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Phylogeny of *Brassica* and allied genera based on variation in chloroplast and mitochondrial DNA patterns: molecular and taxonomic classifications are incongruous

A.K. Pradhan [1], S. Prakash [2], A. Mukhopadhyay [1], and D. Pental [1]

[1] Tata Energy Research Institute, 90 Jor Bagh, New Delhi 110 003, India
[2] Biotechnology Centre, Indian Agricultural Research Institute, New Delhi 110 012, India

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Summary. Chloroplast DNA (cpDNA) variability of 60 taxa of the genus *Brassica* and allied genera comprising 50 species was studied. RFLPs for seven enzymes were generated and F values were estimated from five frequently cutting enzymes. Phenetic clusterings indicated a clear division of *Brassica* coenospecies into two distinct lineages referred to as the *Brassica* and *Sinapis* lineages. Two unexplored genera, *Diplotaxis* and *Erucastrum*, also exhibited two lineages in addition to the genera *Brassica* and *Sinapis*. This finding is inconsistent with the existing taxonomic classification based on morphology. Mitochondrial DNA (mtDNA) variability studied from *Eco*RI RFLP patterns, by hybridizing total DNA with four cosmid clones containing non-overlapping mtDNA fragments, did not show any congruence with cpDNA variation patterns. However, at the cytodeme level, the patterns of genetic divergence suggested by the cpDNA data could be correlated with mtDNA variation. In the *Brassica* lineage, *Diplotaxis viminea* was identified as the female parent of the allotetraploid *D. muralis*. The chloroplast DNAs of *Erucastrum strigosum* and *Er. abyssinicum* were found to be very closely related. In the *Sinapis* lineage, *Brassica maurorum* was found to be the diploid progenitor of autotetraploid *B. cossoneana*. *B. amplexicaulis* showed a very different cpDNA pattern from other members of the subtribe. *Brassica adpressa* was closest to *Erucastrum laevigatum* and could be the diploid progenitor of autotetraploid *Er. laevigatum*. Based on the close similarity of the cpDNA pattern of *Diplotaxis siifolia* with that of *D. assurgens*, we have proposed the retention of this species in the genus *Diplotaxis*. The taxonomic positions of some other species have also been discussed.

Correspondence to: A.K. Pradhan

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Introduction

The subtribe Brassicinae of the family Brassicaceae includes a considerable number of wild and crop species. Based on morphological observations, Schulz (1936) recognized 11 genera and 90 species in this subtribe. Gomez-Campo (1980), however, recognized only nine genera. The relationship of species belonging to the Brassicinae has also been extensively studied cytogenetically. Harberd (1972, 1976) took into consideration the chromosome number, chromosome pairing, and the extent of fertility in the hybrids and established 46 cytodemes which included the following genera – *Brassica*, *Diplotaxis*, *Erucastrum*, *Raphanus*, *Hirschfeldia*, *Trachystoma*, *Sinapis*, *Enarthrocarpus*, *Sinapidendron*, *Eruca* and *Hutera*. He referred to these as *Brassica* coenospecies. The limits of this grouping did not strictly correspond with that of the subtribe Brassicinae as proposed by Schulz. Harberd removed *Reboudia* and transferred two genera, *Raphanus* and *Enarthrocarpus*, from the subtribe Raphaninae to the Brassicinae. Takahata and Hinata (1983) recognized a total of 53 cytodemes in the *Brassica* coenospecies. These studies helped in defining the relationships of different disputed taxa within a species but the relationship between the demes remained obscure. Extensive studies have been conducted on meiotic chromosome pairing of interspecific and intergeneric crosses between species of *Brassica* and related genera (Mizushima 1968; Harberd and McArthur 1980). However, due to wide morphological variations within genera, the evolutionary relationships based on morphological data

(Takahata and Hinata 1986) were not always congruent with the cytological data.

Species relationship can be studied at different levels – morphological, cytogenetical (chromosome structure and extent of pairing), biochemical (isozyme and protein electrophoresis patterns) and molecular (restriction patterns and RFLP analysis of organelle and nuclear genomes). The small, relatively constant, size and conserved nature of the chloroplast (cp) genome makes it an ideal molecule for phylogenetic studies of different plant species (Palmer 1985). In brassicas polymorphism in cpDNA (Erickson et al. 1983; Palmer et al. 1983; Yanagino et al. 1987; Warwick and Black 1991) and nuclear DNA (Song et al. 1988a, b, 1990; Hosaka et al. 1990) have been used to study interspecific relationships. However, most of these studies were confined to a limited number of species. The only exception to-date is the study of Warwick and Black (1991) who established the molecular systematics of 33 taxa (28 species) of the subtribe Brassicinae on the basis of cpDNA polymorphism as detected by the hybridization of cpDNA probes of *B. juncea* with total cellular DNA. However, the phylogenetic relationship of some of the genera closely related to *Brassica*, namely *Erucastrum* and *Diplotaxis*, were not examined in detail.

The present study was undertaken with the objective of establishing the phylogenetic relationship of 60 taxa of the genus *Brassica* and allied genera comprising 43 diploid and seven tetraploid species based on variability in cpDNA and mitochondrial (mt) DNA restriction profiles. This study, therefore, extends the work of Warwick and Black (1991) by including a number of species belonging to *Diplotaxis* and *Erucastrum* and compares the phenetic classification derived from cpDNA and mtDNA analyses. An attempt has been made to relate the present findings to the available data on RFLP analysis of nuclear DNA, and the existing cytogenetic and taxonomic classifications.

Materials and methods

The different species used in the present investigation are listed in Table 1. Healthy, well expanded leaves from 30–45 day-old plants were collected and stored at −70 °C until used.

Chloroplast DNA analysis

Chloroplast DNA was isolated from 10 g of leaf material following Kemble (1987). DNA was digested with *Bam*HI, *Eco*RI, *Cla*I, *Hin*dIII, *Hae*III, *Xho*I and *Pst*I and electrophoresed on either 0.6% or 0.8% agarose gels in TBE (89 m*M* Tris, 2.5 m*M* EDTA and 89 m*M* Boric acid). Gels were photographed on a UV transilluminator.

Mitochondrial DNA analysis

Total DNA was extracted using the method described by Dellaporta et al. (1983). DNAs were digested with *Eco*RI, electrophoresed and transferred to nylon membranes (Hybond N, Amersham). Filters were sequentially hybridized with four cosmid clones containing non-overlapping mitochondrial DNA fragments from a mtDNA library of alloplasmic male-sterile *Brassica campestris* containing *B. oxyrrhina* cytoplasm (a detailed map of these clones will be published elsewhere). These clones, hereafter named as pCos42, 61,88 and 131, have mtDNA inserts of 40.0, 32.7, 32.4 and 34.8 kb, respectively. Probes were labelled with ^{32}P dCTP using a multiprime labelling kit (Amersham). Hybridization was carried out overnight at 42 °C in the presence of 50% formamide (Perbal 1988). Hybridization filters were washed under stringent conditions (2 × 15 min at room temperature and 2 × 15 min at 65 °C in 0.2 × SSC, 0.1% SDS) and exposed to Kodak X-OMAT AR films. For re-probing, membranes were stripped of radioactive probe by washing, for 30 min each at 42 °C, in denaturing (0.4 *M* NaOH) and neutralizing (0.2 *M* Tris, pH 8.0; 0.1 × SSC; 0.5% SDS) solutions.

Data analysis

The F value (an estimate of similarity coefficient, F = 2Nxy/Nx + Ny) was computed according to the method of Nei and Li (1979) where Nx was the number of bands detected in one cytoplasmic type, Ny the number of bands in the other cytoplasmic type, and Nxy the number of bands held in common between the two types. These parameters were computed from photographs of the same size as the original gel in the case of cpDNA and from the bands detected in autoradiograms in the case of mtDNA. F values were computed for each enzyme separately or else pooled for more than one enzyme. Phenograms were constructed according to F values by a computer programme following the unweighted pair-group procedure (Gentzbittel and Nicolas 1989). In case of cpDNA, phenograms were constructed either on the basis of F value estimates from fragment patterns of five enzymes (*Eco*RI, *Bam*HI, *Cla*I, *Hin*dIII and *Hae*III) or three enzymes (*Bam*HI, *Cla*I and *Hin*dIII). Bands of up to 0.5 kb were taken into consideration in all enzymes with the exception of *Cla*I where bands up to 1 kb were considered.

Results and discussion

Analysis of chloroplast DNA

Chloroplast DNAs, isolated from 60 taxa comprising 50 species belonging to *Brassica* and allied genera, were restricted with seven different endonucleases. As an example, the patterns of *Bam*HI-generated fragments are shown in Fig. 1. It was found that the use of frequently cutting enzymes was more informative than rarely cutting enzymes for distinguishing very closely related species. Hence, the phenetic clusterings in the present study were based on the RFLP patterns of the frequently cutting enzymes (*Eco*RI, *Bam*HI, *Cla*I, *Hin*dIII and *Hae*III). Phylogenetic trees, constructed on the basis of length and restriction site mutations of cpDNA, show a close congruence with a phenetic classification based on genetic distance or simple matching similarity coefficient (Dally and Second 1990; Warwick and Black 1991). This rationale was followed in the present study to establish the phylogenetic relationship among *Brassica* coenospecies by a phenetic classification based on cpDNA variation. Species relationship, as determined by phenetic cluster-

Table 1. List of the species of *Brassica* and allied genera included in this study

Taxa[a]	Code no.	Gametic[b] chromosome number
Brassica adpressa Boiss.	Bad	7
B. fruticulosa Cyr.	Bf	8
B. maurorum Durieu	Bm	8
B. spinescens Pomel	Bs	8
B. nigra (L) Koch		
cv. IC257[c]	B	8
B. oleracea L[c]		
var. alboglabra (chinese-kale)	C1	9
var. botrytis (cauliflower)	C2	9
var. capitata (cabbage)	C3	9
var. italica (Broccoli)	C4	9
B. oxyrrhina Coss.	Bo	9
B. barrelieri (L) Janka	Bb	10
B. tournefortii Gouan	Bt	10
B. campestris L[c]		
ssp. *oleifera*		
var. brown sarson	A1	10
var. yellow sarson	A2	10
var. toria	A3	10
ssp. *japonica*	A4	10
ssp. *chinensis*	A5	10
ssp. *parachinensis*	A6	10
ssp. *narinosa*	A7	10
B. gravinae Ten.	Bg	10
B. repanda (Willd.) DC	Br	10
B. amplexicaulis (Desf.) Pomel	Ba	11
B. cossoneana (Boiss. & Reut.) Maire	Bc	16
B. carinata Braun		
cv. IC218[c]	BC	17
B. juncea (L) Czern.		
cv. Pusa bold[c]	AB	18
B. napus L[c]		
cv. Regent	AC1	19
cv. BO-15	AC2	19
Diplotaxis erucoides (L) DC	De	7
D. siettiana Maire	Ds	8
D. virgata (Cav) DC	Dv	9
D. catholica (L) DC	Dc	9
D. assurgens (Del.) Gren	Da	9
D. tenuisiliqua Del.	Dt	9
D. siifolia G. Kunze	Dsi	10
D. viminea (L) DC	Dvm	10
D. tenuifolia (L) DC	Dte	11
D. cretacea Kotov	Dcr	11
D. pitardiana Maire	Dp	11
D. harra (Forsk.) Boiss.	Dh	13
D. muralis (L) DC	Dm	21
Eruca sativa (Mill.) Thell.	Es	11
Eruca vesicaria (L) Cav.	Ev	11
Erucastrum varium Durieu	Erv	7
Er. virgatum Presl.	Eri	7
Er. leucanthum Coss. & Durieu	Ere	8
Er. strigosum (Thunb.) O.E. Schulz	Ers	8
Er. cardaminoides (Webb ex Christ) O.E. Schulz	Erc	9
Er. laevigatum (L) O.E. Schulz	Erl	14
Er. abyssinicum (Rich.) O.E. Schulz	Era	16
Sinapis aucheri (Boiss.) O.E. Schulz	Sa	7
S. arvensis L	Sar	9
S. pubescens L.	Sp	9
S. alba L.	Sal	12
S. flexuosa Poir.	Sf	12
Raphanus raphanistrum L.	Rr	9
Raphanus sativus L.	Rs	9
Hutera leptocarpa Gonzalez-Albo	Hl	12
Rhynchosinapis pseuderucastrum (Brot.) Gz. Campo	Rp	12
Moricandia arvensis (L) DC	Ma	14
Erucaria ollivieri Maire	Ero	8

[a] Seeds from Prof. K. Hinata, Tohoku University, Sendai, Japan and Prof. C. Gomez-Campo, Universidad Politechnica, Madrid, Spain

[b] Chromosome numbers are from Gomez-Compo and Hinata (1980)

[c] Collection from our germplasm

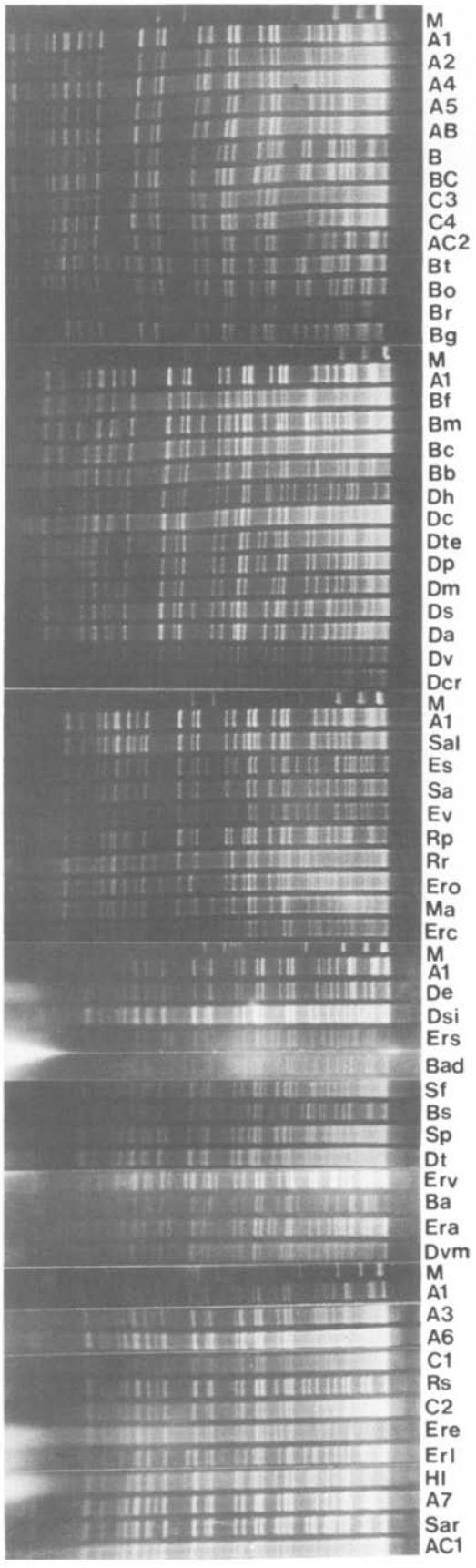
ing using three enzymes (*Bam*HI, *Cla*I and *Hin*dIII), is shown in Fig. 2. Similarly, five enzymes (*Eco*RI, *Hae*III, *Bam*HI, *Cla*I and *Hin*dIII) were used to construct the phenogram for establishing the relationship between the species within a genus (Fig. 3A–D).

Chloroplast DNA analysis shows two distinct evolutionary lineages

Phenetic classification of the cpDNA patterns of different species of *Brassica* and allied genera (Fig. 2) clearly established two evolutionary pathways which we refer to as the *Brassica* and *Sinapis* lineages and which correspond to the *rapa/oleracea* and *nigra* lineages of Warwick and Black (1991). Our results, based on phenetic clustering, confirm the earlier work of Warwick and Black (1991) and extend it in a significant way by showing that two distinct lineages are also present in *Diplotaxis* and *Erucastrum* (Fig. 3B, D). In addition, these molecular analyses (Warwick and Black 1991; this study) show that the generic classifications of Schulz (1936) and Gomez-Campo (1980) do not reflect the natural relationship of species and genera within the subtribe Brassicinae.

Comparison of phenetic classification based on cpDNA with cytodeme classification and chromosome pairing data

The cytodeme classification of Harberd (1972, 1976) and Takahata and Hinata (1983) is primarily based on crossability using high fertility of the F_1 as an important criterion for establishing cytodemes. A high level of congruence was observed between the recognized cytodemes and the cpDNA data. In order to establish the relationships between species belonging to different cytodemes,



chromosome pairing data were considered. However, the pairing data were not congruent with the phenetic classification based on cpDNA, since the extent of chromosome pairing between species across the lineage could be as high as, or even more than, that between species within a lineage. For example, hybrids of *Diplotaxis erucoides* (*Brassica* lineage) × *Brassica nigra* (*Sinapis* lineage) form up to seven bivalents (Quiros et al. 1988) while *D. erucoides* × *B. oleracea* (*Brassica* lineage) form only up to four bivalents (Mizushima 1972). Similarly, *Diplotaxis tenuifolia* belonging to the *Brassica* lineage shows very little homology with *B. oleracea* (0–2 bivalents) but forms 0–6 bivalents with *Hirschfeldia incana* and 0–4 bivalents with *Hutera* (Harberd and McArthur 1980). Significant chromosome homology has also been shown between many other species belonging to the two distinct lineages, e.g., *B. campestris* × *B. nigra* (up to eight bivalents, Prakash 1973), *Sinapis arvensis* × *B. campestris/oleracea* (up to five bivalents, Mizushima 1950) and *Hutera* × *B. oleracea* (up to seven bivalents, Harberd and McArthur 1980). The extent of pairing, therefore, does not reflect the extent of divergence shown by cpDNA analysis. Comparability of pairing between species within a lineage and across lineages could be due to frequent hybridization between species since a large number of them are sympatric in distribution.

Phenetic classification based on cpDNA in comparison with RFLP analysis of nuclear DNA

Song et al. (1988 a, b, 1990) have studied the taxonomy of *Brassica* species based on RFLP analysis of nuclear DNA. Using the RFLP data they were able to establish a relationship between sub-species of *B. oleracea* and *B. campestris* and between the closely related *B. nigra* and *Sinapis arvensis* (Song et al. 1988 b, 1990). However, the cluster analysis of distantly related species is incongruent with the chloroplast data. While cpDNA analysis shows *D. erucoides* to be closely related to *oleracea/campestris* the nuclear RFLP shows *D. erucoides* to be very distant. Thus the nuclear RFLP data is only useful for establishing the relationships of closely related species and may give aberrant results when applied to more diverse species.

Phenetic classification based on mtDNA analysis

For constructing a phenogram based on mtDNA diversity, RFLP patterns were generated by hybridizing *Eco*RI-

Fig. 1. *Bam*HI restriction fragment patterns of cpDNAs from different species of the genus *Brassica* and its allied genera. The restriction pattern of *Er. virgatum* is not included. The code numbers of the species are given in Table 1. M, λ DNA marker restricted with *Hin*dIII

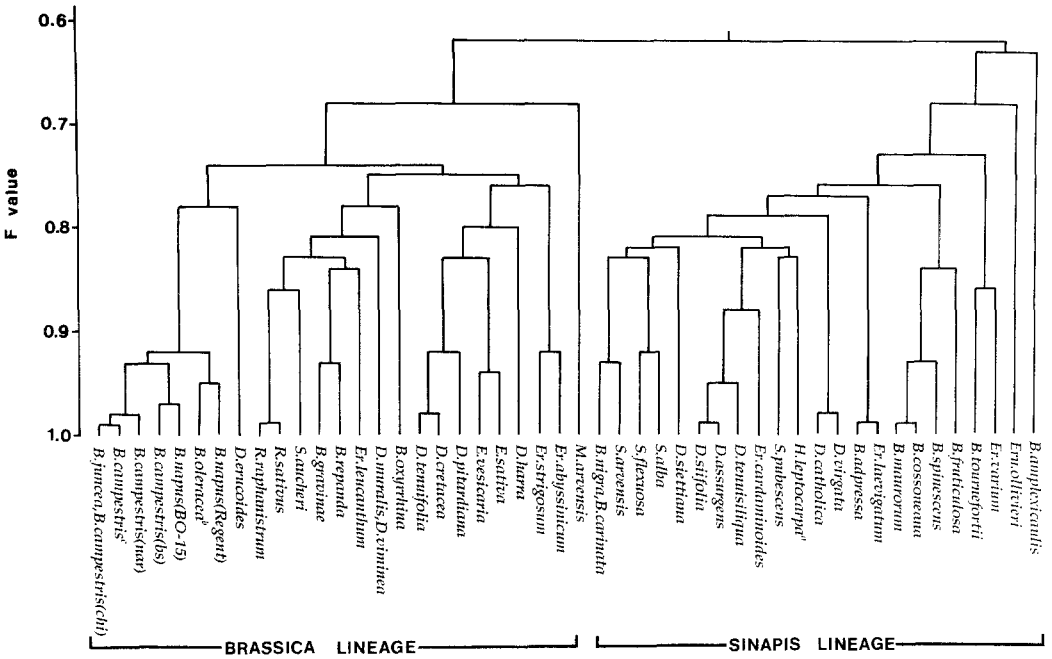


Fig. 2. Phenogram showing relationship among species of the genus *Brassica* and its related genera based on chloroplast DNA variability. F value estimates from the combined data of three enzymes (*Cla*I, *Bam*HI and *Hin*dIII) were used to construct the UPGMA phenogram. *B. barrelieri* and *Er. virgatum* are not included in the construction of the phenogram. *B, Brassica; D, Diplotaxis*; *E, Eruca*; *Er, Erucastrum*; *Eru, Erucaria*; *H, Hutera*; *M, Moricandia*; *R, Raphanus* and *S, Sinapis*. *BO-15*, a synthetic *B. napus* with *B. campestris* cytoplasm; *bs*, brown sarson; *nar*, *narinosa* and *chi*, *chinensis*; [a] includes *Rhynchosinapis pseuderucastrum*; [b] includes alboglabra, botrytis, capitata and italica; [c] includes yellow sarson, toria, *japonica* and *parachinensis*

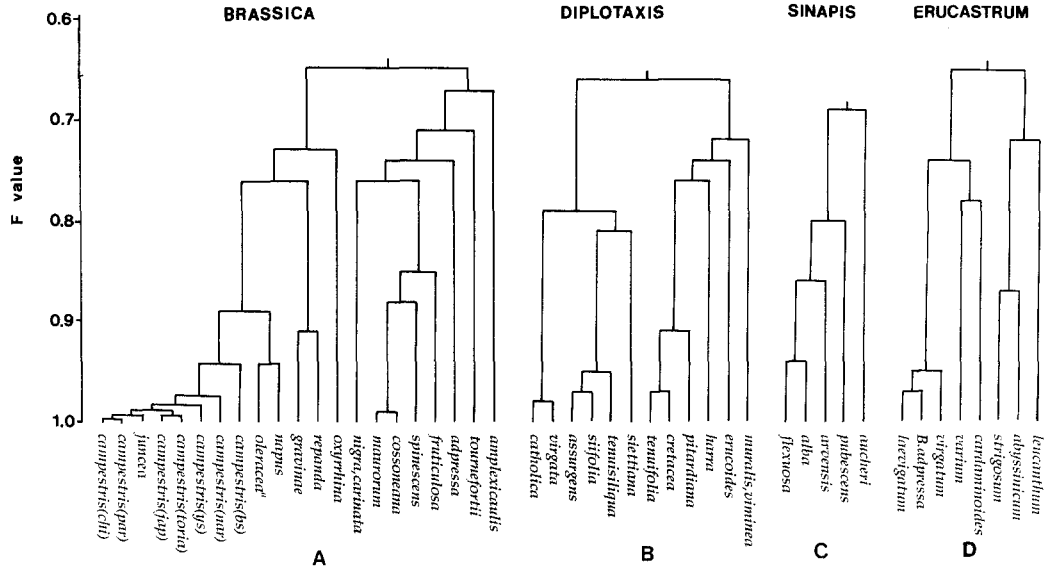


Fig. 3. Phenogram showing cpDNA diversity patterns based on F value estimates using five enzymes (*Eco*RI, *Bam*HI, *Hin*dIII, *Cla*I and *Hae*III) in the genera *Brassica* (*A*), *Diplotaxis* (*B*) and *Sinapis* (*C*) and using four enzymes (*Cla*I, *Hin*dIII, *Eco*RI and *Hae*III) in the genus *Erucastrum* (*D*). *bs*, brown sarson; *nar*, *narinosa*; *ys*, yellow sarson; *jap*, *japonica*; *par*, *parachinensis* and *chi*, *chinensis*

digested total DNA with four heterologous probes of mitochondrial origin. An example of the hybridization pattern with three different probes is shown in Fig. 4. The phenogram based on the RFLP patterns is presented in Fig. 5. In general, the diversity pattern of mtDNA did not show any congruence with the cpDNA diversity pattern (Figs. 2, 5). However, at the cytodeme level the data are congruent. For example, *D. tenuifolia*, *D. cretacea* and *D. pitardiana*, belonging to same cytodeme (Harberd 1976), also show a close relationship in both cp and

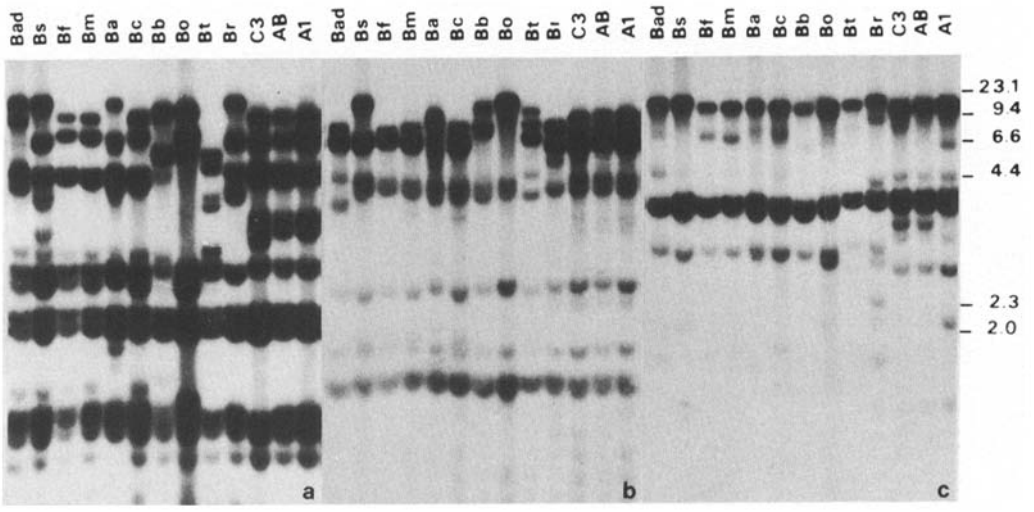


Fig. 4. Autoradiographs of *Eco*RI-digested total DNA probed with the heterologous mtDNA probes pCos42 (**a**), pCos61 (**b**) and pCos131 (**c**) showing variation among some of the *Brassica* species. The code number of the species are given in Table 1. Faint bands were detected by longer exposure of the autoradiograms

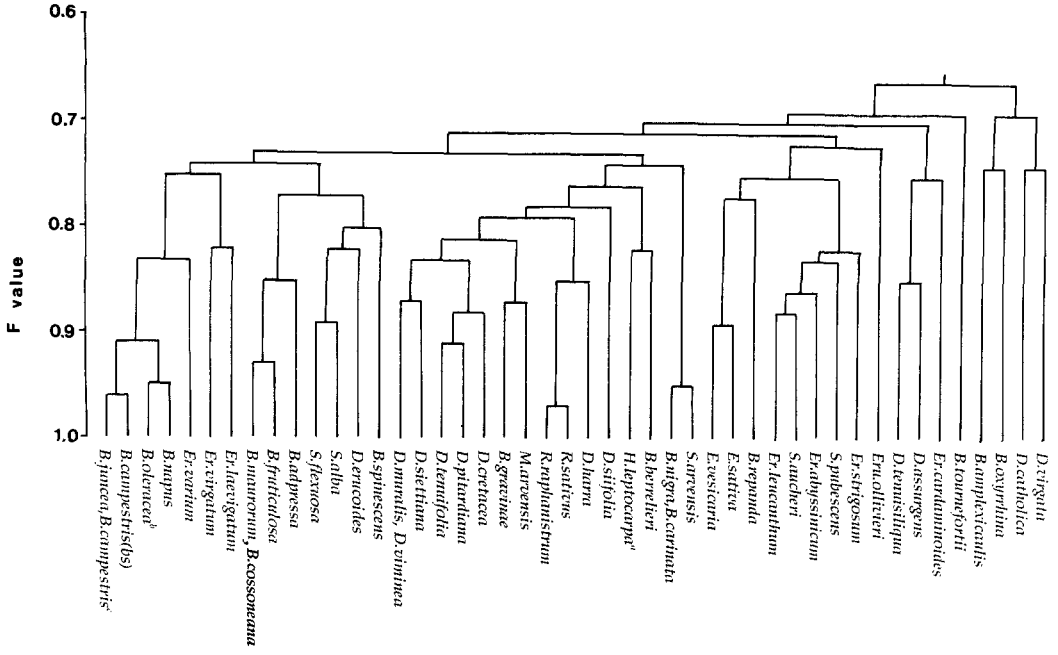


Fig. 5. Phenogram showing mtDNA diversity pattern in the genus *Brassica* and its allied genera based on F value estimates form *Eco*RI RFLP patterns detected from total DNA hybridized to four cosmid clones (pCos42, 61, 88 and 131) containing non-overlapping mtDNA fragments. *bs*, brown sarson; [a] includes *R. pseuderucastrum*; [b] includes alboglabra, botrytis, capitata, italica; [c] includes yellow sarson, toria, *japonica*, *chinensis*, *parachinensis* and *narinosa*

mtDNA patterns (Figs. 2, 3 B and 5). In plants, mtDNA is known to be extremely dynamic and, while its gene sequences are highly conserved, their location changes very rapidly due to intragenomic recombination. Therefore, phenetic classification based on mtDNA RFLP pattern cannot index the divergence in a critical manner. Terachi and Tsunewaki (1986), in their work with *Triticum* and *Aegilops*, pointed out that the mtDNA restriction patterns are useful in clarifying phylogenetic relationship between different accessions of a species, or even between species that have very similar chloroplast genome. On the other hand, mitochondrial genome

variability is so extensive among species with different chloroplast genomes that phylogenetic relationships cannot be inferred from mtDNA.

It can be concluded that the chromosome pairing, nuclear RFLP and mtRFLP data can be used only to ascertain relationships amongst closely related types. These data are difficult to use for developing a natural relationship among all the species in an ascending order of divergence.

Phylogenetic analysis

Phylogeny of Brassica. Cytogenetic evidence has shown that the three basic diploid species, *B. nigra*, *B. oleracea* and *B. campestris*, evolved in ascending order and are secondary balanced polyploids from a common archetype having the chromosome constitution x=6 (Robbelen 1960; Prakash and Hinata 1980). However, our findings clearly discount the monophyletic origin of the diploid species and agree with the proposals of Song et al. (1988a) and Warwick and Black (1991) that the genus is of biphyletic origin (Fig. 3A), where *B. campestris/oleracea* had a common progenitor and *B. nigra* evolved through another pathway. The suggestion that *B. campestris* and *B. oleracea* evolved from the same ancestor is supported by several lines of evidence: close similarities in their cytoplasms (Palmer et al. 1983; Erickson et al. 1983; Yanagino et al. 1987; Warwick and Black 1991; the present study), high chromosome pairing in F_1 hybrids of *B. campestris* × *B. oleracea* (up to nine bivalents, Olsson 1960; Namai 1971), serological similarities in seed proteins (Vaughan et al. 1966), and nuclear DNA studies (Song et al. 1988a, b, 1990; Hosaka et al. 1990; McGrath and Quiros 1991). *Diplotaxis erucoides* appears to be the closest ancestor involved in the origin of *B. oleracea* and *B. campestris*, as earlier proposed by Warwick and Black (1991). High homologies of repeat sequences between *D. erucoides* and *B. campestris/oleracea* (96 and 94% homology to *B. campestris* and *B. oleracea*, respectively, Harbinder and Lakshmikumaran 1990) substantiate the view that *D. erucoides* is related to the ancestor of *B. campestris/B. oleracea.*

Our cp and mtDNA data indicate that *B. nigra* is very close to *Sinapis arvensis* (Figs. 2, 5). This close relationship between *S. arvensis* and *B. nigra* had been suggested earlier from cpDNA data (Yanagino et al. 1987; Warwick and Black 1991) and nuclear RFLP data (Song et al. 1988a). Warwick and Black (1991) proposed that the genus *Sinapis* could be redefined to include three species of *Brassica*, viz., *B. nigra*, *B. fruticulosa* and *B. tournefortii.* Our cpDNA analysis shows that some of the *Diplotaxis* and *Erucastrum* species are more closely related to the *Sinapis* complex than this complex is to *B. fruticulosa* and *B. tournefortii* (Fig. 2). Therefore, only the transfer of *B. nigra* to the genus *Sinapis* can be justified.

Three species, *B. fruticulosa*, *B. maurorum* and *B. spinescens*, all with n=8, have been grouped into one cytodeme by Harberd (1972, 1976) and Takahata and Hinata (1983). The cpDNA and mtDNA analyses in the present study are congruent with this classification. *B. cossoneana* (2n=32) was proposed as an autotetraploid of *B. fruticulosa* (Harberd 1972). However, in view of its close similarities in cp and mtDNA with *B. maurorum*, it would be more reasonable to assume that it originated from *B. maurorum.*

Song et al. (1990) held the view that *B. tournefortii* evolved from a *B. campestris/oleracea* ancestor with introgression from *B. nigra* on the basis of similarity in cpDNA RFLP pattern (only one probe and one enzyme was used) with *B. campestris/oleracea* and high homology in nuclear DNA with *B. nigra.* Our results contradict this view and agree with Warwick and Black (1991) that *B. tournefortii* belongs to the *Sinapis* lineage.

The taxonomic position of *B. amplexicaulis* has long been controversial. It was placed in the section Melanosinapis alongwith *B. dimorpha* and *B. nigra* by Schulz (1936). Takahata and Hinata (1986), however, did not find any justification to group it with *B. nigra.* Harberd (1976) considered it to be an ancient relict since it showed very strong reproductive isolation and proved to be negative in reaction both as male and female parent. The phenetic clustering in the present study puts *B. amplexicaulis* at the base of the *Sinapis* lineage and most distant from the other taxa (Figs. 2, 3A).

Takahata and Hinata (1983) provisionally included *B. gravinae* into the *B. repanda* cytodeme. Our cpDNA data confirms the closeness of these two species and place them in the *Brassica* lineage (Figs. 2, 3A). The *Hin*dIII restriction pattern of *B. barrelieri* was not available. The phenogram constructed on the basis of four enzymes (*Eco*RI, *Bam*HI, *Cla*I and *Hae*III) indicated that *B. barrelieri* is close to *B. oxyrrhina* and belongs to the *Brassica* lineage, as has been reported by Warwick and Black (1991).

Phylogeny of Diplotaxis. The cpDNA data presented in this study clearly divides the different species of *Diplotaxis* into two distinct lineages suggesting a biphyletic origin for this genus (Fig. 3B). A high level of congruence was observed between recognized cytodemes (Harberd 1976; Takahata and Hinata 1983) and clusters defined by cpDNA variation. *D. muralis* (n=21) is a natural allotetraploid between *D. tenuifolia* (n=11) and *D. viminea* (n=10, Harberd 1972). Close similarities of cp and mtDNA between *D. viminea* and *D. muralis* points to *D. viminea* as the cytoplasmic donor in the formation of *D. muralis* and also indicates that this allopolyploid is of relatively recent origin.

The status of *D. siifolia* in the genus *Diplotaxis* has been a point of contention. Gomez-Campo and Tortosa

(1974) and Takahata and Hinata (1986) assigned it to *Brassica*, based on similarities in cotyledon shape. The present study showed that *D. siifolia* has a cpDNA very similar to that of *D. assurgens* and both are known to have sub-biseriate seeds. These facts indicate that *D. siifolia* might have evolved from *D. assurgens* so that it should remain within the genus *Diplotaxis*.

Phylogeny of Erucastrum. Seven species of genus *Erucastrum*, comprising five diploids and two tetraploids, were found to be classified into two distinct groups (Fig. 3D). One group with gametic chromosome numbers of 8 and 16 showed association with the *Brassica* lineage whereas the others, with n=7, 9 and 14, were associated with the *Sinapis* lineage. The status of *Brassica adpressa* (syn. *Hirschfeldia incana*, n=7) is controversial; it was treated as *B. adpressa* by Mizushima (1968) whereas Harberd (1972), Gomez-Campo (1980) and Takahata and Hinata (1983) regarded it as separate from *Brassica*. The cpDNA pattern of this species is very close to those of *Er. laevigatum* and *Er. virgatum*. We suggest that it should be placed under the genus *Erucastrum* and disagree with the proposal of Warwick and Black (1991) for its inclusion in the genus *Sinapis*. *Er. laevigatum* (n=14) has been reported to be an autotetraploid of *Er. virgatum* (n=7) because the hybrid behaves as a typical trivalent-forming triploid (Harberd 1976). It also forms seven bivalents and a high frequency of trivalents in the hybrid with *B. adpressa* (Harberd and McArthur 1980). Our cpDNA analysis indicates that *Er. laevigatum* is closer to *B. adpressa* than to *Er. virgatum* (Fig. 3D) although all three species form a close cluster with a F value of 0.95. On the other hand the mtDNA pattern shows that *Er. laevigatum* is close to *Er. virgatum*. Further work is required to identify the exact diploid progenitor of *Er. laevigatum*.

Er. abyssinicum (n=16) is close to *Er. strigosum* (n=8) in cpDNA. *Er. abyssinicum* has been proposed to be an autotetraploid of an unknown species (Harberd 1976). It is probable that *Er. strigosum*, or else a closely related species, is the diploid parent of *Er. abyssinicum*. At the intrageneric clustering level *Er. cardaminoides* was found to be more closely associated with *Er. varium* than with any other *Erucastrum* species (Fig. 3D). However, at the coenospecies level the former showed a closer association with *Diplotaxis assurgens* and *D. tenuisiliqua* than with *Er. varium* (Fig. 2). A similar situation was observed in terms of mtDNA classification (Fig. 5). This probably indicates a common origin of these taxa.

Phylogeny of other related genera. We believe that the transfer of *Raphanus* into the subtribe Brassicinae is justified. Our cpDNA analysis is in agreement with the earlier report of Warwick and Black (1991) that *Raphanus* is close to *S. aucheri*. *Eruca sativa* and *E. vesicaria*, belonging to same cytodeme (Harberd 1972), are very close in their cpDNA pattern, and are clustered with the *Diplotaxis* species *tenuifolia, cretacea* and *pitardiana* (Fig. 2). Both the *Eruca* and the *Diplotaxis* species have two rows of seeds in each fruit loculus. Therefore, *Eruca* spp. are closely related to *Diplotaxis* spp. of the *Brassica* lineage. Song et al. (1990) proposed a common origin of *Eruca* and *Raphanus*, based on nuclear RFLP data. However, the results of cpDNA analysis (present study) do not agree with this.

Gomez-Campo (1977) studied the clinal variation in the *Hutera-Rhynchosinapis* complex and concluded that both should be placed in the genus *Hutera*. A high fertility of F_1 hybrids between *Rynchosinapis* and *Hutera* has been reported by Harberd (1972) and Vesperinas (1991). Our observations on cp and mtDNA also reveal that both genera have exactly the same type of cytoplasm and hence it is justified to merge them.

Moricandia arvensis belongs to the subtribe Moricandinae. Its cpDNA restriction pattern differs considerably from other species of the subtribe Brassicinae and is aligned at the base of the *Brassica* lineage. Close genetic affinities are reported between *Moricandia arvensis* and the diploid *Brassica* species as revealed by the high degree of chromosome pairing in intergeneric hybrids [a maximum of six bivalents in hybrids with *B. campestris* and *B. oleracea* and five bivalents with *B. nigra* (Takahata 1990; Takahata and Takeda 1990)]. Although there is some evidence favouring the inclusion of *Moricandia* under the subtribe Brassicinae, further studies are required to confirm its taxonomic position. Similarly, the taxonomic classifications of *Erucaria* and *Reboudia* remain controversial (Schulz 1936; Harberd 1976). The cpDNA analyses of *Reboudia* (Warwick and Black 1991) and *Erucaria* (present study) place these near the base of the *Sinapis* lineage. However, the lack of any other supporting evidence makes it difficult to resolve this problem.

Conclusion

We have shown that there are two divergent lineages in the subtribe Brassicinae, which we have termed the *Brassica* and *Sinapis* lineages. This 'two-lineage concept' is reflected even at the genus level. Being highly conserved, the cpDNA analysis can be applied to resolve the taxonomic relationships of a large number and a wider range of taxa, whereas mtDNA, nuclear DNA RFLP, and cytogenetic approaches remain useful only for delineating the relationship of closely related taxa and species. We find that the phenetic classification based on cpDNA analysis is, by and large, incongruent with the taxonomic classification. Due to geographical distribution and breeding behaviour, the morphological characters used to distinguish different genera might have appeared independently in the two lineages, or free genetic exchange

might have occurred between the various species. To seek further confirmation for the presence of two phylogenetic lineages in the Brassicinae we propose the two following lines of work. (1) Use of a large number of species-specific probes of nuclear origin. Our preliminary work with a *B. nigra*-specific probe (probe pBNBH35, Gupta et al. 1992) that does not hybridize with *B. campestris*, and a *B. campestris* probe (Lakshmikumaran and Ranade 1990) that does not hybridize with *B. nigra*, has shown that, under high stringency conditions, the *B. nigra* probe hybridizes predominantly with species of the *Sinapis* lineage, and the *B. campestris* probe with species of the *Brassica* lineage. However, under low stringency there are some overlaps. Therefore, a number of species-specific probes will have to be used on allopatric populations of species belonging to the two lineages so that material with frequent nuclear gene exchange can be avoided. (2) Analysis of the nuclear rDNA non-transcribed spacer and the study of the sequence divergence of some conserved nuclear genes. If the presence of two distinct lineages can also be shown at the nuclear DNA level then a major revision of the taxonomy of the species of the subtribe Brassicinae will be fully justified.

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