Theor Appl Genet (1983) 65:181–189

Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species

J. D. Palmer [1], C. R. Shields [2], D. B. Cohen [3] and T. J. Orton [4]

[1] Carnegie Institution of Washington, Department of Plant Biology, Stanford, CA 94305, USA

[2] Department of Vegetable Crops, [3] Department of Agronomy and Range Science; University of California, Davis, CA 95616, USA

[4] Agrigenetics Corporation, 3375 Mitchell Lane, Boulder, CO 80301, USA

Received January 12, 1983; Accepted January 15, 1983
Communicated by D. von Wettstein

Summary. The origin and evolution of a hybrid species complex in the genus *Brassica* (cabbage, turnip, mustard, rapeseed oil) has been explored through mutational analysis of the maternally inherited chloroplast genome. A detailed chloroplast DNA phylogeny enables identification of the maternal parent for most of the amphidiploids examined and permits quantitative resolution of the relative time of hybridization as well as the relative divergence of the diploid parents. Contradictory chloroplast and nuclear phylogenies obtained for two accessions of the amphidiploid *B. napus* (rapeseed oil) lead to the hypothesis that introgressive hybridization has also figured in their recent evolution.

Key words: Polyploidy – *Brassica* – Evolution of chloroplast DNA – Phylogenetic tree – Restriction endonucleases

Introduction

Interspecific hybridization is a common process among angiosperms and ferns. The origin of a new species directly from a natural hybrid generally requires some mechanism for stabilizing the breeding behavior of the hybrid (Grant 1981). Perhaps the most common mode of hybrid speciation, one which accounts for the majority of polyploid species and 30–50% of all angiosperm species (Grant 1981; Clausen et al. 1945), is amphiploidy (allopolyploidy). In this process fertility is restored in a sterile hybrid by doubling of both chromosome sets – either through the sexual functioning of cytologically non-reduced gametes or via spontaneous somatic chromosome doubling (DeWet 1980).

Abbreviations: kb = kilobase pairs; bp = base pairs

Analysis of hybrid and parent species relationships, particularly where multiple hybridizations have occurred leading to polyploid or hybrid species complexes, is often complicated and has classically been attacked through the combined approaches of comparative morphology, cytogenetics and artificial hybridization and genome analysis (Grant 1981; Clausen et al. 1945). More recently, polyploid complexes have been analyzed at the molecular level using as markers either secondary chemical compounds (Crawford and Giannasi 1982), proteins (Gray 1980; Wildman 1979; Vaughan 1977) or nucleic acids (Verma 1974; Vedel et al. 1978, 1981; Timothy et al. 1979; Lebacq and Vedel 1981; Kung et al. 1982).

In this report, mutational analysis (Palmer and Zamir 1982) of chloroplast DNA variation has been used to explore the evolution of a hybrid species complex in the agriculturally important genus *Brassica*. We present results which demonstrate the potential of chloroplast DNA analysis for providing new insights upon evolutionary relationships within this already extensively-studied and well-understood group of crop plants.

Materials and methods

Chloroplast DNA was purified from flats of two-month old plants of accessions 1–14 and 16–21 (Table 1) according to the DNase I procedure of Kolodner and Tewari (1975) and from accessions 15 and 22 according to the sucrose gradient procedure of Palmer (1982). Restriction endonuclease digestions, agarose gel electrophoresis, bidirectional nitrocellulose filter transfers of DNA, labeling of recombinant plasmids and isolated fragments with ^{32}P by nick-translation, and filter hybridizations were performed exactly as described (Palmer 1982). Chloroplast DNA restriction fragments were prepared from agarose gels according to methods described previously for the isolation of fragments from acrylamide gels (Maxam and Gilbert 1980).

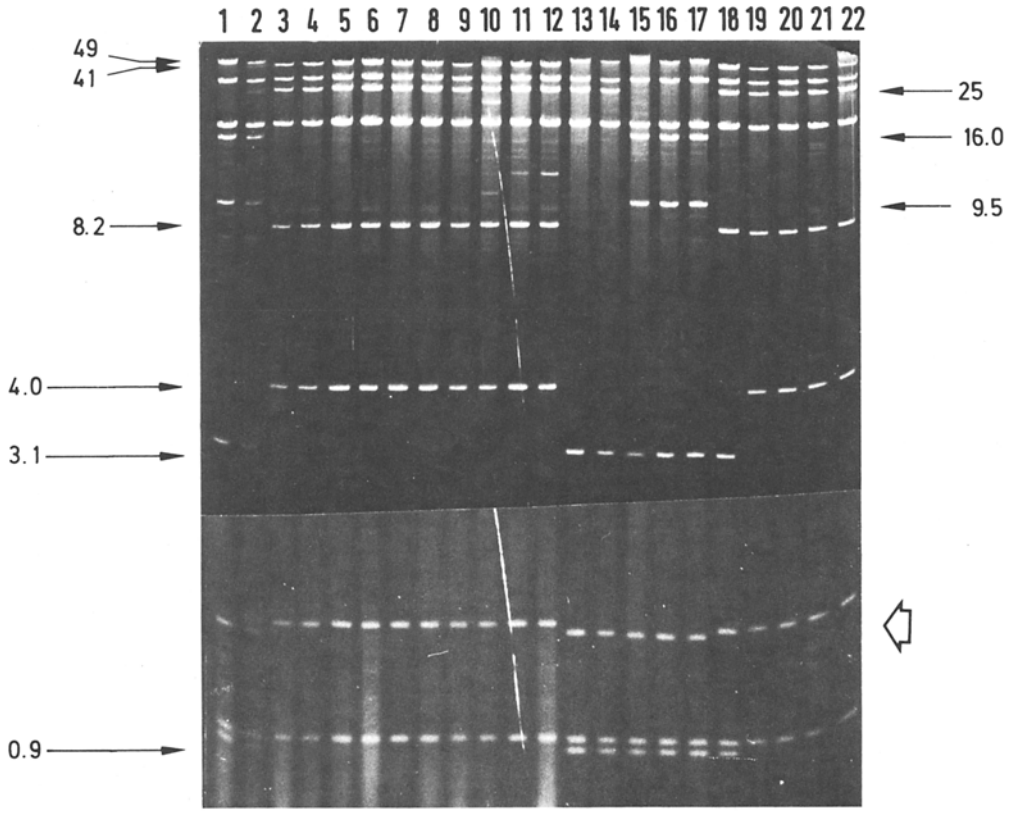

Fig. 1. Sma I chloroplast DNA mutations. Chloroplast DNAs from accessions 1 – 22 (Table 1) were digested with restriction endonuclease Sma I and fragments separated by electrophoresis on a 0.7% agarose gel. Each set of three horizontally staggered arrows indicates fragment size changes ascribable to a specific restriction site mutation (Table 2). Fragment sizes are given in kb. The broad arrow indicates fragment size variation resulting from small deletions/additions. A series of faint bands visible in the region of the gel between 5 kb and 15 kb arise from a small amount of mitochondrial DNA contamination of the chloroplast DNA preparations, as judged by comparison with restriction profiles of purified mitochondrial DNA (data not shown)

Table 1. Source of *Brassica* DNAs. (sps) = seed for plants used were derived from a single plant selection

Species	Origin	Line information
1. *Brassica carinata*	Ethiopa	Plant Introduction 193, 759 (sps)
2. *Brassica carinata*	India	Plant Introduction 033, 181 (sps)
3. *Brassica napus*	Japan	cv. Norin 31 (sps)
4. *Brassica napus*	Poland	cv. Bronowski
5. *Brassica napus*	Canada	cv. Altex (oil seed)
6. *Brassica juncea*	China	Plant Introduction (sps)
7. *Brassica juncea*	India	Plant Introduction NU60039 (sps)
8. *Brassica juncea*	Canada	cv. Domo (oil seed)
9. *Brassica campestris*	China	Plant Introduction
10. *Brassica campestris*	India	Subspecies *trilocularis,* PI180, 412 (sps)
11. *Brassica campestris*	California	Volunteer weed population (sps)
12. *Brassica campestris*	Canada	cv. Torch (oil seed)
13. *Brassica hirta*	Ethiopia	Plant Introduction 195, 922 (sps)
14. *Brassica hirta*	Europe	Commercial white mustard line
15. *Brassica nigra*	California	Volunteer weed population (sps)
16. *Brassica nigra*	Europe	Commercial black mustard line
17. *Brassica nigra*	India	Plant Introduction 179, 860 (sps)
18. *Raphanus sativa*	Korea	Plant Introduction (sps)
19. *Brassica oleracea*	Europe	cv. Oscar (cabbage)
20. *Brassica oleracea*	Europe	cv. Snowball (cauliflower)
21. *Brassica oleracea*	Europe	cv. Cruiser (broccoli)
22. *Brassica oleracea*	Europe	cv. Descicco (broccoli)

Results

Evolution of Brassica chloroplast genome structure

Previous studies have shown that structural rearrangements, particularly frequent small deletions and insertions (Gordon et al. 1982; Fluhr and Edelman 1981; Palmer et al. 1983a), but also rare large inversions, deletions and transpositions (Palmer and Thompson 1982), often distinguish chloroplast DNAs of related taxa and in certain cases (Palmer and Thompson 1982) can be used to infer phylogeny. Comparative analysis of the 22 *Brassica* chloroplast DNAs examined in this study (Table 1) with 28 different restriction enzymes has located a number of small deletions and insertions, ranging in size from 50 bp to 400 bp, in several regions of the *Brassica* chloroplast genome (Fig. 1). However, owing both to the small size of these changes and the inherent and difficulties in accurately assessing fragment size identities (1.6 kb fragment in lanes 14 and 15, and also in lanes 18 and 19; Fig. 1), and also to the tendency for certain regions of the genome to be hotspots (Gordon et al. 1982; Palmer et al. 1983a) for small size changes, we have not considered these events in constructing phylogenies.

No major rearrangements were evident in comparing restriction patterns for the 22 lines tested (Table 2) and in constructing maps for 8 of the enzymes (Fig. 2). Comparison to much more distantly related

Table 2. Chloroplast DNA restriction site mutations. The evolutionary direction of restriction site changes is given with reference to the ancestral pattern for each enzyme based on the root of the tree drawn in Fig. 4a. The direction of mutations at the root of the tree, i.e., those that distinguish samples 3–12 and 18–22 from 1, 2 and 13–17, cannot be determined; such mutations have been arbitrarily assigned to samples 3–12 and 18–22. Enzymes Pvu II through Sac II are Type I enzymes and the rest are Type II enzymes. No mutations were detected with BstEII (12 bands), Kpn I (10 bands) and Sph I (6 bands), while no Type II mutations were detected with Hind III (27 bands), Nco I (20 bands) and Stu I (18 bands). Number of bands scored are given for sample no. 19 and represent all those bands, starting in order from the largest band, that could confidently be separated and analyzed on a series of agarose gels of different percentages

Enzyme	No. bands scored	Changed fragments (kb)		Mutated samples
		Losses	Gains	
Pvu II	12	17.2+8.7	26	10
Sac I	15	1.1+1.05	2.2	18
Bal I	10	21.5+9.9 +5.2[a]	27 +15.1[b]	3–12, 18–22
		15.1[b]	14.2+ 1.1	3–12, 18–22
Bgl I	9	21.9	15.3+ 6.3	3–12, 19–22
		27	19.7+ 7.2	3–12, 18–22
Sal I[c]	6	47 +9.9	58	18
		5.2+4.1	9.4	3–12, 18–22
Tth I	7	27	17.7+ 9.1	1, 2, 17
		22.6	21.5+ 1.0	1, 2, 15–17
Pst I	12	19.2+4.9	24	19–22
		30	28 + 2.0	3–12, 18–22
		12.5+2.6	15.2	3–12, 18–22
Sma I	8	3.1[a]+0.9[a]	4.1[a]	3–12, 19–22
		25	16.0+ 9.5	1, 2, 15–17
		49	41 + 8.2	3–12, 18–22
Xho I	13	6.3+2.2	8.4	3–12, 18–22
		15.3+9.8	16.2+ 8.8	3–12, 18–22
Hpa I	15	20.3	16.6+ 3.5	18
		8.8	5.4+ 3.4	18
		6.6+4.4	11.1	1, 2, 15–17
		25	20.3+ 4.7	3–12, 18–22
Nru I	13	20.3	17.8+ 2.3	18
		3.2[a]	2.1[a]+ 1.1[a]	13, 14
		4.1	2.5+ 1.6	3–12
		25.1	20.3+ 5.2	3–12, 18–22
Sac II	7	30+21.2	52	18
		35	17.4+17.0	3–12, 18–22
		45	32 +12.2	1, 2, 15–17
		45	22.2+22.0	3–12, 19–22
Eco RV	27	12.2	9.4+ 2.8	4, 6–12, 19–22
Mst II	19	6.8+ 5.2	12.2	19–22
Bam HI	31	9.3+ 3.0	12.4	4, 6–12
Xba I	28	17.2	13.4+ 3.8	4, 6–9, 11, 12
Bcl I	27	13.5	11.7+ 1.9	3, 5
Cfo I	39	3.3+ 1.2	4.5	3, 5
		2.3	1.6+ 0.7	19–22
Nci I	29	6.7+ 2.8	9.4	15, 16
		2.2+ 1.6	3.8	19–22
Xmn I	40	4.3[a]+ 0.7[a]	5.0[a]	13, 14, 16
		5.4	3.7+ 1.7	1, 2, 17
Cla I	27	2.6+ 0.6	3.2	4, 6–12, 19–22
		2.9+ 0.5	3.4	3, 5
		2.6	2.2+ 0.4	15, 16
		1.9	1.5+ 0.4	10
Eco RI	33	6.6	4.6+ 1.9	4, 6–12
		1.8+ 1.4	3.2	4, 6–9, 11, 12
		2.1+ 1.9	4.0	15
		8.2	5.4+ 2.7	1, 2, 17

[a] Doublet bands

[b] Fragment predicted to have existed, but destroyed by a second independent mutation within the same region of DNA

[c] Identical Sal I patterns for 6 of these species have been published by Lebacq and Vedel (1981), with the exception that their *B. nigra* pattern was identical to that of both our and their *B. campestris* and *B. juncea*. Based on the large evolutionary distance between *B. nigra* and the latter two species (Fig. 4a) we suggest that their *B. nigra* either was misidentified or had derived its cytoplasm from a foreign source

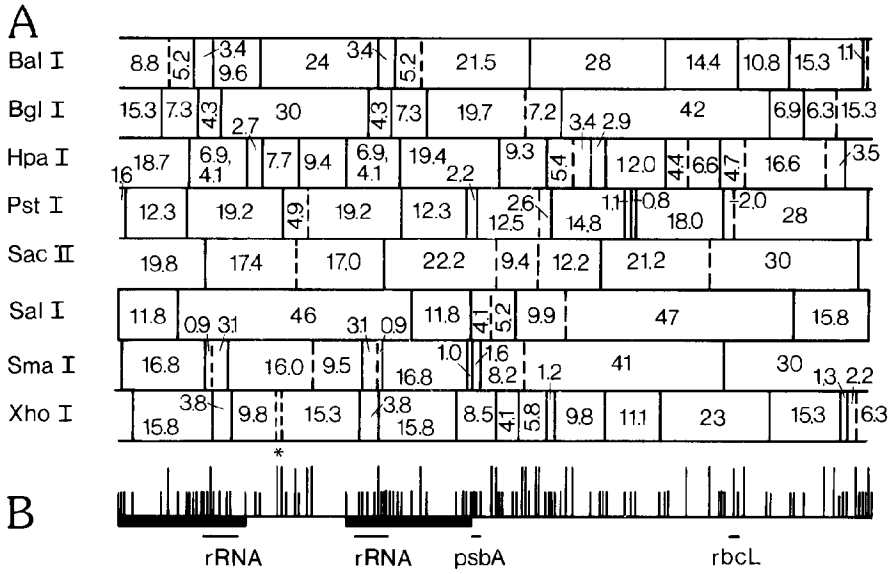

Fig. 2 A, B. Location of mutations on the *Brassica* chloroplast DNA restriction map. **A** Bal I, Bgl I, Hpa I, Pst I and Sma I sites were mapped by heterologous hybridizations using cloned mung bean restriction fragments (Fig. 3), Sac II sites by hybridizations using cloned petunia fragments (Fig. 3) and Sal I and Xho I sites are from Link et al. (1981). In a few places additional mapping information was kindly provided by G. Link (pers. commun.). Sites which are mutated among the 22 DNAs compared are given by a dashed vertical line (Table 2) and conserved sites by a solid line. Fragment sizes are given in kb. The asterisk denotes two putative Xho I restriction site mutations, which may, alternatively, result from a single inversion (see text for discussion). Note that all the maps shown in Figs. 2 and 3 portray the chloroplast genome in only one of the two orientations in which it exists [the single copy regions are flipped relative to one another in half the native molecules (Palmer 1983)]. In addition, each enzyme map was constructed without reference to the others and thus the relative positions of sites within the small single copy region may be reversed for any pair of enzymes. **B** Distribution of *Brassica* chloroplast DNA mutations. Of the 104 mapped restriction sites, 25, representing 23 independent mutations (2 are located within the inverted repeat and thus are shown twice), are variable (long vertical lines) and 79 nonvariable (short vertical lines). Heavy horizontal lines indicate the extent of the inverted repeat. The locations of the *Brassica* rRNA (ribosomal RNA), psbA (32,000 dalton photosystem II polypeptide) and rbcL (large subunit of ribulose-1,5-bisphosphate carboxylase) genes are from Link (1981)

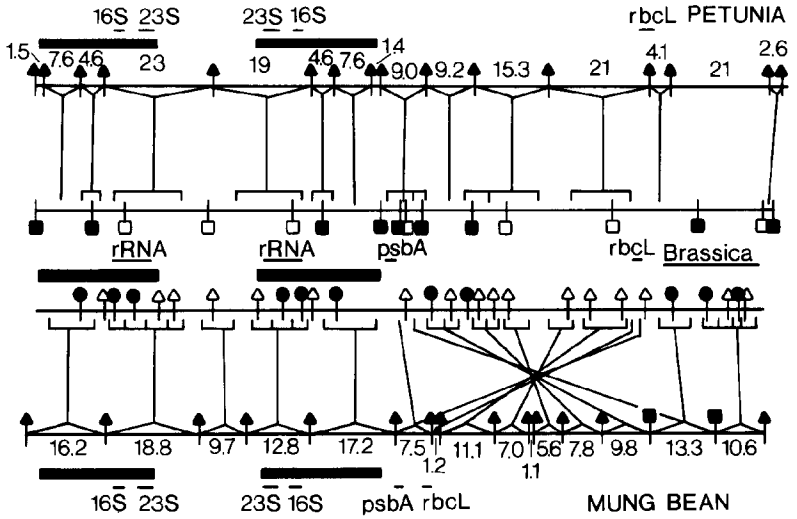

Fig. 3. Evolution of *Brassica* chloroplast genome structure. *Top panel* Twelve nonoverlapping, cloned petunia Pst I chloroplast DNA restriction fragments (J. D. Palmer, K. J. Aldrich and W. F. Thompson, unpublished), which represent 84% of the petunia genome, were each hybridized to replica nitrocellulose filters containing Sac II, Sal I and Sac II-Sal I fragments from DNA 13, Sac II and Sac II-Sal I fragments from DNA 9, and Sac II fragments from DNA 18. The extent of the petunia fragments used as probes is indicated by the two lines that converge below the fragments, while the size of each fragment is given above in kb. The *Brassica* fragments to which the petunia probes hybridize are indicated by the lines leading from the petunia fragments to the *Brassica* fragments. The petunia Pst I fragments were ordered by hybridizing each of 12 cloned petunia Pst I fragments to nitrocellulose filters containing petunia Pst I, Sal I (Bovenberg et al. 1981) and Sal I-Pst I fragments. The petunia 16S and 23S rRNA genes were mapped by Bovenberg et al. (1981) and petunia rbcL gene by J. D. Palmer, (unpublished). Restriction sites shown: (▲) Pst I; (▼) Sal I; (▽) Sac II. *Bottom panel* Fourteen nonoverlapping, cloned (Palmer and Thompson 1981a) mung bean restriction fragments were each hybridized to replica nitrocellulose filters containing Bgl I fragments from DNA 6 and Hpa I fragments from DNA 18. Mung bean mapping data are from Palmer and Thompson (1981b) and Palmer et al. (1982). Restriction sites shown: (▲) Pst I; (▼) Sal I; (●) Bgl I; (△) Hpa I

chloroplast DNAs by heterologous filter hybridizations reveals that, as a group, these *Brassica* chloroplast DNAs are colinear with petunia chloroplast DNA and differ from that of mung bean by the same large inversion of approximately 50 kb found also in petunia, spinach and cucumber (Palmer and Thompson 1982) (Fig. 3).

Comparative restriction analysis reveals, that in addition to numerous small deletions-insertions and point mutations (see next section), one small inversion may have occurred during the evolution of the *Brassica* chloroplast genome. DNAs 1, 2 and 13–17 possess adjacent Xho I fragments (Link et al. 1981) of 15.3 kb and 9.8 kb which are replaced in DNAs 3–12 and 18–22 by fragments of 16.2 kb and 8.8 kb (Table 2, Fig. 2a). This result is equally consistent with two independent Xho I restriction site mutations 1 kb apart from one another or with a single inversion of 1–3 kb. For the present purposes of phylogenetic analysis we have considered these fragment changes to result from two independent site changes (Table 2 and Fig. 4).

Mutational analysis of Brassica chloroplast DNA variation

Analysis of the restriction fragment patterns produced with Sma I reveals a broad range of evolutionary divergence among the 22 *Brassica* chloroplast DNAs (Fig. 1). However, direct inspection of these gel profiles, involving the successive analysis of DNAs which differ at only 3 band positions, allows one to directly reconstruct the pathway of specific restriction site mutations which differentiate even the most divergent DNAs. For example, DNAs 3–12 and 19–22 are identical to DNA 18 except for the presence of a 4.0 kilobase pair (kb) band which is replaced in DNA 18 by bands of 3.1 kb and 0.9 kb. The most likely explanation for these differences is a mutation, probably a single base substitution, that has created a new Sma I site within the 4.0 kb fragment, or alternatively, has abolished the preexisting site between the 3.1 kb and 0.9 kb fragments. Similar logic suggests that DNA 18 differs from DNAs 13 and 14 only by a single Sma I restriction site mutation (49 kb = 41 kb and 8.2 kb) and, in turn, that DNAs 13 and 14 differ from 1, 2 and 15–17 by a single site change (25 kb = 16.0 kb + 9.5 kb) (Fig. 1). Thus, the 9 fragment differences which distinguish DNAs 1, 2 and 15–17 from DNAs 3–12 and 19–22 can be explained by postulating mutations at three different Sma I sites. It is important to emphasize that these inspectional inferences concerning the map positions of mutated fragments were confirmed by constructing a complete map of *Brassica* Sma I fragments (Fig. 2a).

Variation among these 22 chloroplast DNAs was analyzed using a total of 28 different restriction enzymes (Table 2). 15 of the restriction enzymes are classified as Type I enzymes. These cut the DNA rarely enough so that restriction fragment changes could be analyzed for the entire group of 22 DNAs (Table 2). The patterns for 7 of these enzymes, 3 of which are invariant for the 22 DNAs, were analyzed solely by inspection, while for 8 enzymes an independent mapping approach (Fig. 2a) was used to confirm the inspectional analysis (Table 2). The 23 independent mutations mapped in Figs. 2a, b are distributed throughout the genome, with a very slight tendency to cluster at the ends of the large single copy region (Gordon et al. 1982; Kung et al. 1982; Palmer et al. 1983a) and in the middle of the small single copy region. Conversely, mutations tended to be slightly underrepresented in the inverted repeat (Palmer and Zamir 1982) and central portion of the large single copy region.

A total of 31 restriction site mutations were observed at 155 6-bp sites sampled with the 15 Type I enzymes (Table 2). Four major lineages are defined solely on the basis of these Type I mutations (Fig. 1). These are: 1) *B. carinata* and *B. nigra*, 2) *B. hirta*, 3) *R. sativus*, and 4) *B. oleracea*, *B. campestris*, *B. juncea* and *B. napus* (Fig. 4a). 13 enzymes, designated as Type II enzymes, an additional 19 mutations were was not possible to satisfactorily analyze all the variation observed between these four major lineages, but were extremely useful in providing increased resolution within the four lineages (Table 2, Fig. 4a). Using Type II enzymes, and additional 19 mutations were observed at 365 sites (2083 bp) within each of the four major lineages defined by Type I mutations (Table 2, Fig. 4a).

In order to determine the evolutionary direction for the Type II mutations, the largest changed fragment for each mutation was isolated by preparative agarose gel electrophoresis, labeled with ^{32}P by nick-translation and hybridized to a nitrocellulose filter blot containing the mutated DNAs plus one or more DNAs from each of the other three major lineages (as defined by Type I mutations) to serve as outside reference groups (outgroups). For example, using the Type II enzyme EcoRV, DNAs 3 and 5 were observed to differ from DNAs 4, 6–12 and 19–22 at three fragment positions (Table 2). The direction of this mutation, and also the physical relationships of the altered fragments, was determined by isolating the 12.2 kb EcoRV fragment and hybridizing it to filter-bound EcoRV-digested chloroplast DNAs from accessions 3, 8, 13, 17 and 18. The 12.2 kb fragment hybridized to a fragment of similar size in the three outgroup DNAs (13, 17, 18) and to fragments of 9.4 kb and 2.8 kb in DNA 8 (data not shown). Thus, it was concluded that these fragment changes result from a site gain in DNAs 4, 6–12 and 19–22, rather than a site loss in DNAs 3 and 5.

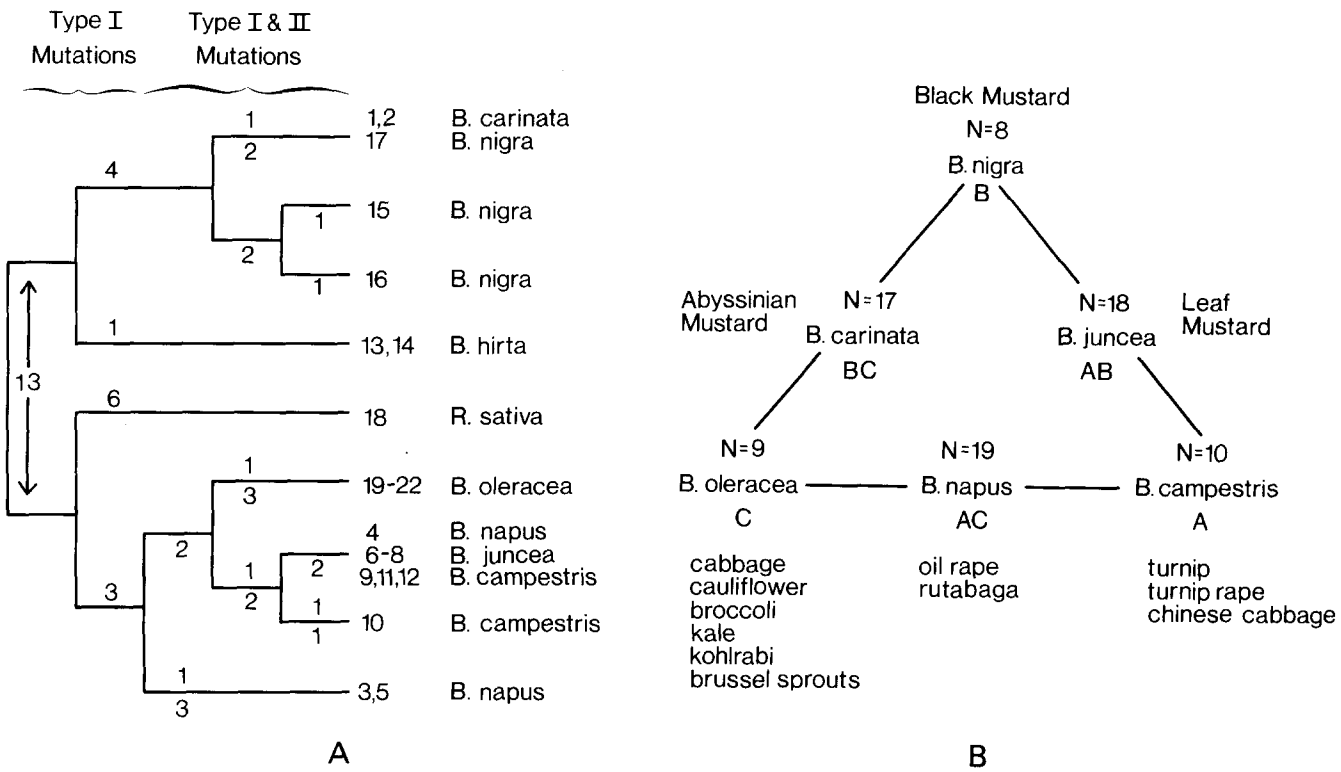

Fig. 4. **A** *Brassica* chloroplast DNA phylogeny. Numbers at termination of branches indicates accession (Table 1). Numbers above branches indicate the number of Type I mutations specific to each branch, while numbers below branches indicate the number of Type II mutations (Table 2; see text). **B** Classical phylogeny for cultivated diploid and tetraploid species of *Brassica* based on cytogenetic analysis (U 1935)

Brassica chloroplast DNA phylogeny

40 of the 50 Type I and II mutations are phylogenetically informative – i.e. are shared by two or more DNAs – and were used to construct a chloroplast DNA phylogeny according to principles of parsimony analysis (Ferris et al. 1981; Palmer and Zamir 1982). The most parsimonious tree (Fig. 4a) requires a minimum of 41 independent mutations to account for the observed distribution of the 40 phylogenetically informative DNA phenotypes. This tree postulates the independent occurrence twice, once in DNAs 3 and 5 and once in DNAs 4 and 6–12, of a Nru I mutation (4.1 kb = 2.5 kb + 1.6 kb) shared by DNAs 3–12. An alternative tree, less parsimonious by one mutation, would exchange DNAs 19–22 with DNAs 3 and 5 in Fig. 4a, thereby postulating 2 cases of parallelisms (Eco RV and Cla I mutations, Table 2) involving DNAs 4 and 6–12 and DNAs 19–22. Another instance of apparent parallelism in restriction site mutations, which does not, however, impinge upon the analysis presented in Fig. 4a, was observed with an Xmn I mutation shared by DNAs 13, 14 and 16 (Table 2).

In order to root our tree we have assumed that the chloroplast genome is evolving at a fairly constant, clocklike rate (Wilson et al. 1977). This assumption results in a tree (Fig. 4a) in which the numbers of mutations are most nearly equal in all lineages. Formal cladistic analysis would use the best outgroup available, in this case radish (*R. sativus*), in order to root such a tree. This would result in an extremely lopsided phylogeny, with 14–18 rooted mutations for DNAs 1, 2 and 13–17 as compared to only 4–5 rooted mutations for DNAs 3–12 and 19–22. In similar phylogenetic analysis of chloroplast DNA variation in the genus *Lycopersicon* (Palmer and Zamir 1982) it was found that the same root was obtained when analysis was based either on the clock assumption or on outgroup comparisons. In the present case, we note the rather weak fertility barriers between radish and the *Brassica* species examined (Karpechenko 1924), indicating not only much stronger genetic affinities than in the *Lycopersicon* case (Palmer and Zamir 1982), but also increasing the likelihood of cytoplasmic transfer between species via introgression, as appears to have happened with DNAs 3 and 5 (see below).

Evolution of amphiploidy in Brassica

A great diversity of taxonomic approaches (Prakash and Hinata 1980) – including comparative anatomy

(Berggren 1962), cytogenetics (U 1935), artificial resynthesis of hybrids (Olsson 1960a, 1960b), and molecular analysis of secondary chemical compounds (Vaughan 1977; Dass and Nybom 1967), proteins (Uchimiya and Wildman 1978; Gatenby and Cocking 1978; Vaughan 1977) and nuclear DNA (Verma and Rees 1974) – are compatible with the so-called "triangle of U" (U 1935; Fig. 4b), hypothesized to explain relationships among the six agriculturally important diploid and amphidiploid species of *Brassica*. The only evidence concerning the direction of these crosses comes from isoelectric focusing studies (Uchimiya and Wildman 1978) on the chloroplast-encoded (Link 1981), maternally-inherited (Palmer et al. 1983b) large subunit polypeptide of ribulose-1,5-bisphosphate carboxylase. Uchimiya and Wildman found two types of large subunit patterns – one common to *B. nigra*, *B. carinata*, and *B. hirta* and one shared by the other 5 species examined (Table 1). They concluded that *B. nigra* and *B. campestris* were the maternal parents for *B. carinata* and *B. juncea*, respectively, while no assignment could be made for *B. napus*.

Chloroplast DNAs from both accessions of *B. carinata* examined are identical at 3013 bp sampled to that of *B. nigra* no. 17, while DNAs from all three *B. juncea* and from *B. napus* no. 4 are identical to those of *B. campestris* no. 9, no. 11 and no. 12 (Fig. 4a). Thus one can not only identify the maternal parent species (and by subtraction, the paternal parent as well; Fig. 4a, b) for each of these amphidiploids, but can also implicate specific types within each of these diploids as the likely maternal parent. Quite surprisingly, chloroplast DNA analysis indicates that *B. napus* no. 3 and no. 5 branched off from a lineage containing their putative parents, *B. oleracea* and *B. campestris*, prior to the divergence of the two diploids from one another (Fig. 4a). This result is contradictory to all published analyses (see above references) for a large number of diverse accessions from these 3 species (Fig. 4b). In addition, analysis of nuclear ribosomal DNA sequences from the very plants examined in this study reveals that the nuclear genomes of all 3 *B. napus* contain equal amounts of *B. oleracea*- and *B. campestris*-specific sequences (J. D. Palmer, unpublished).

This contradiction between maternal, chloroplast DNA-based and biparental, nuclear-DNA based phylogenies very strongly implicates introgressive hybridization – the repeated backcrossing of a natural hybrid to one or both parental populations (Anderson 1953) – in the origin of *B. napus* no. 3 and no. 5. We postulate that at some point either *B. oleracea*, *B. campestris* or their F_1 hybrid derivative served as the paternal parent in a cross with some unknown species. Following this initial cross the new hybrid served as the maternal parent in a series of backcrosses to the original paternal parent population, ultimately leading to the completely new combination of cytoplasm and nucleus now found in *B. napus* no. 3 and no. 5.

Table 3. Percent sequence divergence values for different chloroplast DNA pairs. Values are calculated as 100p, where p is the estimated substitutions per base pair from equation 3 of Brown et al. (1979). Panel A is based on Type I mutations only (Fig. 4a) and represents comparisons of 930 base pairs (155 six-base pair restriction sites). Panels B and C are based on both Type I and Type II mutations (Fig. 4a) and represent comparisons of 3013 base pairs (452 six-base pair sites, 29 five-base pair sites and 39 four base-pair sites)

A		1, 2, 17	13, 14	18	3–9, 11, 12, 19–22
	1, 2, 17	–			
	13, 14	0.65	–		
	18	2.59	2.15	–	
	3–9, 11, 12, 19–22	2.37	1.94	1.08	–
B		1, 2, 17	15	16	
	1, 2, 17	–			
	15	0.20	–		
	16	0.20	0.07	–	
C		19–22	4, 6–8 9, 11, 12	10	3, 5
	19–22	–			
	4, 6–8 9, 11, 12	0.30	–		
	10	0.30	0.13	–	
	3, 5	0.33	0.37	0.37	–

The large number of mutations analyzed in this study allows quantitative conclusions concerning both the relative time of hybridization and also the relative divergence of the diploid parents (Table 3, Fig. 4a). The identity of chloroplast DNAs from all assigned amphidiploids (*B. carinata, B. juncea, B. napus* no. 4) to those of their respective maternal parents indicates that all these hybridizations occurred very recently. Clearly, no equivalent conclusion can be reached for *B. napus* no. 3 and no. 5. *B. oleracea* and *B. campestris* are much more closely related (0.3% sequence divergence) to each other than either diploid is to *B. nigra* (2.4% sequence divergence), both now and also at the time of their various hybridizations.

On the basis of cytoplasmic relationships, both radish (*R. sativus*) and white mustard (*B. hirta;* syn. *Sinapis alba* in Europe) clearly belong in the genus *Brassica*. Radish is fairly closely related (1.1% divergence, Table 3) to the *oleracea-napus-juncea-campestris* complex and white mustard to the *nigra-carinata* complex (0.6% divergence). It is possible that introgression has also affected cytoplasmic relationships for these species; examination of this issue requires detailed phylogenetic analysis of nuclear DNA variation and is currently in progress.

Discussion

The wealth of taxonomic information available on evolutionary relationships in the genus *Brassica* makes this an ideal case study group to examine the additional evolutionary insights that can be gained by mutational analysis of chloroplast DNA variation. Our findings indicate that, even in this well-characterized group, chloroplast DNA analysis can yield new information concerning the origin of polyploid species – including the revelation of an unexpected case of introgressive hybridization – and generally provides previously unattainable quantitative information on species relationships.

The total range of sequence variation encountered in *Brassica* (2.4%) is over 3 times that found in *Lycopersicon* (Palmer and Zamir 1982). Although this greater variation necessitated a two-tier system, including more tedious blot hybridizations, for evaluating mutations, it was still possible to delineate overall relationships for the entire group while at the same time making critical fine distinctions within terminal subgroups. The level of sequence divergence observed within *Brassica* is still much less than the range of divergence values encountered in interspecific studies of animal mitochondrial DNA variation (Avise et al. 1979a, 1979b; Brown and Simpson 1981) and is consistent with the notion that the chloroplast genome is evolving relatively slowly (Palmer and Zamir 1982). However, more comparative data relating chloroplast and nuclear evolutionary rates, particularly in plant groups with good fossil records, are needed before this conclusion can be made with any certainty.

The results presented in this and previous studies (Palmer and Zamir 1982; Timothy et al. 1979; Kung et al. 1982; Vedel et al. 1978, 1981; Lebacq and Vedel 1981) demonstrate that chloroplast DNA analysis can be used to study a wide range of problems in plant evolution and systematics. A major question which remains is: What are the practical limits, both in terms of base sequence divergence and also in terms of the taxonomic range of plants that can be studied, to which chloroplast DNA analysis can be extended? In general, the major factor which limits phylogenetic interpretation of macromolecular sequence data, nucleic acid or protein, is the incidence of parallel and back mutations. So far, the incidence of these mutations is very low, 4.0% in *Brassica* and 2.5% in *Lycopersicon* (Palmer and Zamir 1982), which is to be expected given the extremely low absolute rate of base change observed. Adaptation of these techniques to study older and larger taxonomic groups, where one can expect to find a significantly greater amount of base change, accompanied by a higher rate of parallelisms, will probably involve direct sequence analysis (Brown et al. 1982; Zurawski et al. 1981; Tohdoh and Sugiura 1982) of a small, relatively slowly evolving portion of the genome.

Acknowledgements. We are grateful to William F. Thompson, in whose laboratory this research was done, to B. Osorio for expert technical assistance, to G. Link for communication of unpublished mapping data, and to R. Jorgensen, N. Polans, D. Stern and M. Zolan for helpful discussion. This is CIW-DPB publication no. 805.

References

Anderson E (1953) Introgressive hybridization. Biol Rev 28: 280–307

Avise JC, Giblin-Davidson C, Laerm J, Patton JC, Lansman RA (1979a) Mitochondrial DNA clones and matriarchal phylogeny within and among geographic populations of the pocket gopher, *Geomys pinetis.* Proc Natl Acad Sci USA 76:6694–6698

Avise JC, Lansman RA, Shade RO (1979b) The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. 1. Population structure and evolution in the genus *Peromyscus.* Genetics 92:279–295

Berggren G (1962) Reviews on the taxonomy of some species of the genus *Brassica*, based on their seeds. Sv Bot Tidskr 56:65–135

Bovenberg WA, Kool AJ, Nijkamp HJJ (1981) Isolation, characterization and restriction endonuclease mapping of the *Petunia hybrida* chloroplast DNA. Nucleic Acids Res 9:503–517

Brown GG, Simpson MV (1981) Intra- and interspecific variation of the mitochondrial genome in *Rattus norvegicus* and *Rattus rattus:* restriction enzyme analysis of variant mitochondrial DNA molecules and their evolutionary relationships. Genetics 97:125–143

Brown WM, George MJr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76:1967–1971

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 18:225–239

Clausen J, Keck DD, Hiesey WM (1945) Plant evolution through amphiploidy and autoploidy with examples from the Madiinae. Carnegie Inst Washington Publ 564:1–174

Crawford DJ, Giannasi DE (1982) Plant chemosystematics. Bioscience 32:114–124

Dass H, Nybom H (1967) The relationships between *Brassica nigra, B. campestris, B. oleracea,* and their amphidiploid hybrids studied by means of numerical chemotaxonomy. Can J Genet Cytol 9:880–890

Dewet JMJ (1980) Origin of polyploids. In: Lewis WH (ed) Polyploids. Biological relevance. Plenum, New York, pp 3–15

Ferris SD, Wilson AC, Brown WM (1981) Evolutionary tree for apes and humans based on cleavage maps of mitochondrial DNA. Proc Natl Acad Sci USA 78:2432–2436

Fluhr R, Edelman M (1981) Conservation of sequence arrangement among higher plant chloroplast DNAs: molecular cross hybridization among the Solanaceae and between *Nicotiana* and *Spinacia.* Nucleic Acids Res 9: 6841–6853

Gatenby AA, Cocking EC (1978) The evolution of fraction 1 protein and the distribution of the small subunit polypeptide coding sequences in the genus *Brassica.* Plant Sci Lett 12:299–303

Gordon KHJ, Crouse EJ, Bohnert HJ, Herrmann RG (1982) Physical mapping of differences in chloroplast DNA of the five wild-type plastomes in *Oenothera* subsection *Euoenthera*. Theor Appl Genet 61:373–384

Grant V (1981) Plant speciation. Columbia University, New York, pp 1–563

Gray JC (1980) Fraction 1 protein and plant phylogeny. In: Bisby FA, Vaughan JG, Wright CA (eds) Systematics association – special vol 16, Chemosystematics: principles and practice. Academic Press, London New York, pp 167–193

Karpechenko GD (1924) Hybrids of *Raphanus sativus* × *Brassica oleracea* L. J Genet 14:375–396

Kolodner R, Tewari KK (1975) The molecular size and conformation of the chloroplast DNA from higher plants. Biochim Biophys Acta 402:372–390

Kung SD, Zhu YS, Shen GF (1982) *Nicotiana* chloroplast genome. 3. Chloroplast DNA evolution. Theor Appl Genet 61:73–79

Lebacq P, Vedel F (1981) Sal I restriction enzyme analysis of chloroplast and mitochondrial DNAs in the genus *Brassica*. Plant Sci Lett 23:1–9

Link G (1981) Cloning and mapping of the chloroplast DNA sequence for two messenger RNAs from mustard (*Sinapis alba* L.). Nucleic Acids Res 9:3681–3694

Link G, Chambers WE, Thompson JA, Falk H (1981) Size and physical organization of chloroplast DNA from mustard (*Sinapis alba* L.) Mol Gen Genet 181:454–457

Maxam AM, Gilbert W (1980) Sequencing end-labeled DNA with base-specific chemical cleavages. In: Grossmann L, Moldave K (eds) Methods in enzymology, vol 65 I Academic Press, New York, pp 499–560

Olsson G (1960a) Species crosses within the genus *Brassica*. 1. Artificial *Brassica juncea* Coss. Hereditas 46:171–222

Olsson G (1960b) Species crosses within the genus *Brassica*. 2. Artificial *Brassica napus* L. Hereditas 46:351–396

Palmer JD (1982) Physical and gene mapping of chloroplast DNA from *Atriplex triangularis* and *Cucumis sativa*. Nucleic Acids Res 10:1593–1605

Palmer JD (1983) Chloroplast DNA exists in two orientations. Nature 301:92–93

Palmer JD, Thompson WF (1981a) Clone banks of the mung bean, pea and spinach chloroplast genomes. Gene 15: 21–26

Palmer JD, Thompson WF (1981b) Rearrangements in the chloroplast genomes of mung bean and pea. Proc Natl Acad Sci USA 78:5533–5537

Palmer JD, Thompson WF (1982) Chloroplast DNA rearrangements are more frequent when a large inverted repeated sequence is lost. Cell 29:537–550

Palmer JD, Zamir D (1982) Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*. Proc Natl Acad Sci USA 79:5006–5010

Palmer JD, Edwards H, Jorgensen RA, Thompson WF (1982) Novel evolutionary variation in transcription and location of two chloroplast genes. Nucleic Acids Res 10:6819–6832

Palmer JD, Singh GP, Pillay DTN (1983a) Structure and sequence evolution of three legume chloroplast DNAs. Mol Gen Genet (in press)

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983b) An unusual mitochondrial DNA plasmid in the genus *Brassica*. Nature 301:725–728

Prakash S, Hinata K (1980) Taxonomy, cytogenetics and origin of crop *Brassica*, a review. Opera Bot 55:1–57

Timothy DH, Levings CS III, Pring DR, Conde MF, Kermicle JL (1979) Organelle DNA variation and systematic relationships in the genus *Zea:* Teosinte. Proc Natl Acad Sci USA 76:4220–4224

Tohdoh N, Sugiura M (1982) The complete nucleotide sequence of a 16S ribosomal RNA gene from tobacco chloroplasts. Gene 17:213–218

UN (1935) Genomic analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn J Bot 7:389–452

Uchimiya H, Wildman SG (1978) Evolution of fraction 1 protein in relation to origin of amphidiploid *Brassica* species and other members of the Cruciferae. J Hered 69:299–303

Vaughan JG (1977) A multidisciplinary study of the taxonomy and origin of *Brassica* crops. Bioscience 27:35–40

Vedel F, Quetier F, Dosba F, Doussinault G (1978) Study of wheat phylogeny by EcoRI analysis of chloroplastic and mitochondrial DNAs. Plant Sci Lett 13:97–102

Vedel F, Quetier F, Cauderon Y, Dosba F, Doussinault G (1981) Studies on maternal inheritance in polyploid wheats with cytoplasmic DNAs as genetic markers. Theor Appl Genet 59:239–245

Verma SC, Rees H (1974) Nuclear DNA and the evolution of allotetraploid *Brassica*. Heredity 33:61–68

Wildman SG (1979) Aspects of Fraction 1 protein evolution. Arch Biochem Biophys 196:598–610

Wilson AC, Carlson SS, White TJ (1977) Biochemical evolution. Ann Rev Biochem 46:573–639

Zurawski G, Perrot B, Bottomley W, Whitfeld PR (1981) The structure of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase from spinach chloroplast DNA. Nucleic Acids Res 9:3251–3270