eveshum Giant)	WGB
C6		italica	Broccoli (Nine Star Perennial)	WGB
C7		italica	Broccoli (Packman)	Olds
C8		italica	Broccoli (Green Sprouting)	Olds
C9		palmifolia	Jersey kale	WGB
C10		medullosa	Marrow stem	WGB
C11		selensia	Borecole (Vates, Curled)	Olds
C12		alboglabra	Chinese kale (Large leaf kailan)	WGB
C13		ramosa	Thousand head kale (Dwarf)	WGB
C14		ramosa	Thousand head kale	WGB
C15		costata	Portugese cabbage	CrGC
C16		sabellica	Collards (Georgia)	Olds
C17		capitata	Cabbage (Wis. Golden Acre)	CrGC
C18		capitata	Cabbage (Brunswick)	CrGC
AB1	*B. juncea*	multiceps	Tillering mustard	CrGC
AB2		tsatsai	Big stem mustard	CrGC
AB3		multisecta	Shoot mustard	CrGC
AB4		oleifera	Indian mustard	WGB
AB5		rugosa	Leaf mustard (Southern Giant Curled)	CrGC
AB6		oleifera	Domo	CrGC
AC1	*B. napus*		Wild species	WGB
AC2			Asparagus kale	WGB
AC3		oleifera	Oilseed rape (Westar)	CrGC
AC4		oleifera	Oilseed rape (Altex)	CrGC
AC5		rapifera	Rutabaga (Laurentian)	CrGC
BC1	*B. carinata*		Abyssinian cabbage (Karate)	WGB
BC2			Abyssinian cabbage (Tex-sel)	CrGC
[b]S.a	*Sinapis arrensis*			CrGC
[b]B.a	*Brassica adpressa*			CrGC
[b]B.f	*Brassica fruticulosa*			CrGC
[c]B.t	*Brassica tournefortii*			CrGC
[c]R	*Raphanus sativus*			CrGC

[a] WGB = Wellesbourne Gene Bank in England, UCD = University of California-Davis, Olds = Olds Seeds Co., CrGC = Crucifer Genetics Cooperative; [b] Outgroups; [c] Species not included in PAUP analysis

The development of nuclear restriction fragment length polymorphism (RFLP) technology has been useful for the genetic analysis of various species and is of potential value in crop improvement (Beckmann and Soller 1986; Helentjaris et al. 1985; Burr et al. 1985; Landry et al. 1987; Nienhuis et al. 1987; Osborn et al. 1987). RFLP analysis also has considerable potential for exploring the evolutionary relationships among species and populations (Saghai-Maroof et al. 1984). In this study, RFLP methods have been used to examine nuclear DNA variation in a number of species and subspecies comprising the U-triangle. A phylogenetic tree was constructed based on RFLP data. New information concerning the origin of diploid species and more insight into the evolution of amphidiploid species have been obtained.

Materials and methods

Plant materials

Fifty-two cultivated brassicas from most of the subspecies within the six cultivated *Brassica* species, and three wild species were selected as representatives of a wide range of morphotypes. These accessions and their sources are listed in Table 1.

Detection of RFLPs

Plant DNA was isolated from lyophylized leaf tissue and restriction endonucleases *Eco*RI, *Hind*III (BRL) and *Eco*RV (Promega) were used to digest the crude DNA samples. DNA isolation, restriction endonuclease digestion, electrophoresis, Southern blotting, hybridization and autoradiography have been described previously (Osborn et al. 1987). Thirteen cloned DNA probes were used in combination with one, two or three restriction enzymes for a total 21 probe-enzyme combinations (Table 2). Two probes, pC1 and pN2, are cloned genes of the seed storage proteins, cruciferin and napin, respectively (Simon et al. 1985; Crouch et al. 1983). The remaining probes were from genomic DNA libraries of cabbage (*B. oleracea* cv. Wisconsin Golden Acre) and Chinese cabbage (*B. campestris* cv. Michihili). These libraries were constructed by digesting total genomic DNA with the methylation sensitive restriction endonuclease *Pst*I, recovering 1.0–2.0 kb fragments from an agarose gel, cloning these fragments into the plasmid vector pTZ18R (Pharmacia) and transforming the recombinants into *E. coli* strain DH5 α. Recombinant DNA clones from these libraries were screened by slot blot hybridization to total genomic, mitochondrial, and chloroplast DNAs. Nine clones containing low copy number nuclear DNAs (1–5 restriction fragments on Southern blots), one clone containing middle repetitive nuclear DNA and one chloroplast DNA (cpDNA) clone were selected for use as probes in this study.

Data handling and phylogenetic analysis

For each probe-enzyme combination, different size restriction fragments across all accessions were assigned numbers (1, 2, 3, ... n) according to decreasing molecular weights. A total of 166 nuclear DNA fragments were identified by 20 probe-enzyme combinations. Since most probes hybridized to several different sequences and gave complex RFLP patterns, it was difficult to use the conventional restriction site mutation analysis which is suitable for cpDNA or a single nuclear gene. To solve this problem, we assumed that common restriction fragments among different accessions reflect shared-derived restriction sites and thus they are indicative of relative homologies in nuclear DNA sequences. Based on this assumption, each fragment was treated as an unit character and the computer program "Phylogenetic Analysis Using Parsimony" (PAUP version 2.4) developed by D. L. Swofford (Illinois Natural History Survey, 607 East Peabody Dr., Champaign, Illinois 61820) was used to generate the phylogenetic tree. The basic principles and the application of this parsimony analysis were described by Felsenstein (1982), Fink (1986) and Sytsma and Gottlieb (1986). Three wild species, *B. fruticulosa, B. adpressa* and *Sinapis arvensis,* were used as the outgroups for rooting of the tree because these three species are more primitive than the six cultivated species according to morphological classification (Prakash and Hinata 1980; Mizushma 1980).

Table 2. Probes and enzymes used for detecting RFLPs

Probe	Probe source	Plant source	Enzymes used
pC1	Cruciferin (seed protein)	*B. napus*	*Hind*III, *Eco*RI and *Eco*RV
pN2	Napin (seed protein)	*B. napus*	*Eco*RV
pK4A3	Nuclear DNA	Cabbage	*Hind*III, *Eco*RI, *Eco*RV
pK4G11	Nuclear DNA	Cabbage	*Eco*RV
p21D8	Nuclear DNA	Chinese cabbage	*Hind*III, *Eco*RI
p21E8	Nuclear DNA	Chinese cabbage	*Eco*RV
p21B9	Nuclear DNA	Chinese cabbage	*Eco*RV, *Hind*III
p21H2	Nuclear DNA	Chinese cabbage	*Hind*III, *Eco*RV
p21G4	Nuclear DNA	Chinese cabbage	*Hind*III, *Eco*RV
p21E5	Nuclear DNA	Chinese cabbage	*Hind*III
p21E3	Nuclear DNA	Chinese cabbage	*Eco*RV
p21C8	Nuclear DNA	Chinese cabbage	*Eco*RV
p21B5	CpDNA	Chinese cabbage	*Eco*RI

Results and discussion

A large amount of genetic variation was detected among accessions at the species, subspecies and variety levels. In this study, we report on the variation among species and the use of these data to provide additional information on the origin of diploid and amphidiploid species. The relationships within species, especially within *B. campestris* and *B. oleracea,* will be addressed elsewhere.

Phylogeny of Brassica

Among 166 nuclear DNA restriction fragments recorded, 15 fragments were unique among accessions (9.0% of the total), 31 fragments were common to all accessions (18.7%) and the remaining 120 fragments were phylogenetically informative (72.3%). Based on

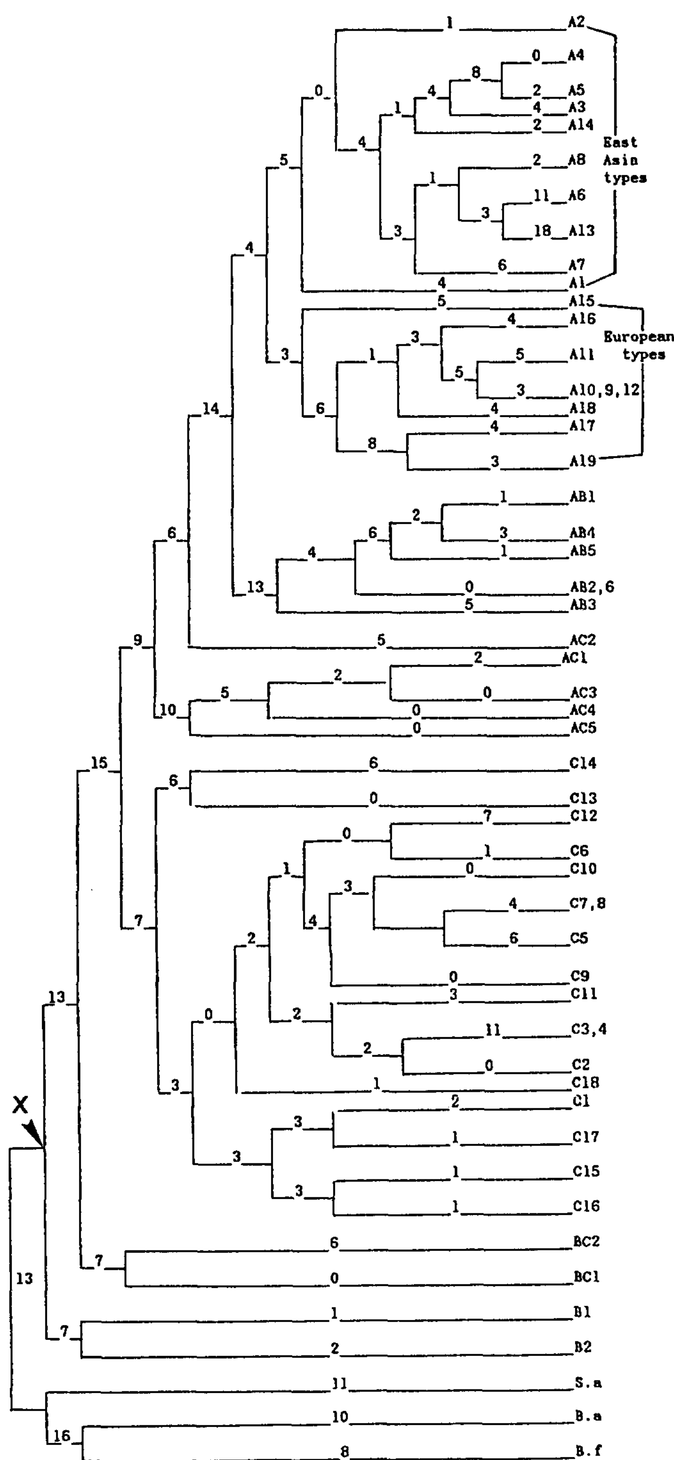

Fig. 1. Phylogenetic tree of *Brassica* based on RFLP data. The tree length = 421, consistency index = 0.31 and root = outgroups (*S. arvensis, B. adpressa* and *B. fruticulosa*). The point X (*arrow*) indicates the hypothetical common ancestor of *B. nigra* and other cultivated species (see text). Letters at termination of branches indicate accessions (Table 1). Numbers on the branches indicate the minimum number of mutation steps (see text). The length of branch is not proportional to the number

these data, the phylogenetic trees were generated by the PAUP program using "Hold = 10 Swap = Global Mulpars Blrange" functions. The shortest or most parsimonious trees had lengths of 421 and consistency indices of 0.31. Twenty different trees with the same length were examined and all of these had quite similar topologies (tree output format). There were only slight differences within species in the formats and in the branch lengths. One tree was selected (Fig. 1), which represented the most common topology (8 out of the 20 trees) and fitted better than other tree topologies with the results from direct RFLP comparisons. A simplified version of this tree is shown in Fig. 2.

The phylogenetic tree clusters closely related accessions and the topology of the tree presents how the accessions are related. The number on each branch represents the minimum number of mutational steps which occurred during the evolution of a particular accession. The number reflects the degree of divergence of an accession from its closest ancestor, the branch joint or node on the tree. For instance, *B. nigra* and other cultivated *Brassica* species have a hypothetical common ancestor at point X of the tree, and 13 characters (restriction fragments) distinguish them from the outgroups (Figs. 1 and 2). This means that *B. nigra* and other cultivated brassicas are "sister groups" and are equally distant from the outgroups in terms of evolutionary time. Also, *B. nigra* has diverged much less from the common ancestor than has either *B. campestris* or *B. oleracea,* as indicated by the numbers on the horizontal branches (Figs. 1 and 2). Unequal rates of molecular divergence among species have also been found in nuclear rDNA (Sytsma and Schaal 1985) and in cpDNA restriction pattern analyses (Jansen and Palmer 1987). Our observation of this phenomenon will be discussed later.

The phylogenetic tree has clearly separated the taxonomically defined species and the subgroups within a particular species. For instance, all subspecies in *B. campestris* are grouped separately from all subspecies in *B. oleracea.* Within *B. campestris,* the two subgroups, European types including turnip, turnip rape and sarson and East Asian types including Chinese cabbage and other vegetable forms, are separated from each other. As expected, the three amphidiploid species are located between their constituent diploid species (Figs. 1 and 2). The species relationships revealed by these trees agree with results using other taxonomic methods. However, they provide new details and evolutionary insights.

Origin of diploid species

Based on cytogenetic studies, previous researchers have proposed that the three basic diploid species are

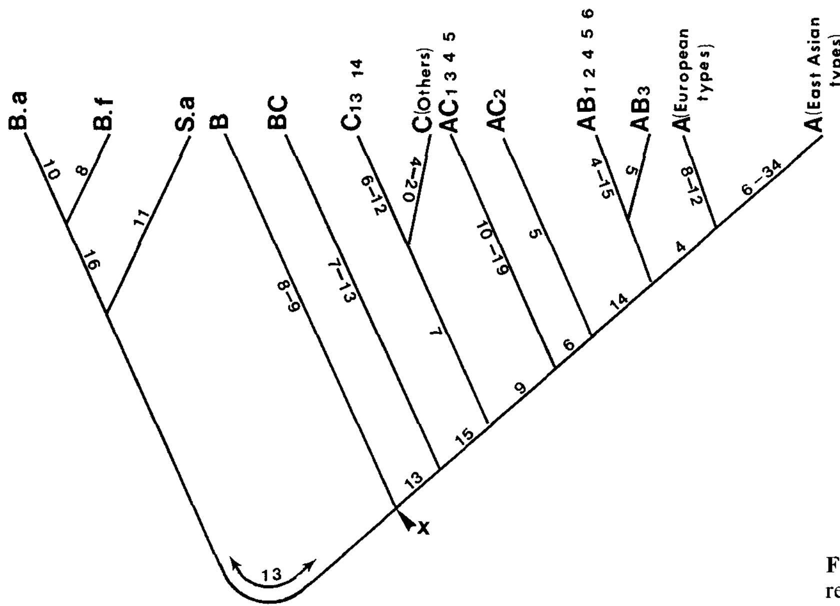

Fig. 2. Simplified tree of Fig. 1, illustrating the relationships between species

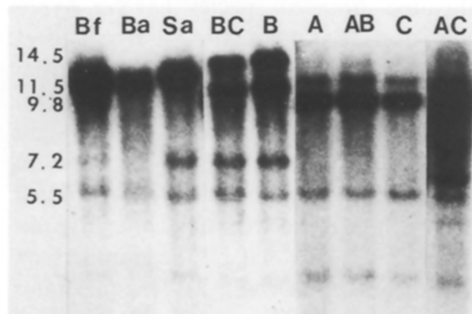

Fig. 3. Autoradiograph of *Eco*RV digested DNAs probed with pK4A3 showing species relationships. RFLP patterns common to all accessions within a species are labelled with the species letter symbols (Table 1). The sizes of major RFLP fragments are given in kilobases. *B. oleracea* and *B. campestris* have the same pattern but *B. nigra* has a different pattern. The 14.5 kb and 11.5 kb fragments are B genome-specific, and the 7.2 kb fragment is common to *B. nigra* and *S. arvensis*

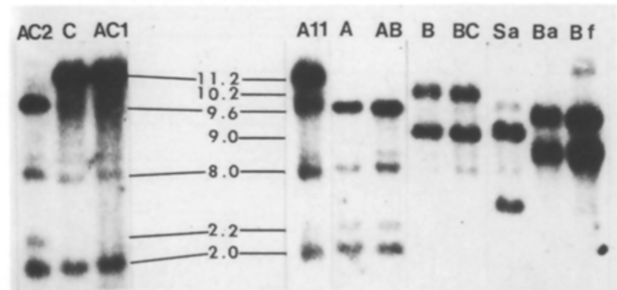

Fig. 4. Autoradiograph of *Hin*dIII digested DNAs probed with pK4A3 showing difference between species. RFLP patterns specific to accessions within species are labelled with accession letter(s) and number (Table 1). The 9.6 kb and 2.2 kb fragments are A genome-specific, the 11.2 kb fragment is C genome-specific, the 10.2 kb fragment is B genome-specific and the 9.0 kb fragment is common to *B. nigra* and *S. arvensis*

aneuploids evolved in ascending order from a common ancestor which probably had a chromosome number of x=6 (Mizushima 1980; Prakash and Hinata 1980). However, there is little evidence to support this speculation. From our RFLP analysis, two evolutionary pathways appear to be involved in the origin of the diploid species; *B. nigra* originated from one, while *B. oleracea* and *B. campestris* came from another. For most probe-enzyme combinations, *B. nigra* had very different RFLP patterns from those of *B. oleracea* and *B. campestris*, whereas *B. oleracea* and *B. campestris* had similar RFLP patterns. Using species-specific probes, many common restriction fragments were observed in *B. oleracea* and *B. campestris* which were absent in *B. nigra* (Figs. 3 and 4). Furthermore, when the cpDNA probe (p21B5) from the Chinese cabbage library was used to detect cytoplasmic differentiation in *Eco*RI digests of *Brassica* species, it was found that *B. nigra*, *S. arvensis*, *B. fruticulosa* and *B. adpressa* had the same cpDNA RFLP pattern, whereas *B. oleracea*, *B. campestris* and two species not included in the PAUP analysis, *Raphanus sativus* and *B. tournefourtii*, had another distinct cpDNA pattern (Fig. 5).

Previous studies also suggest that *B. nigra* is distant from *B. oleracea* and *B. campestris*. Chromosome paring in amphihaploids of AB and BC is poor with a maximum number of bivalents of 3 or 4; whereas the bivalent number in AC amphihaploids is usually 9

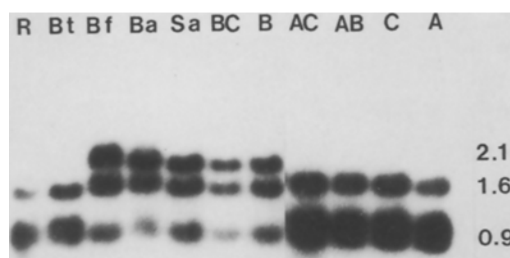

Fig. 5. Autoradiograph of *Eco*RI digested DNAs probed with p21B5 showing differentiation of cpDNAs. The type B cytoplasm, represented by *B. nigra,* has a unique 2.1 kb fragment and has fewer copies of the 0.9 kb fragment than the A/C type cytoplasm, represented by *B. campestris* and *B. oleracea*

(Attia and Robbelen 1986a, b; Fan and Tai 1985; Inomata 1985; Prakash and Hinata 1980). *B. nigra* also is distinct from *B. oleracea* and *B. campestris* in containing smaller and more uniform chromosomes. However, there is considerable similarity in size and morphology between *B. oleracea* and *B. campestris* chromosomes (K.M. Song et al., unpublished data). Serological analysis of seed proteins (Vaughan 1977) and isozyme studies (Takahata and Hinata 1986) suggest that *B. oleracea* and *B. campestris* are phylogenetically closer to each other than either is to *B. nigra.* The chloroplast DNA restriction pattern of *B. nigra* is distinct from *B. oleracea* and *B. campestris,* whereas the latter two species have closely related cpDNA patterns (Palmer et al. 1983). Morphologically, both *B. oleracea* and *B. campestris* exist in a variety of cultivated forms, whereas *B. nigra* is found only as a leafy form (Williams and Hill 1986). On the phylogenetic tree, *B. nigra* has changed less than *B. campestris* and *B. oleracea* from the common ancestor. The lack of morphological variation and the low frequency of DNA sequence mutation in *B. nigra* may be due to the absence of selection pressure during domestication. Alternatively, factors inherent in the genetic constitution of B genome may have limited its rate of change compared to the A and C genomes. The above observations together with the divergent RFLP patterns support the hypothesis that *B. nigra* has had a different origin from that of *B. campestris* and *B. oleracea.*

Results from RFLP analysis of nuclear and chloroplast DNA suggest that *Sinapis arvensis* is a likely progenitor of *B. nigra.* For example, probe pK4A3 identified a 7.2 kb *EcoRV* fragment in *B. nigra* and *S. arvensis,* but not in any *B. campestris* and *B. oleracea* accessions (Fig. 3). Also, pK4A3 identified a 9.0 kb fragment in *Hind*III digests of *B. nigra* and *S. arvensis,* but not in other diploid accessions (Fig. 4). The total homology of RFLPs between *B. nigra* and *S. arvensis* is high and is reflected in the phylogenetic tree by the close distance of 13 units between the two species (Fig. 2). *B. adpressa* and *B. fruticulosa* are also closely related to each other and to *B. nigra.* However, based on differences in RFLP patterns (Figs. 4 and 6) and the greater distance to *B. nigra* in the phylogenetic tree (Fig. 2), neither *B. adpressa* nor *B. fruticulosa* appears to be as close a progenitor of *B. nigra* as *S. arvensis.* Evidence that *S. arvensis* is the progenitor of *B. nigra* was also found in previous studies by Mizushima (1980) who reported a high degree of homoeologous pairing in a *B. nigra* × *S. arvensis* interspecific hybrid and by Prakash and Hinata (1980) who indicated that both species have the same natural distribution.

Although from this study we can not determine the direct progenitor of *B. oleracea* and *B. campestris,* evidence from other taxonomic research suggests that the progenitor of *B. oleracea* exists in the 9 chromosome wild *B. oleracea* (Snogerup 1980). A primitive cultivated *B. oleracea* might have evolved directly from one of the wild *B. oleracea* forms. Our RFLP analysis indicated that such a primitive cultivated *B. oleracea* could be thousand head kale (C14) or a close relative. In the phylogenetic tree, thousand head kale was separated from all other *B. oleracea* forms and was close to the ancestor at the nearest branch joint (Figs. 1 and 2). This suggests that thousand head kale is the most ancient form of the cultivated *B. oleracea* we studied.

At least two hypotheses are possible concerning the origin of *B. campestris.* One is that primitive *B. campestris* forms were derived from one of the wild 9 chromosome *B. oleracea* groups. Another is that *B. campestris* evolved from a primitive cultivated *B. oleracea* since according to classical taxonomy, *B. oleracea* is an older species (Prakash and Hinata 1980). Our RFLP analysis also provided evidence that *B. oleracea* is the older species since some probes identified fragments in *B. oleracea* which were also present in the three wild species but not in *B. campestris* (Fig. 6). In either situation, chromosome rearrangement or duplication would have been required to produce a ten chromosome species.

The hypothetical evolutionary relationships among the diploid species is depicted in the upper portion of Fig. 7. According to this hypothesis, two groups including different genera and species were differentiated from a distant ancestor species (common ancestor 1 in Fig. 7) by mutations in both cytoplasmic and nuclear genomes. These two groups can be distinguished by our cpDNA probe (p21B5) and are designated as the type B cytoplasm group and the type A/C cytoplasm group (Fig. 5). *B. nigra* originated from the former group with *S. arvensis* as a likely progenitor, whereas *B. campestris* and *B. oleracea* evolved from the latter

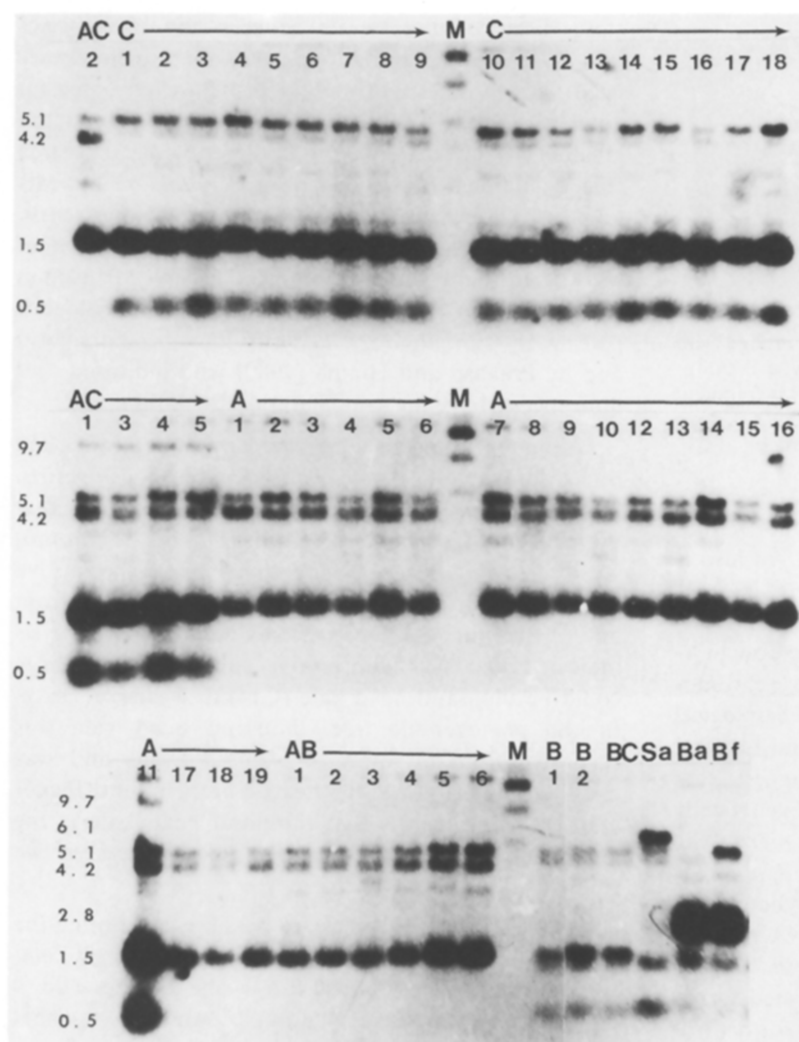

Fig. 6. Autoradiograph of *Eco*RI digested DNAs probed with pK4A3. The 0.5 kb fragment is common for all species except *B. campestris* and *B. juncea*. The 4.2 kb fragment is A genome-specific. The 9.7 kb fragment distinguishes *B. napus* from all other species, but asparagus kale (AC2) does not have this fragment. Spring broccoli raab (All) has the 0.5 kb and 9.7 kb fragments suggesting it has C genome introgression and may be the A genome donor of *B. napus* (see text)

group with a possible common ancestor from wild *B. oleracea*. Although our RFLP analysis provides evidence for the above hypothesis, further studies involving more wild species are needed to give more details on the origins of the diploid species.

Origin of amphidiploid species

RFLP analysis has confirmed the widely accepted theory that the three amphidiploid species are interspecific hybrids derived from the three basic diploid species. For example, probe p21G4 showed *B. napus* and *B. carinata* to have the combined RFLP patterns of A+C and B+C genomes, respectively (Fig. 8). Probe pK4A3 identified both A and C genome-specific *Eco*RI fragments in *B. napus* (Fig. 6). Other probe-enzyme combinations, such as pCl-*Eco*RV, p21B9-*Hind*III and p21H2-*Eco*RV gave similar results. In the phylogenetic tree, the amphidiploid species are located between their parental diploid species indicating their hybrid origins. Within amphidiploids, however, considerable variation, e.g. gain or loss of specific restriction fragments, was found. Previous studies have suggested the possibility of polyphyletic or multiple origins of the amphidiploid species from their parental diploids (Prakash and Hinata 1980). Our RFLP results provide evidence for the multiple origins of some amphidiploid species.

a) B. napus. When different forms of *B. napus*, including oil rape, rutabaga, asparagus kale and a wild form were analyzed for RFLPs, striking differences were found between asparagus kale (AC2) and the other *B. napus* accessions. Though asparagus kale has 2n=38 chromosomes and the typical morphology of *B. napus*, very different RFLP patterns were obtained with some probes. For example, probe pK4A3 identified 9.7 kb and 0.5 kb *Eco*RI fragments in all *B. napus* lines except asparagus kale (Fig. 6). Asparagus kale is deficient in a C genome-specific 11.2 kb *Hind*III frag-

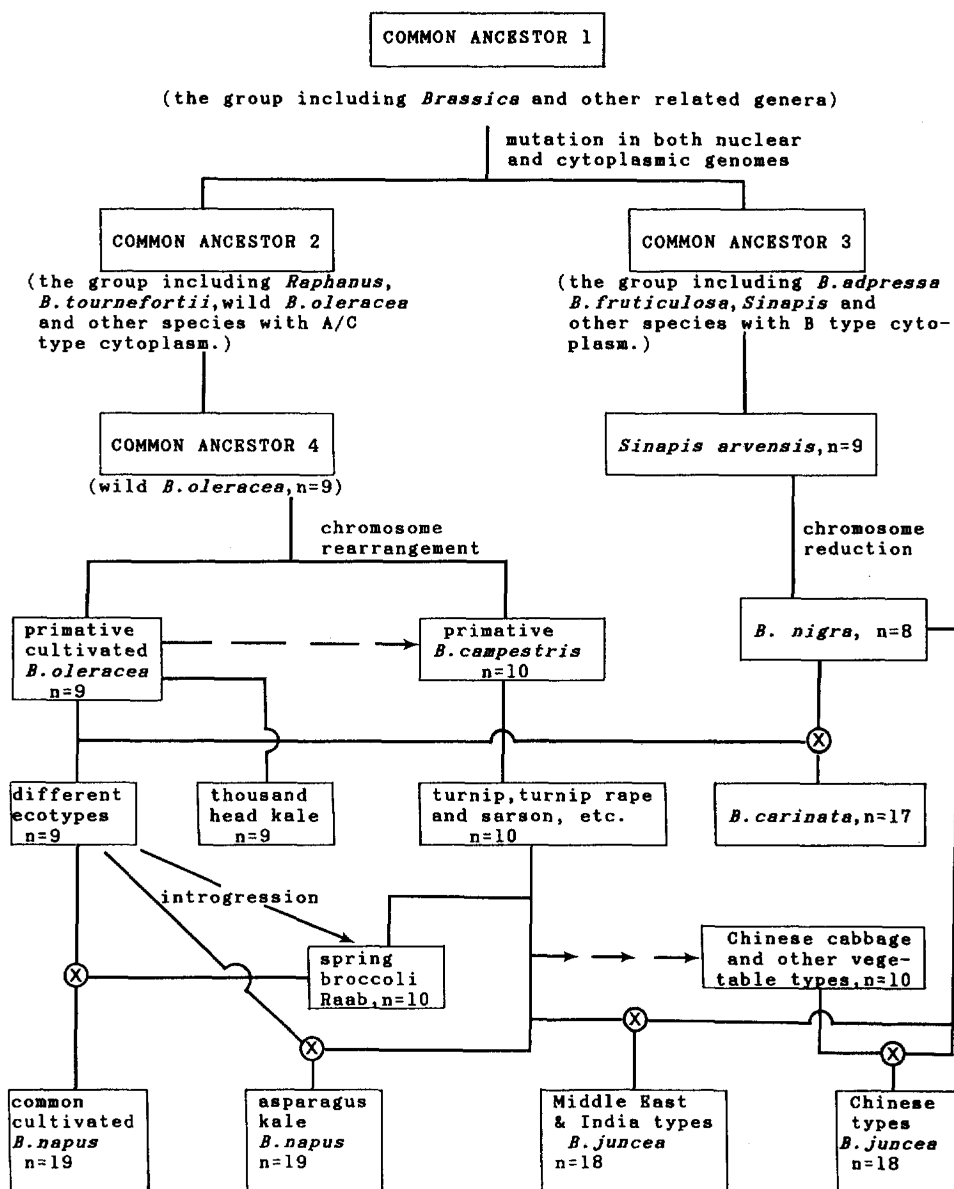

Fig. 7. Hypothetical scheme for genome evolution of *Brassica* (see text for details)

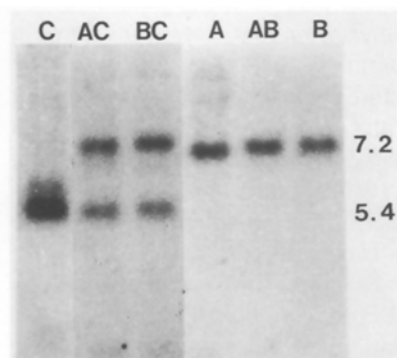

Fig. 8. Autoradiograph of *Hin*dIII digested DNAs probed with p21G4 showing amphidiploids containing the combined RFLP pattern of their diploid parents. The 7.2 kb fragment is common to the A and B genomes, while the 5.4 kb fragment is C genome-specific. *B. napus* and *B. carinata* have both 7.2 kb and 5.4 kb fragments

ment, but contains 9.6 kb and 2.2 kb *Hind*III fragments which were absent in other *B. napus* lines (Fig. 4). In the phylogenetic tree, asparagus kale is separated from other *B. napus* lines (Figs. 1 and 2). These observations suggest that asparagus kale evolved from a different A×C combination than the other *B. napus* types examined.

Although artificial synthesis of *B. napus* has been carried out by many researchers, no one knows which accessions of *B. campestris* and *B. oleracea* are the original A genome and C genome donors of the natural *B. napus.* Our data suggest that the *B. campestris* accession spring broccoli raab (All) was the possible A genome donor of common cultivated *B. napus.* Probe pK4A3 detected in spring broccoli raab a 9.7 kb *Eco*RI fragment which appears in most *B. napus* lines including the wild form (AC1) (Fig. 6). Since this probe detects highly conserved sequences, presence of the 9.7 kb *Eco*RI fragment in both *B. napus* and spring broccoli raab is unlikely due to convergence. It is possible that the 9.7 kb fragment exists in the A or C genome donors of *B. napus* and in a primitive *B. napus.* Among the possible diploid donors including all accessions of *B. campestris* and *B. oleracea* examined, only spring broccoli raab has the 9.7 kb *Eco*RI fragment. Probe pK4A3 also detected a 0.5 kb *Eco*RI fragment (Fig. 6) and a 11.2 kb *Hind*III fragment (Fig. 4) in spring broccoli raab. These two fragments were present in all *B. oleracea* accessions examined but not present in any *B. campestris* accession except spring broccoli raab. This suggests that spring broccoli raab was derived from partial introgression of the C genome into the A genome. Phylogenetically, spring broccoli raab is closely related to turnip (Fig. 1) and therefore it may have developed from an interspecific cross between turnip and broccoli, followed by backcrosses to turnip or other *B. campestris* accessions.

On the phylogenetic tree, common cultivated *B. napus* is closer to the C genome than to the A genome, suggesting that the C genome comprises a larger component in these *B. napus* lines (Fig. 2). This might be the case if the A genome donor were derived from spring broccoli raab. Also, one might predict that with C genome introgression, spring broccoli raab would cross more easily with *B. oleracea* than would the other *B. campestris* accessions and this could facilitate the natural interspecific hybridization between A and C genomes. *B. napus* asparagus kale, on the other hand, is located at the center between C and A genomes (Fig. 2), suggesting that it may have a different A genome donor without C genome introgression. Although the above evidence supports the hypothesis that spring broccoli raab was the A genome donor of *B. napus,* it is possible that the 9.7 kb and 0.5 kb *Eco*RI fragments were introduced into spring broccoli raab from *B. napus* by interspecific hybridization followed by backcrossing.

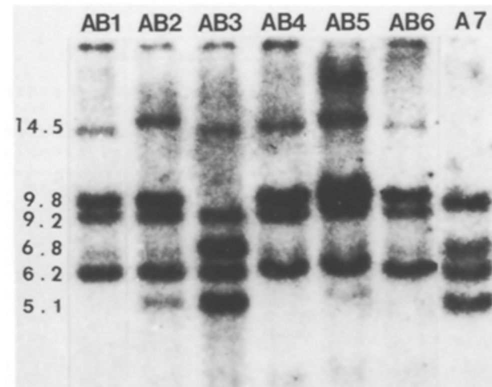

Fig. 9. Autoradiograph of *Eco*RV digested DNAs probed with pCl showing the particular relationship of AB3 to A7 (see text). The 9.8 kb fragment is missing in AB3 while the 6.8 kb and 5.1 kb fragments are present in both AB3 and A7

b) B. juncea. Reports from interspecific hybridization and studies of comparative morphology suggested that *B. juncea* has polyphyletic origins (Ramanujam and Srinivasachar 1943; Mukherijea 1975; Prakash and Hinata 1980). A number of interspecific hybridizations involving different morphotypes of diploids might have occurred independently and resulted in different forms of *B. juncea.* Parallel variation between *B. campestris* and *B. juncea* can be seen clearly in East Asian vegetable forms where bush loose head, heading, savoy, and stalked types occur in both species (Chen 1982; Prakash and Hinata 1980).

In our study, six *B. juncea* accessions having different morphotypes were analyzed and parallel variation between *B. campestris* and *B. juncea* was found at the DNA level. For example, using probe pCl and *Eco*RV digests, the RFLP pattern of AB3, an endemic cultivar of shoot mustard from China, was different from all other *B. juncea* accessions, but very similar to that of A7, an accession of *B. campestris* ssp. *narinosa* (Fig. 9). Since both AB3 and A7 originated from China, *B. campestris* ssp *narinosa* may be the A genome donor of AB3, whereas other *B. juncea* accessions have a different A genome donor(s).

Our RFLP data and phylogenetic evidence (Figs. 1 and 2) together with a knowledge of the natural distributions of *B. campestris* and *B. juncea* (Chen 1982; Prakash and Hinata 1980) support the idea that *B. juncea* has polyphyletic origins (Olsson 1960). There appears to be at least two centers of origin of *B. juncea;* one in the Middle East and India and another in China. The Middle East and India center contains the oldest *B. juncea* group from which many derivatives,

including AB1, AB2, AB4, AB5 and AB6, have originated. The Chinese center contains a variety of vegetable types, including AB3, which may have originated from independent hybridization events between different morphotypes of *B. campestris* and *B. nigra*. RFLP studies involving more ecotypes are needed to provide further information on the multiple origin of *B. juncea*.

C) B. carinata. Based on rDNA analysis, Quiros et al. (1985) suggested that *B. carinata* is an amphidiploid of recent origin and may have multiple origins. Our RFLP study has confirmed that *B. carinata* is derived from the cross of *B. nigra* × *B. oleracea*. Some probes detected the combined RFLP patterns of the diploid species in *B. carinata* (Fig. 8) and the phylogenetic analysis placed *B. carinata* between the two diploid species (Figs. 1 and 2). However, some probes detected RFLP patterns that were similar to those of *B. nigra* and excluded *B. oleracea* fragments (Figs. 3 and 4). The implication of these observations will be discussed below. Since only two *B. carinata* accessions were analyzed, we do not have enough data to address the issue of multiple origins of *B. carinata*.

Based on our RFLP data, a hypothetical scheme summarizing the origins of amphidiploid species was developed and is presented in the lower portion of Fig. 7. This hypothesis proposes that multiple origins could be common among the amphidiploids of *Brassica*, especially for *B. napus* and *B. juncea*. A number of interspecific hybridization events may have taken place between different diploid parents and therefore, different morphotypes of amphidiploids may have evolved at different times and places through combinations of various diploid morphotypes.

The role of cytoplasm in evolution of amphidiploid species

Analysis of our data indicate that the nuclear DNA compositions of the amphidiploid species are more closely related to their cytoplasm donors than to the pollen parents. This was particularly noticeable in *B. juncea* and *B. carinata* where rather than showing the combined RFLP patterns of their constituent diploid species, the amphidiploid patterns were more similar to those of their cytoplasm donors. For example, using probe pK4A3 with *Eco*RI, *Hind*III and *Eco*RV digestions, all of the *B. juncea* accessions showed the same RFLP patterns as those of *B. campestris;* and the B genome-specific fragments such as the 7.2 kb *Eco*RV fragment and the 10.2 kb and 9.0 kb *Hind*III fragments, were absent (Figs. 3 and 4). *B. carinata* had the same *Eco*RI, *Hind*III and *Eco*RV patterns as *B. nigra,* but was missing the 11.2 kb, 8.0 kb and 2.0 kb *Hind*III fragments which were present in C genome (Fig. 4). In the phylogenetic tree (Fig. 2), *B. juncea* was located very close to *B. campestris* with unit distance of four and far away from *B. nigra,* whereas *B. carinata* was closely related to *B. nigra* with unit distance of thirteen (Figs. 1 and 2). However, in *B. napus* one accession (asparagus kale) was close to *B. campestris* and the others were close to *B. oleracea*. The overall phylogeny of *Brassica* based on our nuclear RFLP analysis is quite similar to that based on cpDNA restriction patterns (Erickson et al. 1983; Palmer et al. 1983).

It appears that the cytoplasm has a profound influence on the evolution of the nuclear genome in the amphidiploid species. When the parental diploid species of an amphidiploid have highly differentiated cytoplasms, as reflected by different cpDNA restriction patterns, the nuclear genome from the male donor has been altered considerably more than the nuclear genome from the female side. As mentioned previously, the B cytoplasm is quite distinct from A and C cytoplasms, while A and C cytoplasms are similar. *B. juncea* has the A cytoplasm and *B. carinata* has the B cytoplasm (Fig. 5, Erickson et al. 1983; Palmer et al. 1983). During the evolution of *B. juncea*, the A nuclear genome has remained intact whereas the B nuclear genome has changed considerably. In *B. carinata,* the B genome has remained almost unchanged, whereas the C genome has changed considerably. In *B. napus,* however, both the A and C genomes have evolved with similar rates of change. These observations imply a coevolution of the nuclear genome with cytoplasmic genome. The native cytoplasm may provide selection pressure on portions of the foreign or introgressed nuclear genome, which helps to stabilize the newly synthesized amphidiploid by establishing a "harmonious relationship" between cytoplasmic and nuclear genomes. On the other hand, if there is little difference between cytoplasms of parental diploid species, as is the case of the A and C genomes in *B. napus,* the selection pressure of the cytoplasm will be minimized and the two nuclear genomes in the amphidiploid will change with similar frequencies during the course of evolution.

The results presented above indicate that RFLP technology is very useful for studying nuclear genome evolution. Unlimited probe-enzyme combinations allow both qualitative and quantitative analysis of homology and diversity among species. This feature provides a tool for resolving taxonomically perplexing problems which cannot be resolved by conventional methods. Also, combining nuclear DNA and cpDNA analysis using RFLP methods, one can look at the coevolution of nuclear and cytoplasmic genomes, which should provide new insights into plant evolution and speciation.

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