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Systematic Implications of Isozyme Number Variation in Tribe Brassiceae (Brassicaceae)

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ABSTRACT

Tribe Brassiceae (haploid chromosome number, n = 6-75) is generally recognized as one of the few natural (monophyletic) groups in the Brassicaceae. It is divided into seven subtribes on the basis of morphological characteristics and includes some 220 species and 54 genera. Recent molecular analyses, however, have questioned the validity of this subtribal classification and have proposed new generic and subtribal circumscriptions. The present study examined isozyme number, which is usually highly conserved in diploid plants, in order to assess the evolution of chromosome number and systematic relationships in the tribe. Ten enzyme systems were surveyed for 108 species in 35 genera of tribe Brassiceae and for eleven species from seven other tribes of the family. Taxa with n = 7-18 were found to exhibit basically diploid isozyme profiles, refuting the widespread belief that taxa with n = 14-18 evolved by way of entire genome duplication from taxa with lower base chromosome numbers. These results suggest that aneuploidy has played a more significant role than entire genome duplication in the evolution of base chromosome numbers in the tribe.

The observed variation in isozyme number among taxa was phylogenetically informative. Duplications for

cytosolic phosphoglucomutase (*Pgm*-2) and plastid triose-phosphate isomerase (*Tpi*-1) were evident in 33 of the 35 Brassiceae genera examined, supporting the monophyletic nature of the tribe, with inclusion of *Orychophragmus* and exclusion of *Calepina* and *Conringia*. Duplications for cytosolic isocitrate dehydrogenase (*Idh*-2) were observed in members of *Brassica*, *Diplotaxis*, *Moricandia* and *Pseuderucaria*, confirming that subtribes Moricandiinae and Brassicinae are artificially separated, as indicated by restriction site analysis of chloroplast DNA and hybridization data. The only subtribe clearly supported as monophyletic by the isozyme data was the Cakilinae which displayed duplications for cytosolic glucose-6-phosphate isomerase (*Gpi*-2) and *Idh*-2 in several taxa. Duplications for fructose-bisphosphatase (*Fbp*-1, *Fbp*-2) and *Gpi*-2 were also phylogenetically informative at lower taxonomic ranks.

The present study also compared allozyme patterns of select allopolyploids and their proposed parental species. These analyses verified *Diplotaxis tenuifolia* (n = 11) and *D. viminea* (n = 10), and *D. erucoides* (n = 7) and *Erucastrum nasturtiifolium* (n = 8) as respective parental taxa of *D. muralis* (n = 10+11) and *E. gallicum* (n = 7+8). In addition, inheritance studies conducted on *Sinapis arvensis* confirmed Mendelian inheritance at seven isozyme loci.

RÉSUMÉ

La tribu Brassiceae (nombre de chromosomes haploïde, n = 6-75) est généralement reconnue comme l'un des seuls groupes naturels (monophylétiques) de la Brassicaceae. Elle est divisée en sept sous-tribus sur la base de caractères morphologiques et inclut quelques 220 espèces et 54 genres. Pourtant, certaines analyses moléculaires ont récemment mis en question la classification sous-tribale et ont proposé de nouvelles circonscriptions taxonomiques aux niveaux sous-tribal et générique. L'étude actuelle a examiné le nombre d'isozymes, lequel est normalement très conservé dans les plantes diploïdes, afin d'évaluer l'évolution du nombre de chromosomes et les relations systématiques dans la tribu. Dix systèmes enzymatiques ont été essayés pour 108 espèces dans 35 genres de la tribu Brassiceae et pour onze espèces provenant de sept autres tribus de la famille. Les taxons avec n = 7-18 ont montré des profils isozymatiques essentiellement diploïdes, réfutant l'hypothèse voulant que les taxons avec n = 14-18 aient évolué à partir de taxons dont le nombre de chromosome de base est moins élevé par voie de duplication entière des génomes. Ces résultats suggèrent que l'aneuploïdie a joué un rôle plus important que la duplication entière des génomes dans l'évolution des nombres de chromosomes de base dans la tribu.

La variation en nombre isozymatique parmi les taxons a fourni des renseignements phylogénétiquement utiles. Des duplications pour phosphoglucomutase cytosolique (*Pgm*-2) et triose-phosphate isomérase plastide (*Tpi*-1) ont été évidentes dans 33 des 35 genres examinés de la tribu Brassiceae, appuyant une origine monophylétique pour la tribu avec inclusion d'*Orychophragmus* et exclusion de *Calepina* et *Conringia*. Des duplications pour isocitrate déhydrogénase cytosolique (*Idh*-2) ont été observées dans certains membres des genres *Brassica*, *Diplotaxis*, *Moricandia* et *Pseuderucaria*, affirmant que les sous-tribus Moricandiinae et Brassicinae sont artificiellement séparées, tel qu'indiqué par des études des sites de restriction de l'ADN chloroplastique et de l'hybridisation. La seule sous-tribu que les données isozymatiques ont clairement établie comme monophylétique s'agit de la Cakilinae, dont certains membres ont démontré des duplications pour glucose-6-phosphate isomérase (*Gpi*-2) et *Idh*-2. Des duplications pour fructose-bisphosphatase (*Fbp*-1, *Fbp*-2) et *Gpi*-2 ont également été phylogénétiquement utiles, cependant à des niveaux taxonomiques plus bas.

L'étude actuelle a aussi comparé les patrons allozymatiques entre certaines espèces allopolyploïdes et leurs parents proposés. Ces analyses ont vérifié *Diplotaxis tenuifolia* (n = 11) et *D. viminea* (n = 10), et

D. erucoides (n = 7) et *Erucastrum nasturtiifolium* (n = 8) comme parents respectifs de *D. muralis* (n = 10+11) and *E. gallicum* (n = 7+8). De plus, des analyses de l'héritance chez *Sinapis arvensis* ont confirmé que l'héritance à sept lieux isozymatiques conforme aux normes Mendéliennes.

Knowledge is simply a kind of fuel;
it needs the motor of understanding
to convert it into power.

- John Wyndham

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INTRODUCTION

Background

The Brassicaceae (= Cruciferae) contains approximately 3350 species and 340 genera (Al-Shehbaz 1984), and is recognized as a well-defined, natural family on the basis of its remarkably uniform basic floral and fruit structures (Hedge 1976; Rich 1991). The vast majority of Brassicaceae species exhibit four sepals, four equal cruciform petals, six tetradynamous stamens (*i.e.*, a short outer pair and two longer inner pairs), a single ovary with two parietal placentae and a distinctive fruit consisting of a capsule with a false septum (Hedge 1976; Rich 1991). The most contentious aspect of the taxonomy of the family concerns the number of tribes into which it is subdivided (Al-Shehbaz 1984). The last comprehensive taxonomic treatment recognized 19 tribes (Schulz 1919, 1924, 1936) and is regarded as "an excellent system for identification purposes" (Hedge 1976). It has been criticized, however, with suggestions that some of its tribes do not form natural groups (*e.g.*, Euclidieae) and that others are artificially separated (*e.g.*, Romanschulzieae, Stanleyeae and Streptantheae) (Hedge 1976; Al-Shehbaz 1984). Various authors have proposed reducing the number of tribes to 15 (Janchen 1942, cited in Hedge 1976), 13 (Hedge 1976; Al-Shehbaz 1984) and three (Avetisian 1983), although none

of these alternative classifications is viewed as fully satisfactory (Al-Shehbaz 1984). The tribal classification of Schulz is still the most widely followed and will be used in this thesis.

Tribe Brassiceae is undoubtedly the most important tribe in the family in terms of economic value (Rich 1991). Among its many cultivated plants, are *Brassica napus* L. (canola), *B. rapa*[a] (canola), *B. oleracea* (colecrops), *B. juncea* (L.) Czern. & Coss. (brown mustard), *B. nigra* (black mustard), *Sinapis alba* (white mustard) and *Raphanus sativus* (radish). The tribe also includes a number of important agricultural weed species with *Brassica rapa* (wild rape), *Raphanus raphanistrum* (wild radish) and *Sinapis arvensis* (wild mustard) as examples.

The tribe is principally distributed in the Old World, centred in the western Mediterranean region, where some 41 of its 54 genera are either endemic or exhibit their greatest species diversity (Hedge 1976; Al-Shehbaz 1985). The tribal range extends southward to South Africa and eastward to India and Pakistan, with the monotypic genus *Orychophragmus* disjunct and endemic to China (Al-Shehbaz 1985). The tribe is very poorly represented in the New World (23 species) with only *Cakile* and possibly *Sinapis*

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[a] Nomenclatural authorities not given in text are provided in Tables 1 and 2.

arvensis [=*Brassica kaber* (DC.) Wheeler][b] believed to be indigenous (Al-Shehbaz 1985; Jacobson *et al.* 1988).

Systematic aspects

Tribe Brassiceae is widely recognized as one of the few natural or monophyletic groups in the family (Hedge 1976; Al-Shehbaz 1984; Rollins 1993), although this evolutionary relationship remains untested. It is generally distinguished by two distinctive morphological features which are unknown elsewhere in the family: heterocarpic fruits, fruits which consist of two distinct segments (Figure 1); and/or conduplicate cotyledons, cotyledons which are longitudinally folded around the radicle (Figure 2) (Hedge 1976; Gómez-Campo 1980; Al-Shehbaz 1985). Either or both of these features are present in the majority of Brassiceae species, with the exception of *Ammosperma*, *Conringia*, *Orychophragmus*, *Pseuderucaria* and possibly *Calepina* (Gómez-Campo 1980; Al-Shehbaz 1985).

Despite lacking the typical features, *Ammosperma* and *Pseuderucaria* have always been associated with *Moricandia* and are widely considered to be legitimate members of the tribe (Gómez-Campo 1980; Al-Shehbaz 1985). Given the placement of these genera in the tribe, Al-Shehbaz (1985)

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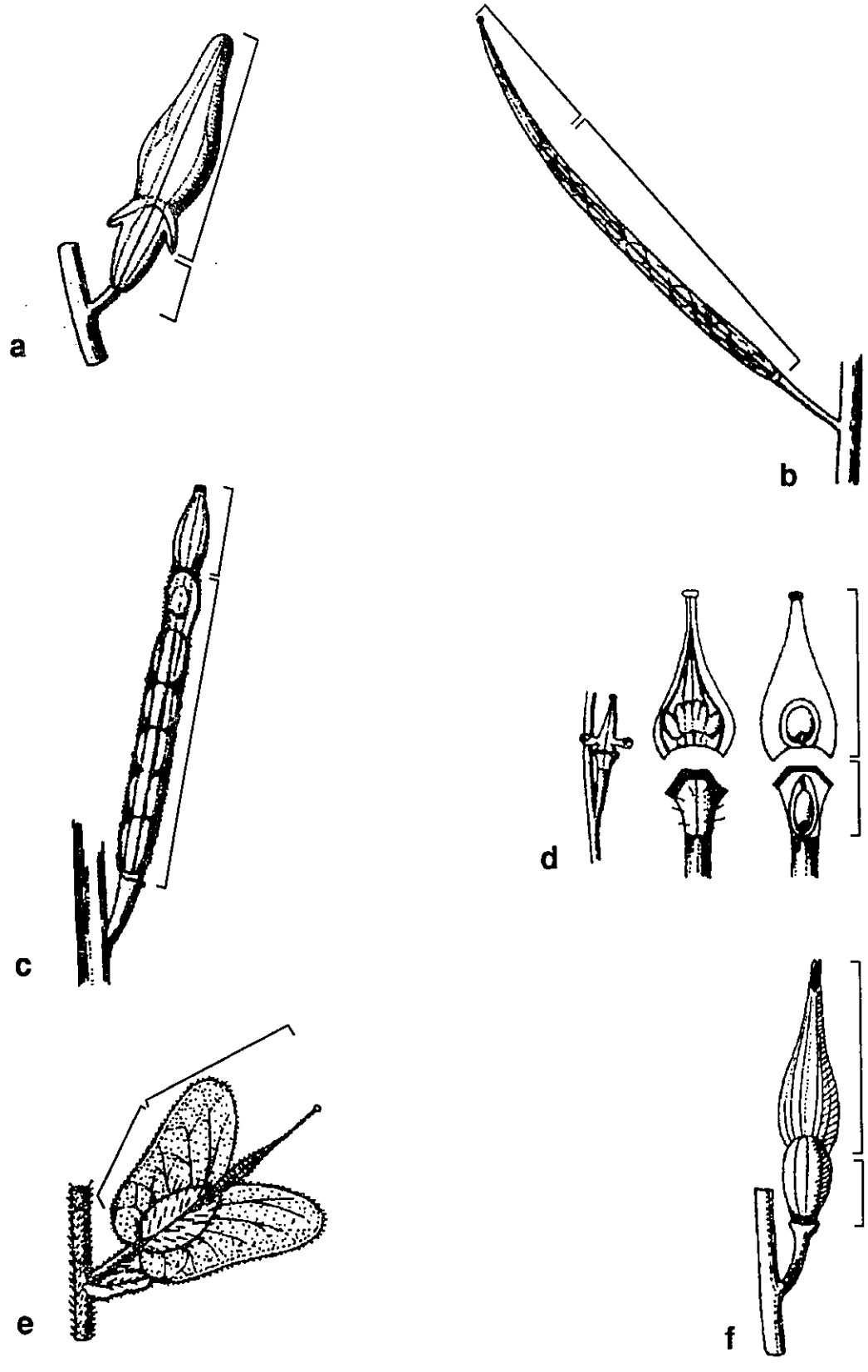
[b] Taxonomic synonymy provided in square brackets when appropriate.

Figure 1. Heterocarpic fruits of (a) *Cakile maritima*; (b) *Coincya monensis* [=*Rhynchosinapis cheiranthos* (Vill.) Dandy], (c) *Hirschfeldia incana*, (d) *Otocarpus virgatus* Durieu, (e) *Psychine stylosa*, and (f) *Rytidocarpus moricandioides* with upper and lower segments indicated. From Clemente Muñoz 1980.


a
b
c
d
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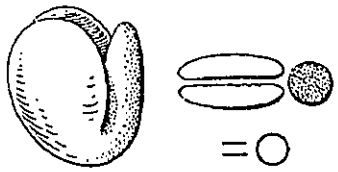
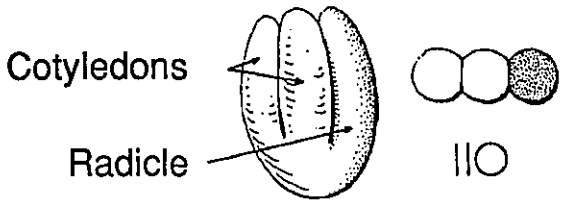
f

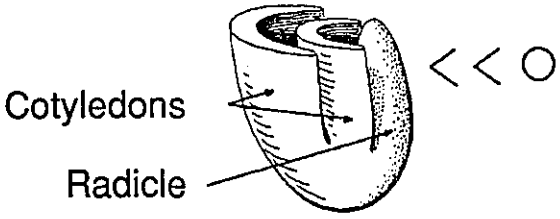
Figure 2. Cotyledon morphology of *Brassica* (Brassiceae) contrasted with that of *Barbarea* (Arabideae) and *Sisymbrium* (Sisymbrieae). From Taxonomy of Flowering Plants, by Porter. Copyright (c) 1967 by W.H. Freeman and Company. Used with permission.



Cotyledons accumbent
(*Barbarea*)



Cotyledons incumbent
(*Sisymbrium*)



Cotyledons conduplicate
(*Brassica*)

believed "there [to be] no solid grounds" to support the exclusion of *Conringia*, as proposed by Botschantzev (1966, cited in Al-Shehbaz 1985). Al-Shehbaz (1985) further supported retaining the genus in the tribe based on the nearly conduplicate cotyledons of one of its members, *C. planisiliqua* Fischer & C.A. Meyer. The taxonomic position of the monotypic genus *Calepina* is also controversial. Gómez-Campo (1980) proposed excluding it from the tribe since it lacked the typical morphological features. However, Al-Shehbaz (1985) recommended retaining it, since it is has "somewhat conduplicate cotyledons", and is without close relatives elsewhere in the family. Its taxonomic position remains, therefore, unresolved. Several authors have supported excluding *Orychophragmus* from the tribe based on its atypical geographical distribution (China) and lack of the typical morphological features (Gómez-Campo 1977, 1980; Al-Shehbaz 1985). Botschantzev (1966, cited in Gómez-Campo 1977) believed it to be closer to tribe Hesperideae. Because the evidence supporting their exclusion is weak, *Calepina*, *Conringia* and *Orychophragmus* will be treated in this study as members of the Brassiceae as proposed by Schulz (1936).

The last comprehensive taxonomic treatment of the tribe (Schulz 1919, 1924, 1936) recognized 203 species and 52 genera, including *Brassicella* Fourr. ex O.E. Schulz and *Reboudia* Coss. & Durieu which are now included in *Coincya*

and *Erucaria* respectively (Leadlay and Heywood 1990; Greuter *et al.* 1986). Since 1936, several new species and genera (*viz.*, *Dolichorhynchus*, *Pseudofortuynia*, *Quezeliantha* and *Quidproquo*) have been described, bringing the number of species and genera to 220 and 54, respectively, if *Calepina*, *Conringia* and *Orychophragmus* are included (Table 1) (Warwick and Francis 1994).

Within the tribe, Schulz (1919, 1924, 1936) recognized seven subtribes: Brassicinae Hayek, Cakilinae (DC.) O.E. Schulz, Moricandiinae Hayek, Raphaninae Hayek, Savignyinae Hayek, Vellinae Hayek and Zillinae DC.. He distinguished the subtribes using only a few morphological characters, placing particular emphasis on fruit morphology (*e.g.*, degree of fruit reduction and segmentation). Subtribes Brassicinae and Moricandiinae are characterized by dehiscent, elongated fruits and are differentiated by two main features: median floral nectaries (present in Brassicinae, absent in Moricandiinae) and beak morphology (usually seeded in Brassicinae, seedless in Moricandiinae) (Al-Shehbaz 1985). In contrast, fruits in the remaining five subtribes are reduced or nucamentaceous (nut-like) (Gómez-Campo and Tortosa 1974). Subtribe Raphaninae has indehiscent, usually strongly-segmented fruits with seeds in both segments or in the upper one only (Al-Shehbaz 1985). Subtribes Vellinae and Zillinae both have two-segmented fruits with beaked upper segments. Fruit

Table 1. Genera of tribe Brassiceae followed by number of species[a].

Ammosperma Hook. f.	2	*Hemicrambe* Webb	2
Boleum Desv.	1	*Henophyton* Coss. & Durieu	1
Brassica L.	35	*Hirschfeldia* Moench	2
Cakile Miller	7	*Kremeriella* Maire	1
Calepina Adans.	1	*Moricandia* DC.	9
Carrichtera DC.	1	*Morisia* Gay	1
Ceratocnemum Coss. & Bal.	1	*Muricaria* Desv.	1
Chalcanthus Boiss.	2	*Orychophragmus* Bunge	1
Coincya Porta & Rigo ex Rouy	6	*Otocarpus* Durieu	1
Conringia Adans.	6	*Physorrhynchus* Hook.	2
Cordylocarpus Desf.	1	*Pseuderucaria* (Boiss.) O.E. Schulz	2
Crambe L.	25	*Pseudofortuynia* Hedge	1
Crambella Maire	1	*Psychine* Desf.	1
Didesmus Desv.	2	*Quezeliantha* H. Scholz	1
Diplotaxis DC.	28	*Quidproquo* Greuter & Burdet	1
Dolichorhynchus Hedge et Kit Tan	1	*Raffenaldia* Godr.	2
Douepia Cambess.	1	*Raphanus* L.	2
Enarthrocarpus Labill.	5	*Rapistrum* Crantz	2
Eremophyton Bég.	1	*Rytidocarpus* Coss.	1
Eruca Miller	3	*Savignya* DC.	1
Erucaria Gaertner	9	*Schouwia* DC.	1
Erucastrum Presl.	20	*Sinapidendron* Lowe	4
Euzomodendron Coss.	1	*Sinapis* L.	5
Fezia Pitard	1	*Succowia* Medik.	1
Foleyola Maire	1	*Trachystoma* O.E. Schulz	3
Fortuynia Shuttl. ex Boiss.	2	*Vella* L.	5
Guiraoa Coss.	1	*Zilla* Forssk.	1

[a] Modified from Warwick and Francis (1994).

morphology differs between the two subtribes with the upper segment sterile in the Vellinae and the lower segment normally sterile in the Zillinae (Schulz 1936). Subtribe Savignyinae is distinguished by its smooth, broadly winged seeds and its strongly dorsally-compressed fruits which are not distinctly two-segmented (Schulz 1936). Subtribe Cakilinae is distinguished by its lack of conduplicate cotyledons (cotyledons, lanceolate or linear, accumbent or incumbent) and strongly segmented siliques (Schulz 1936; Al-Shehbaz 1985).

Based on reports of hybridization between species with divergent fruit morphologies (*e.g.*, *Brassica oleracea* × *Raphanus sativus*), Gómez-Campo and Tortosa (1974) contended "that the fruit should not be given an excessive phylogenetic importance". They suggested that the many instances of reduced or segmented fruits in the tribe have arisen by way of convergent evolution, and advocated conducting additional studies on other characters in order to gain a better understanding of evolutionary relationships within the tribe (Gómez-Campo and Tortosa 1974). Recent studies based on morphological, hybridization and molecular data sets have also suggested that Schulz's classification does not truly reflect phylogenetic relationships. These studies, as summarized below, have provided support for alternative generic and subtribal circumscriptions.

Restriction site analysis of chloroplast DNA has shown subtribe Brassicinae to be composed of two distinct lineages referred to as the Rapa/Oleracea and Nigra lineages (Warwick and Black 1991, 1993b; Warwick *et al.* 1992) or as the Brassica and Sinapis lineages (Pradhan *et al.* 1992), respectively. These lineages are proposed to have "diverged very early in the evolution of the Brassicinae, and prior to the evolution of distinct cytodemes in the subtribe" (Warwick and Black 1991). Within the Brassicinae, several genera (*Brassica*, *Diplotaxis*, *Erucastrum*, *Sinapis*, *Trachystoma*) have been shown to have polyphyletic origins since their members were present in both lineages and/or in distinct groups within a lineage (Pradhan *et al.* 1992; Warwick and Black 1991, 1993b; Warwick *et al.* 1992). Chloroplast DNA studies have also identified as members of subtribe Brassicinae several genera which were previously assigned to other subtribes, as discussed below.

Restriction site analysis of chloroplast DNA did not support separate subtribal ranking for the Moricandiinae, providing evidence for the inclusion of *Moricandia* and *Pseuderucaria* in the Rapa/Oleracea lineage of subtribe Brassicinae, and for the inclusion of *Foleyola* in the Zillinae (Warwick and Black 1994). These findings were consistent with suggestions that the Moricandiinae is "a heterogeneous association of genera" and that the lines

separating subtribes Moricandiinae and Brassicinae are "undoubtedly artificial" (Al-Shehbaz 1985). The genetic affinity of *Moricandia* to subtribe Brassicinae was previously established by hybridization studies (reviewed in Takahata *et al.* 1993). Similarly, Gómez-Campo (1980) suggested that *Foleyola* was more closely aligned with the Zillinae on the basis of fruit and cotyledon morphology.

Chloroplast DNA studies have provided support for the monophyletic origin of subtribe Zillinae with the inclusion of *Schouwia* (Vellinae) and *Foleyola* (Moricandiinae, see above) (Warwick and Black 1994). The inclusion of these genera in the Zillinae is consistent with observations of stigma and cotyledon morphology, flower colour and chromosome number (reviewed in Warwick and Black 1994).

Chloroplast DNA studies have also supported the monophyletic origin of subtribe Vellinae with the inclusion of *Euzomodendron* (Savignyinae) (see below), and the exclusion of *Schouwia* (see above) and *Rytidocarpus* (Warwick and Black 1994). *Rytidocarpus* was found to form part of the Rapa/Oleracea lineage of subtribe Brassicinae, aligned with *Moricandia* at the base of the lineage (Warwick and Black 1994). The affinity of *Rytidocarpus* to *Moricandia* was previously recognized by Gómez-Campo (1980) and is consistent with their common base chromosome number[c],

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[c] Base chromosome number refers to the ancestral haploid number of a given taxonomic grouping.

x = 14 (Warwick and Anderson 1993).

Based on observations of fruit and cotyledon morphology, Gómez-Campo (1980) proposed including members of subtribe Savignyinae in the Vellinae. Molecular data have only partially supported this proposal, identifying *Euzomodendron* as a member of subtribe Vellinae, and leaving unresolved the taxonomic position of *Henophyton* (Warwick and Black 1994). The chloroplast genome of the only other genus of the Savignyinae, *Savignya*, remains to be studied.

Subtribe Raphaninae is considered to be the most heterogeneous of all the subtribes (Al-Shehbaz 1985). Indeed, two of its genera, *Enarthrocarpus* and *Raphanus*, have been shown to be so closely related to the crop Brassicas and other members of subtribe Brassicinae as to be capable of artificial hybridization with them (reviewed in Warwick and Black 1993a). Chloroplast DNA studies have shown the subtribe to be polyphyletic with 12 of its 15 genera aligned with taxa in either of the two lineages of subtribe Brassicinae (Warwick and Black 1991, in prep.). Notable exceptions are *Crambe* which formed a distinct entity from the other subtribes, and *Didesmus* and *Crambella* which were found aligned with taxa in subtribe Cakilinae (see below) (Warwick and Black in prep.). The uniqueness of *Crambe* was previously recognized by Gómez-Campo (1980), who suggested that it may warrant separate subtribal status. A number of morphological features (reviewed in

Warwick and Black, in prep.) and a unique base chromosome number (x = 15) set *Crambe* apart from other taxa of the subtribe.

Chloroplast DNA studies confirmed subtribe Cakilinae as a monophyletic group with the inclusion of *Didesmus* and *Crambella*, both formerly ascribed to the Raphaninae (Warwick and Black, in prep.). The affinity of *Didesmus* to members of subtribe Cakilinae is evidenced by the extensive synonymy which exists between its species and those of *Cakile* and *Erucaria* (reviewed in Schulz 1919). Similarly, the affinity of *Crambella* to *Erucaria* spp. (Cakilinae) has also been recognized on the basis of their similar growth habit, flower colour, and leaf and fruit morphologies (Warwick and Black, in prep.).

Chromosome number

Chromosome number has been particularly well documented within the Brassiceae, with counts reported for nearly 80% of the species (Gómez-Campo and Hinata 1980; Warwick and Anderson 1993; Warwick *et al.* 1993, 1994). Gametic chromosome number has been found to vary substantially from n = 6 in *Erucaria cakiloidea* (DC.) O.E. Schulz to n = 75 in *Crambe gordjaginii* Sprygin & Popov. (Warwick and Anderson 1993). No single chromosome number is dominant within the tribe as evidenced by the wide range of base numbers, x = 6-13, 15 and 17, proposed for various

genera (Al-Shehbaz 1985).

Hybridization studies have identified eleven genera (*Coincya*, *Diplotaxis*, *Enarthrocarpus*, *Eruca*, *Erucastrum*, *Hirschfeldia*, *Moricandia*, *Raphanus*, *Sinapidendron*, *Sinapis*, *Trachystoma*) "which are sufficiently related to the crop Brassicas to be potentially capable of exchanging gene material with them" (Harberd 1972; 1976; Takahata *et al.* 1993). These genera, termed *Brassica* coenospecies, correspond closely to subtribe Brassicinae with the inclusion of *Enarthrocarpus* and *Raphanus* of subtribe Raphaninae and *Moricandia* of subtribe Moricandiinae. Assuming $n = 7$ to be the lowest gametic chromosome number for the *Brassica* coenospecies, Harberd (1976) contended that "tetraploids would have a gametic number of at least 14, and therefore all cytodemes of $n = 13$ or less should be diploid". Although Harberd stated that taxa with $n \geq 14$ are not necessarily polyploid and limited his speculations to the *Brassica* coenospecies, his hypothesis has been expanded and applied to the tribe as a whole. That is, Brassiceae taxa with $n \geq 14$ are widely believed to be polyploid (Gómez-Campo and Hinata 1980; Al-Shehbaz 1985), having evolved from taxa with lower base chromosome numbers by way of entire genome duplication. If this hypothesis is true, polyploidy then characterizes some 37% of the species in the tribe (Warwick and Anderson 1993), such that many genera would appear to be entirely polyploid: *Boleum*

($n = 3x = 51$), *Crambe* ($x = 15$), *Euzomodendron* ($x = 17$), *Moricandia* ($x = 14$), *Rytidocarpus* ($x = 14$), *Savignya* ($x = 15$), *Schouwia* ($x = 18$), *Succowia* ($x = 18$), *Vella* ($x = 17$) and *Zilla* ($x = 16$) (Gómez-Campo and Hinata 1980; Al-Shehbaz 1985). Further studies are necessary to confirm this hypothesis.

Polyploidy

Autopolyploidy has been suggested as playing a significant role in the evolution of some taxa within the tribe (Al-Shehbaz 1985). Euploid series have been reported for several species and genera, reaching the octoploid level in *Erucastrum* (*E. meruense* Jonsell, $2n = 8x = 64$) and the hexaploid level in *Moricandia* (*M. spinosa*, $2n = 6x = 84$) (Sobrino Vesperinas 1978; Jonsell 1979).

Several allopolyploid species have also been reported in the tribe. The most familiar examples are the crop species *Brassica carinata* A. Braun (genome BBCC, $n = 8+9$), *B. juncea* (genome AABB, $n = 10+8$) and *B. napus* (genome AACC, $n = 10+9$), formed by natural hybridization from *B. nigra* (BB, $n = 8$), *B. oleracea* (CC, $n = 9$) and *B. rapa* (AA, $n = 10$) (Prakash and Hinata 1980). These relationships have been corroborated by extensive cytological, hybridization and molecular investigations (reviews: Prakash and Hinata 1980; Palmer *et al.* 1983; Song *et al.* 1988; Prakash and Chopra 1991). Allotetraploids have also

been reported among non-cultivated members of the tribe, including *Brassica balearica* Pers., *Erucastrum elatum* (Ball) O.E. Schulz, *Diplotaxis muralis* and *Erucastrum gallicum* (Harberd and McArthur 1980; Al-Shehbaz 1985).

In comparison to other allotetraploids in the tribe, the origins of *Brassica balearica* and *Erucastrum elatum* have been relatively unexplored. Analysis of allosyndetic pairing in hybrids of *B. balearica* (n = 16) and *B. insularis* (n = 9) has suggested that *B. insularis* (or another member of Section *Brassica* L.) may represent one of the parental taxa (Snogerup and Persson 1983). The identity of the other parent remains to be determined. Based on morphological observations, Gómez-Campo (1983) has suggested the diploid parents of *Erucastrum elatum* (n = 7+8) to be *E. littoreum* Maire (n = 8) and either *E. virgatum* (n = 7) or *Hirschfeldia incana* (n = 7). Data from seed protein electrophoresis have confirmed *E. littoreum* (*sensu lato*) as one of the parental species, but have presented inconclusive evidence regarding the identity of the n = 7 parent, suggesting both *H. incana* (Sánchez-Yélamo 1992) and *E. virgatum* (Sánchez-Yélamo *et al.* 1992). Chloroplast DNA studies, however, have provided evidence for *E. virgatum* as the maternal parent and not *H. incana* (Warwick and Black 1993b).

The origin of *Diplotaxis muralis* (n = 10+11) from *D. tenuifolia* (n = 11) and *D. viminea* (n = 10) was first

proposed by Harberd and McArthur (1972). Their hypothesis was based on the morphological and cytological examination of *D. tenuifolia*, *D. viminea* and *D. muralis*, and the synthetic hybrids *D. muralis* × *D. viminea* and *D. muralis* × *D. tenuifolia*. The hybrids were highly fertile and displayed 10 and 11 allosyndetic pairs, respectively, strongly supporting *D. tenuifolia* and *D. viminea* as the parental taxa. This hypothesis has been confirmed by seed electrophoresis (Sánchez-Yélamo and Martínez-Laborde 1991; Sanchez-Yélamo *et al.* 1992), flavonoid data (Sánchez-Yélamo and Martínez-Laborde 1991) and cluster analysis of morphological characteristics (Takahata and Hinata 1986). Evidence for *D. viminea* as maternal parent has been provided by chloroplast DNA studies (Pradhan *et al.* 1992; Warwick *et al.* 1992) and by isoelectric focusing of nuclearly and maternally inherited Rubisco subunits (Mummenhoff *et al.* 1993).

In the case of *Erucastrum gallicum* (n = 7+8), *E. nasturtiifolium* (n = 8) has been proposed to be one of the parental taxa based on the presence of eight allosyndetic bivalents in their triploid hybrids (Harberd and McArthur 1980; Takahata and Hinata 1983). Although the identity of the n = 7 parent was previously unknown, recent chloroplast DNA studies have identified it as either of the two members of the *Diplotaxis erucoides* cytodeme, *D. erucoides* or *D. cossoniana* (Reut. ex Boiss.) O.E. Schulz (Warwick and Black

1993b). Although the authors did not indicate which of these species represented the most likely maternal parent of *E. gallicum*, they did note that *D. erucoides* and *E. gallicum* have similar widespread distributions "in Europe and the southwest Mediterranean region [...], while *D. cossoniana* is restricted to Algeria and Morocco" (Warwick and Black 1993b).

Chromosome evolution

Because of its economic value, the genus *Brassica* has been the focus of more cytological investigations than any other genus in the tribe. Diploid species of this genus display a range of chromosome numbers (n = 7-11) (Warwick and Anderson 1993) which has been proposed as representing an aneuploid series (Manton 1932). Considerable controversy surrounds the base number of the series, with three base numbers proposed, x = 6 (Catcheside 1934; Röbbelen 1960), x = 5 (Sikka 1940) and x = 3 (Hussein and Abobakr 1976; Chen and Heneen 1991). While all three hypotheses acknowledge the importance of aneuploidy in the evolution of *Brassica* species, the latter two differ markedly by contending that aneuploidy occurred only after the base number was increased by amplification of the entire genome. In many instances, support for these evolutionary hypotheses (discussed in detail below) is drawn from studies of chromosomal pairing. The validity of

the latter evidence should be evaluated carefully, however, since chromosomal pairing may result from factors other than homology (reviewed in Armstrong and Keller 1981, 1982).

The $x = 6$ hypothesis proposes that *Brassica* species represent an aneuploid series of "secondarily-balanced polyploids originating from a common prototype with $x = 6$" (Prakash and Hinata 1980). This model proposes that aneuploidy progressed in ascending order from $x = 6$ to $x = 11$. However, it is also equally possible that there were numerous ascending and decending aneuploid series during the evolution of the genus. Diploid members of *Brassica* ($n = 7$-11) are considered to be secondary polyploids "since presumably they have some of the basic chromosome types forming part of their genome in duplicate or even in triplicate", the duplicated chromosome segments believed to be the result of chromosomal rearrangement and differentiation during the process of aneuploid evolution (Quiros *et al.* 1987; Hosaka *et al.* 1990). Support for the hypothesis of $x = 6$ has been gained from studies of chromosome pairing during meiosis which have established the presence of six basic types of chromosomes in *B. napus* ($n = 19$) (Catcheside 1934), *B. nigra* ($n = 8$) (Prakash 1973), *B. oleracea* ($n = 9$) (Armstrong and Keller 1982), *B. rapa* ($n = 10$) (Armstrong and Keller 1981), *B. tournefortii* ($n = 10$) (Prakash 1974) and the hybrid *B. napus* × *B. rapa*

(n = 29) (Nwankiti 1970). The hypothesis of x = 6 has also been supported by analyses of pachytene morphology (Röbbelen 1960; Venkateswarlu and Kamala 1971) which established the presence of two bivalents in *B. nigra* (ABCDDEFF), of three bivalents in *B. oleracea* (ABBCCDEFF) and of two bivalents and one trivalent in *B. rapa* (AABCDDEFFF) (genomic formulae following Röbbelen 1960). The validity of x = 6 as base number is questionable since no n = 6 *Brassica* species has ever been recorded. In proposing an ascending aneuploid series for the genus, the model, therefore, presumes that n = 7 taxa are the most ancient extant members of the genus (Prakash and Hinata 1980). Molecular-based phylogenetic studies do not support the ancestral status of n = 7 taxa, further questioning the validity of x = 6 as base number (Warwick and Black 1991, 1993b; Warwick *et al.* 1992).

The hypothesis of base number x = 5 for genus *Brassica* was proposed by Sikka (1940), based on his observation of five basic chromosomes during meiosis in *B. nigra*, *B. monensis* Huds. [=*Coincya monensis* (L.) Greuter and Burdet] and *B. sinapistrum* Boiss. [=*Sinapis arvensis* L.]. He proposed that *Brassica* species with n = 10 were derived by tetraploidy from an ancestral species with x = 5, and that other members of the aneuploid series "resulted by reduplication or loss of certain chromosomes from the tetraploid number". Though not mentioned by the author,

aneuploid decreases may also have resulted by way of chromosome fusion. Sikka derived further support for his hypothesis from chromosome numbers in other members of the tribe (*Crambe* and *Erucastrum*) which are multiples of five. He explained the discrepancy between his results and the reported observations of six basic chromosomes by suggesting that the material studied by other researchers may have been "of very remote origin, so that structural differentiation of some homologous chromosomes had occurred to such an extent that their affinity was no longer enough to cause attraction". Aside from an electrophoretic analysis of seed proteins in *Brassica* and *Sinapis* which suggested that *B. rapa* (n = 10) might be the closest to the archetype of the genus (Vaughan and Denford 1968), the x = 5 hypothesis has received little support. Furthermore, subsequent cytological investigations have identified a broader range of chromosome numbers in diploid species of *Erucastrum* (n = 7, 8, 9) and have proposed that n = 15 *Erucastrum* species arose by allopolyploidy from taxa with n = 7 and n = 8 (*cf.* pp. 18-19).

The hypothesis of x = 3 for genus *Brassica* was proposed by Hussein and Abobakr (1976) based on the observation of three basic types of chromosomes (one bivalent, one trivalent and one tetravalent) during meiosis in *B. oleracea*. Expanding on this hypothesis, Chen and Heneen (1991) suggested that the diploid species of

Brassica (n = 7-11) arose by aneuploidy "from a triplicate of the basic x = 3 genome or from a combination of two ancient archetypes with x = 3 and x=6" (Chen and Heneen 1991). Support for the x = 3 hypothesis (reviewed in Chen and Heneen 1991) has been derived from studies of chromosome pairing showing the presence of trivalents in haploids of *B. oleracea* and *B. rapa* (Armstrong and Keller 1981, 1982), of hexavalents in *B. napus* (Newell *et al.* 1984, in Chen and Heneen 1991) and of pentavalents in amphihaploids of *B. napus* (Attia and Röbbelen 1986). Further support was derived from the relatively higher frequency of *Brassica* species with n = 9 (50%) than with n = 8 (20%) and n = 10 (30%) (Chen and Heneen 1991). Isozyme studies showing the presence of three copies of the cytosolic isozyme of phosphoglucomutase in *B. oleracea* and *B. rapa* (Chen *et al.* 1989; Chen *et al.* 1990) have also been cited as supporting the x = 3 hypothesis since only one copy is normally found in diploid plants. This evidence seems doubtful, however, since the majority of isozyme studies conducted on *Brassica* species have only indicated the presence of two cytosolic isozymes of phosphoglucomutase (reviewed in Arús *et al.* 1991; McGrath and Quiros 1992). Weaker evidence supporting the x = 3 hypothesis was derived from studies of the hybrid *B. napus* × *B. rapa* (AAC, $2n$ = 29) in which gametes were found to display a non-random distribution of chromosome numbers

between 10 and 19 (Nwantiki 1970). The preponderance of gametes with n = 13 and n = 16 was interpreted by Chen and Heneen (1991) as "possibly reflecting the preferential survival of 'balanced' gametes carrying certain groups of three or six chromosomes from the C genome". More recently, the x = 3 hypothesis was supported by cytological studies showing the presence of three chromosomal satellites and three nucleolar organizing regions in *B. nigra* and *Sinapis arvensis* (Cheng and Heneen 1994). Molecular studies have also shown that the gene encoding acyl-CoA-binding protein is present in six copies in the tetraploid species *Brassica napus* (n = 10+9), with three copies derived from each parental species, *B. oleracea* (n = 9) and *B. rapa* (n = 10) (Hills *et al.* 1994). While the x = 3 hypothesis is currently supported by the greatest diversity of evidence, it would be premature to rule out either of the alternate hypotheses.

More recently, x = 15 has been proposed as base number for the tribe. Comparisons of detailed genomic maps of *Brassica* and *Arabidopsis thaliana* (L.) Heynh. (x = 5) (Lagercrantz *et al.* 1994; Kowalski *et al.* 1994) indicated that "the *Arabidopsis* genome appears to be represented in three somewhat rearranged copies in the diploid genomes [A, B and C] of *Brassica*" (Lagercrantz *et al.* 1994). This would suggest triplication of the *Brassica* genomes from an ancestor with n = 5, resulting in a base number of x = 15

for tribe Brassiceae (Lydiate, pers. comm.). This model further proposes that other chromosome numbers in the tribe evolved by way of aneuploidy, including chromosome fusion, from $x = 15$.

Support for some degree of secondary polyploidy has been provided by DNA-based marker mapping studies which have shown *Brassica* genomes to be highly duplicated with up to 50% loci duplication in diploid species (McGrath *et al.* 1990; McGrath and Quiros 1991; Kowalski *et al.* 1994; Lagercrantz *et al.* 1994). Isozyme studies on *Brassica* and allied genera (*Diplotaxis*, *Hirschfeldia*, *Raphanus*) have also supported some degree of secondary polyploidy by demonstrating isozyme duplications for some commonly assayed enzyme systems in species with chromosome number as low as $n = 7$-10 (Quiros 1987; Quiros *et al.* 1988; Ellstrand and Devlin 1989; Arús *et al.* 1991; Gotoh and Ikehashi 1992).

Isozyme electrophoresis

Starch gel electrophoresis provides an economical means for examining genetic phenomena at the molecular level (Murphy *et al.* 1990). The technique permits proteins of differing net electrical charge to be separated and visualized as bands on the gel using enzyme-specific histochemical stains (Murphy *et al.* 1990). These bands correspond to different forms of the same enzyme and are

interpreted as allozymes (encoded by alternate alleles at the same locus) or isozymes (encoded by genes at distinct loci) (Crawford 1989). Nuclearly encoded allozymes are inherited in a simple Mendelian fashion and are free of the epistatic effects which often complicate the interpretation of the genetic basis of traditional morphological characteristics (Crawford 1989; Weeden and Wendel 1989). Consequently, electrophoretic studies yield powerful data with a strong genetic basis. This type of data has been used to address numerous population genetic and evolutionary considerations as described in recent reviews (Tanksley and Orton 1983; Soltis and Soltis 1989; Murphy *et al.* 1990).

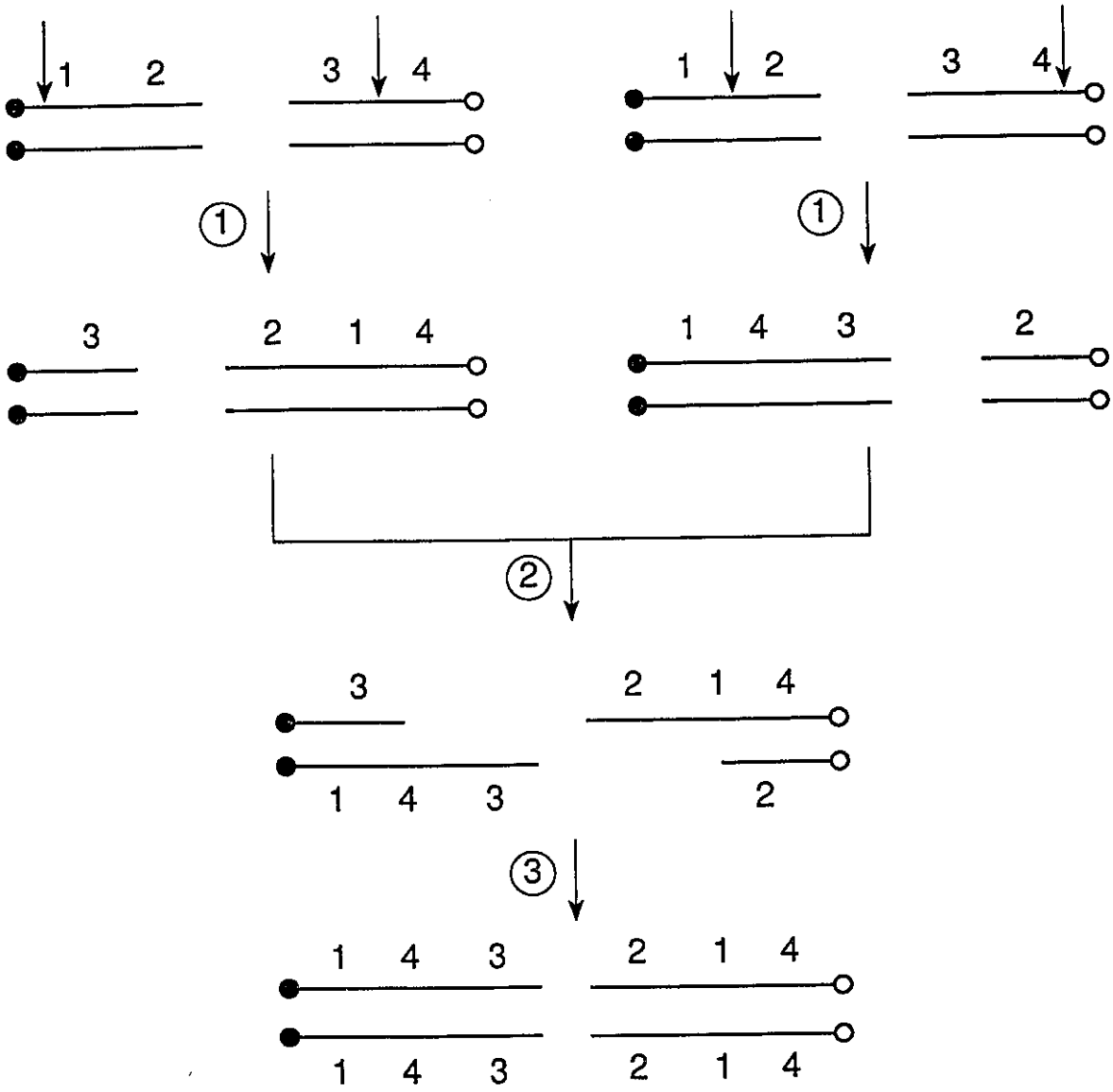
The plant enzymes most commonly studied by isozyme electrophoresis are those of the glycolytic and citric acid cycles (Gottlieb 1983). Electrophoretic studies on numerous plant species have shown that a minimum number of isozymes exists for each enzyme, and that this number is broadly conserved within diploid plants (Gottlieb 1982). Further studies using cell fractionation techniques have revealed that this minimum number corresponds to the different subcellular locations of each isozyme and results from the endosymbiotic nature of chloroplasts and other organelles (Gottlieb 1982, 1983; Weeden and Wendel 1989; Kephart 1990).

Increases above the conserved minimum isozyme number

are uncommon in diploid plants and arise by the duplication of short chromosomal segments resulting from various cytological events (Weeden and Wendel 1989). Gene duplications detected in plant isozyme studies have generally been attributed to unequal crossing-over or overlapping reciprocal translocations (Gottlieb 1983; Weeden and Wendel 1989). The possibility of gene duplications arising from viral transduction or transposon-mediated processes (Ohno 1970) has not been addressed by plant isozyme studies. Unequal crossing-over produces tightly-linked, tandemly-repeated segments, whereas overlapping reciprocal translocations produce homologous segments on separate chromosomes (Weeden and Wendel 1989). The two can be differentiated by analyzing linkage relationships in lines which are heterozygous for both copies of the duplicated gene (Pichersky and Gottlieb 1983). Gottlieb (1983) believed unequal crossing-over to occur frequently and to be of little systematic importance. He considered overlapping reciprocal translocations to be of much greater systematic value since they result from a series of rare cytological events involving "the occurrence of simultaneous multiple chromosome breaks and the production of true-breeding (homozygous) duplicate progeny which must be viable and fertile" (Figure 3) (Gottlieb 1983).

Regardless of their mode of origin, isozyme

Figure 3. Diagram to illustrate "how a cross between two overlapping reciprocal translocations can produce a progeny which is duplicated for the chromosome segments between the translocation break points": *Step 1.* Independent translocations occur at different sites on homologous chromosomes in distinct plant lines. *Step 2.* When the two plant lines are crossed, "a chromosomally-heterozygous type is produced. One of four progeny classes is illustrated; it carries two duplicated segments". *Step 3.* Structurally homozygous individuals carrying both duplications are produced by self-fertilization or crosses with siblings. Because the duplicate genes are located on non-homologous chromosomes, they "are expected to assort independently". Modified from Proteins and Nucleic Acids in Plant Systematics, by Jensen and Fairbrothers (see Gottlieb 1983).


1 2
3 4
①
3
2 1 4
1 4 3
2
②
3
2 1 4
1 4 3
2
③
1 4 3
2 1 4
1 4 3
2 1 4

duplications in diploid plants "are sufficiently unusual that they have received considerable attention when discovered" (Werth *et al.* 1993). Based on the numerous isozyme studies conducted on diploid plant species, the probability of the same isozyme duplication occurring more than once in a single lineage is very low (Gottlieb 1983). Consequently, taxa sharing an isozyme duplication must have inherited it from a common ancestor and must therefore constitute a monophyletic group (Gottlieb 1983). By defining monophyletic groups, isozyme duplications are considered to be of phylogenetic significance (Gottlieb 1983). Isozyme duplications have been used to address numerous questions in plant systematics (see review, Weeden and Wendel 1989), most notably relationships within the genus *Clarkia* Pursh. (Onagraceae) (Gottlieb and Weeden 1979) and subtribe Coreopsidinae Less. (Heliantheae, Asteraceae) (Crawford *et al.* 1990).

Because isozyme number remains largely unchanged following aneuploidy but increases significantly following polyploidy, it can also be used to study evolution of chromosome number (Gottlieb 1981). This technique has proven useful in determining ploidy level in the tribe Astereae (n = 2-9; Asteraceae) (Gottlieb 1981) and in subtribe Oncidiinae Bentham (n = 5-30; Vandeae, Orchidaceae) (Chase and Olmstead 1988). Isozyme electrophoresis can also serve as a tool in the

identification of the parental species of allopolyploids since the homoeologous loci inherited from each parental species are generally expressed additively in the allopolyploid (Gottlieb 1977). The effectiveness of this technique in determining the parentage of allopolyploids depends on two principal factors: the degree of genome divergence between the parental species since closely related taxa may not exhibit differentially migrating allozymes (Gottlieb 1983); and the length of time since the formation of the allopolyploid, since its genetic similarity to the parental taxa is likely to decrease over time (Gottlieb 1977).

It is important to note that detection of gene duplications in diploid and polyploid species can be limited by gene silencing (*viz.*, the mutation of a coding sequence such that the resulting protein is non-functional), with the extent of gene silencing expected to increase over evolutionary time (Ferris and Whitt 1977). Such gene silencing is well documented in tetraploid fish species (Ferris and Whitt 1977; Bailey *et al.* 1978; Stoneking *et al.* 1981) and in *Chenopodium* (Chenopodiaceae) (Wilson *et al.* 1983). Gene silencing has also been proposed as one hypothesis to explain the apparent diploid isozyme profiles of homosporous ferns with chromosome numbers as high as $n = 108$ (Haufler and Soltis 1986; Haufler 1987).

Goals

Previous isozyme studies conducted on diploid species of tribe Brassiceae have established the presence of greater than expected numbers of isozymes for several enzyme systems (Quiros 1987; Quiros *et al.* 1988; Ellstrand and Devlin 1989; Arús *et al.* 1991; Gotoh and Ikehashi 1992). These findings suggested that isozyme number variation might represent a useful data set for investigating systematic relationships in the tribe.

The present research undertakes to investigate chromosome number evolution and species relationships in tribe Brassiceae (Brassicaceae) using isozyme number variation. This study includes 35 genera and 108 species from tribe Brassiceae, making it the largest systematic survey of isozyme number variation. This research tests the following hypotheses: (1) Taxa with $n \leq 13$ display the minimum number of isozymes expected for diploid taxa; (2) Taxa with $n \geq 14$ display more isozymes than the expected minimum number, consistent with a polyploid origin; (3) Isozyme duplications support the monophyletic groups proposed for tribe Brassiceae; (4) The allozyme patterns of allopolyploid species *Diplotaxis muralis* and *Erucastrum gallicum* correspond to the additive expression of the homoeologous loci contributed by each of the proposed parental species. In addition, the research establishes the genetic basis of the electrophoretic banding patterns for one member of the tribe, *Sinapis arvensis*.

MATERIALS AND METHODS

Plant material

A total of 108 species from 35 genera in the tribe Brassiceae were examined (Table 2); these included representatives from all seven subtribes. Viable accessions of the other 19 Brassiceae genera were not available for study. The species examined ranged in chromosome number from n = 7-60 (Table 2) based on previously reported counts (Gómez-Campo and Hinata 1980; Warwick and Anderson 1993) and counts conducted on eight accessions included in this study (see Table 2 for references). Eleven species from seven additional tribes (Alysseae, Arabideae, Euclidieae, Hesperideae, Lepidieae, Lunarieae, Sisymbrieae) were included as outgroup taxa in analyses of isozyme number variation (Table 2). In selecting accessions for this study, preference was given to accessions examined by Dr. S. I. Warwick (Agriculture and Agri-Food Canada) in previous systematic studies. When such accessions were not available, other representative accessions were used. Seeds were obtained from the germplasm collection of Dr. S.I. Warwick. Most accessions were originally acquired from the wild germplasm collection of Dr. C. Gómez-Campo (E.T.S.I.A., Universidad Politécnica, Madrid, Spain) or from wild collections made by Dr. S.I. Warwick in 1992 and Dr. G. Baillargeon in 1983. The

Table 2. Taxa, chromosome number (*n*) and source of seed for plant material used in this study.

Taxa[a]	*n*[b]	Source[c]
Tribe Brassiceae		
Brassica		
B. barrelieri (L.) Janka	10	PGRC No. 13218 (BCN 3232)
B. cretica Lam.	9	GCC 6344 (BCN 8006A)
B. deflexa Boiss.	7	GCC No. 3713-75 (BCN 3210)
B. desnottesii Emb. & Maire	10	GCC 4404-76 (BCN 8083)
B. elongata Ehrh.	11	GAT BRA1146/84 (BCN 3661)
B. fruticulosa Cyr.		
ssp. *fruticulosa*	8	GCC 4068-76 (BCN 8008)
ssp. *cossoniana* (Boiss. & Reut.) Maire	16	GCC 0912-66 (BCN 8009)
B. gravinae Ten.	10,20	
Accession 1		BGL No. 387 (BCN 3179)
Accession 2		NEU No. 197 (BCN 3467)
Accession 3		BGL No. 373 (BCN 3549)
B. hilarionis Post	9	GCC 7344 (BCN 8010)
B. incana Ten.	9	GCC 6567 (BCN 8205)
B. insularis Moris	9	PAR No. 89-43 (BCN 8258)
B. macrocarpa Guss.	9	BGP (BCN 3022)
B. maurorum Durieu	8	GCC 1966-71 (BCN 8014)
B. montana Pourret	9	GCC 6835 (BCN 8096)
B. nigra (L.) Koch	8	BGP (BCN 3023)
B. oleracea L.	9	
Accession 1		BGL No. 374 (BCN 3550)
Accession 2 [=*B. alboglabra* L.H. Bailey]		PGRC No. 7784 (BCN 3017)
B. oxyrrhina (Coss.) Willk. & Lange	9	BIC (BCN 3141)
B. procumbens (Poiret) O.E. Schulz	9*	GCC 6507-84 (BCN 8089)

Table 2 (*cont.*)

Taxa	*n*	Source
B. rapa L.	10	
Accession 1		PRI UPM6464 (BCN 8107)
Accession 2		PRI UPM6652 (BCN 8108)
B. repanda (Willd.) DC.	10	GCC 1554-72 (BCN 8063)
B. rupestris Raf.	9	BGP (BCN 3025)
B. souliei (Batt.) Batt.		
ssp. "*dimorpha*" (Coss. & Durieu) Salmeen	22†	GCC 5016-82 (BCN 8086)
ssp. *souliei*	11	GAT 991/78 (BCN 3659)
B. spinescens Pomel	8	GCC 1800-70 (BCN 8015)
B. tournefortii Gouan	10	WGB No. 4531 (BCN 3439)
B. villosa Biv.	9	
Accession 1		BGP (BCN 3026)
Accession 2 [=*B. drepanensis* (Caruel) Damanti]		BGP (BCN 3019)
Accession 3 [=*B. drepanensis* (Caruel) Damanti]		PAR No. 90-27 (BCN 8257)
Cakile		
C. arctica Pobedimova	9	BGR No. 0354/06-93-06 (BCN 8256)
C. maritima Scop.	9	BGG (BCN 3564)
Calepina irregularis (Asso) Thell.	7,14,21	
Accession 1		BGP (BCN 3018)
Accession 2		CD No. 1002 (BCN 3087)
Accession 3		SKO No. 444 (BCN 3182)
Carrichtera annua (L.) DC.	8,16	CD No. 1021 (BCN 3088)
Ceratocnemum rapistroides Coss. & Bal.	8	GCC 1088-67 (BCN 3636)

Table 2 (*cont.*)

Taxa	*n*	Source
Coincya		
C. longirostra (Boiss.) Greuter & Burdet	12	BGC 191 (BCN 3150)
C. rupestris Porta & Rigo ex Rouy	12	BGC 10763-85 (BCN 3601)
Conringia		
C. orientalis Andrzejowski ex DC.	7	BGM No. 502 (BCN 3167)
C. perfoliata (C.A. Meyer) N. Busch	7	GCC No. 3703-77 (BCN 8077)
Crambe		
C. abyssinica Hochst. ex O.E. Schulz	45	
Accession 1		BGN No. 221 (BCN 3472)
Accession 2		CD No. 1016 (BCN 3593)
C. cordifolia Steven	60	BGU No. 224 (BCN 3477)
C. filiformis Jacq.	15	BGC No. 192 (BCN 3151)
C. hispanica L.	15,30	NOV No. 507 (BCN 3428)
C. kralikii Coss. ex Reboud	15,30,45	Rich/Ncala River Ravine, Morocco (BCN 8177)
C. maritima L.	15,30	HAL (BCN 3576)
C. orientalis L.	15,30	BGV No. 1739 (BCN 3507)
C. sventenii B. Petters ex. Bramwell & Sundell	15	OCS No. 57 (BCN 3641)
C. tataria Sebeók	15,30,60	HAL (BCN 3579)
Diplotaxis		
D. brachycarpa Godr.	9**	GCC 6467-84 (BCN 7017)
D. catholica (L.) DC.	9	GCC 1390-68 (BCN 7018)
D. cretacea Kotov	11	BGV No. 1742 (BCN 3510)
D. erucoides (L.) DC.	7	
Accession 1		BOR No. 872 (BCN 3129)
Accession 2		BGS No. 330 (BCN 3463)
Accession 3		BGV No. 1740 (BCN 3508)
Accession 4		GCC 1235-67 (BCN 7009)

Table 2 (*cont.*)

Taxa	*n*	Source
D. gomez-campoi Martínez-Laborde	8**	GCC 4065-76 (BCN 7014)
D. harra (Forssk.) Boiss.	13	
Accession 1		GCC 1472-68 (BCN 7005)
Accession 2		GCC 1831-70 (BCN 7006)
D. muralis (L.) DC.	21	
Accession 1		CD No. 1028 (BCN 3594)
Accession 2		BGC No. 153 (BCN 3644)
Accession 3		GCC 0990-68 (BCN 7004)
D. siifolia G. Kunze	10	GCC 1447-68 (BCN 7023)
D. simplex (Viv.) Spreng.	11	GCC 1931-71 (BCN 7002)
D. tenuifolia (L.) DC.	11	
Accession 1		BGD (BCN 3031)
Accession 2		SAL No. 332 (BCN 3465)
Accession 3		BGV No. 1741 (BCN 3509)
Accession 4		HAL (BCN 3581)
Accession 5		GCC 0980-66 (BCN 7000)
D. viminea (L.) DC.	10	
Accession 1		BGA No. 897 (BCN 3517)
Accession 2		GCC 2108-76 (BCN 7003)
Enarthrocarpus lyratus (Forssk.) DC.	10	CD No. 1061 (BCN 3089)
Eruca vesicaria (L.) Cav. *s. lat.*	11	
ssp. *sativa* (Mill.) Thell.		
Accession 1		TOR No. 404 (BCN 3080)
Accession 2		OXF No. 406 (BCN 3126)
Accession 3		BGN No. 024 (BCN 3474)
ssp. *pinnatifida* (Desf.) Emb. & Maire		GCC 1813-70 (BCN 8064)
ssp. *vesicaria*		

Table 2 (*cont.*)

Taxa	*n*	Source
Erucaria		
E. erucarioides (Coss. & Durieu) C. Mueller	8*	GCC 1944-71 (BCN 8067)
E. hispanica (L.) Druce	8	GCC 2055-72 (BCN 8071)
E. microcarpa Boiss.	8	CD No. 1138 (BCN 3093)
E. ollivieri Maire	8	GCC 2983-74 (BCN 8075)
E. pinnata (Viv.) Täckh. & Boulos	8	GCC 1851-70 (BCN 8065)
Erucastrum		
E. abyssincum (A. Rich.) O.E. Schulz	16	BGD (BCN 3033)
E. brevirostre (Maire) Gómez-Campo	9	GCC 1503-68 (BCN 8052)
E. canariense Webb & Berthel.	9	CD No. 1065 (BCN 3091)
E. gallicum (Willd.) O.E. Schulz	15	
Accession 1		Shawinigan, QC, Canada (BCN 2565)
Accession 2		GCC 1209-69 (BCN 8021)
Accession 3		MAN (BCN 8106)
E. leucanthum Coss. & Durieu ex Coss.	8,16	GCC 1474-68 (BCN 8027)
E. littoreum (Pau & Font Quer) Maire		
ssp. *brachycarpum* (Maire & Weiller) Gómez-Campo	24†	GCC 3018-74 (BCN 8025)
ssp. *glabrum* (Maire) Gómez-Campo	8†	GCC 3016-76 (BCN 8024)
E. nasturtiifolium (Poiret) O.E. Schulz	8,16	
Accession 1		PAR 224 (BCN 3057)
Accession 2		GUY 64 (BCN 3546)
Accession 3		GCC 1220-67 (BCN 8028)
E. rifanum (Emb. & Maire) Gómez-Campo	8	GCC 2040-73 (BCN 8029)
E. strigosum (Thunb.) O.E. Schulz	8	GCC 4048-76 (BCN 8030)
E. varium Durieu	7	GCC 3654-75 (BCN 8031)
E. virgatum (J.C. Presl) C. Presl	7	
ssp. *pseudosinapis* (Lange) Gómez-Campo		GCC 4116-76 (BCN 8026)
ssp. *virgatum*		BGP No. 231 (BCN 3609)

Table 2 (*cont.*)

Taxa	*n*	Source
Euzomodendron bourgaeanum Coss.	17	BGC No. 205 (BCN 3155)
Fortuynia bungei Boiss.	16†	GCC No. 3764-75 (BCN 3634)
Guiraoa arvensis Coss.	8	GCC 1550-68 (BCN 3635)
Henophyton deserti (Coss. & Durieu) Coss. & Durieu ssp. *zygarrhenum* (Maire) Greuter & Burdet	42*	GCC No. 1468-68 (BCN 8061)
Hirschfeldia incana (L.) Lagrèze-Fossat	7	Djebel Thaya, Algeria (BCN 2700)
Kremeriella cordylocarpus (Coss. & Durieu ex Coss.) Maire	12	CD No. 1103 (BCN 3092)
Moricandia		
M. arvensis (L.) DC.	14	
Accession 1		MUN (BCN 3039)
Accession 2		GCC 4071-76 (BCN 8113)
Accession 3		Meski, Morocco (BCN 8138)
M. foetida Bourgeau ex Coss.	14	Rioja, Spain (BCN 8140)
M. foleyi Batt.	11*	GCC 5549-80 (BCN 8117)
M. moricandioides (Boiss.) Heywood	14	Onteniente, Spain (BCN 8145)
M. nitens (Viv.) Durieu & Barr.	14	ISR No. 95 (BCN 3614)
M. spinosa Pomel	42	MAD No. 566+ (BCN 3541)
M. suffruticosa (Desf.) Coss. & Durieu	28	
Accession 1		BGF No. 479 (BCN 3461)
Accession 2	28‡	GCC 1833-70 (BCN 8125)
Orychophragmus violaceus Bunge	12	TOR No. 412 (BCN 3081)

Table 2 (*cont.*)

Taxa	*n*	Source
Pseuderucaria teretifolia (Desf.) O.E. Schulz	14*	OSN No. 88-2000410 (BCN 8132)
Pseudofortuynia esfandiarii Hedge	7†	GCC 3760-75 (BCN 3626)
Psychine stylosa Desf.	15	BGB No. 133 (BCN 3515)
Raffenaldia primuloides Godr.	7	GCC 4386-76 (BCN 3622)
Raphanus		
R. raphanistrum L.	9	St.-Victoire-de-Sorel, QC, Canada (BCN 3449)
R. sativus L.	9	ZBG No. 181 (BCN 3520)
Rapistrum		
R. perenne (L.) All.	8	HAL (BCN 3586)
R. rugosum (L.) All.	8	Bejaia, Algeria (BCN 2680)
Rytidocarpus moricandioides Coss.	14	
Accession 1		CD No. 1141 (BCN 3094)
Accession 2		BGM No. 533 (BCN 3173)
Accession 3	14†	GCC 0708-67 (BCN 8126)
Accession 4		OSN 86-1507974 (BCN 8135)
Schouwia purpurea (Forssk.) Schweinf.	18*	GCC No. 5780-81 (BCN 8087)
Sinapidendron		
S. angustifolium (DC.) Lowe	10	GCC 3620-75 (BCN 8035)
S. frutescens (Aiton) Lowe	10	GCC 5471-79 (BCN 8036)

Table 2 (*cont.*)

Taxa	*n*	Source
Sinapis		
S. alba L.	12	GAT No. SIN20/81 (BCN 2962)
S. arvensis L.	9	
accession 1		Dunvegan, AB, Canada (BCN 8220)
accession 2	9*	Grandview, MB, Canada (BCN 8221)
accession 3		Tantah, Egypt (BCN 2657)
accession 4		Morden, MB, Canada (BCN 8156)
accession 5		Saskatoon, SK, Canada (BCN 8157)
S. aucheri (Boiss.) O.E. Schulz	7	GCC No. 3735-75 (BCN 3211)
S. pubescens L., *s. lat.*		
ssp. *pubescens*	9	Setif, Algeria (BCN 2713)
ssp. *virgata* (Battand.) Baillarg.	9	Cap-Carbon, Algeria (BCN 2691)
S. boivinii Baillarg.	18	Chabet el Akra, Algeria (BCN 2695)
Succowia balearica (L.) Medik.	18	BGB No. 134 (BCN 3516)
Trachystoma		
T. aphanoneurum (Maire & Weiller) Maire & Weiller	8	GCC 1504-68 (BCN 3200)
T. ballii O.E. Schulz	8	GCC 1488-68 (BCN 3199)
T. labasii Maire	8*	GCC 3014-74 (BCN 3208)
Vella		
V. anremerica (Litard. & Maire) Gómez-Campo	17	GCC No. 4397-76 (BCN 8082)
V. pseudocytisus L.	17,34	MAD No. 576+ (BCN 3544)
Zilla spinosa (L.) Prantl	16	
ssp. *macroptera* (Coss.) Maire & Weiller		GCC No. 1096-67 (BCN 8058)
ssp. *spinosa*		GCC No. 0731-67 (BCN 8055)

Table 2 (*cont.*)

Taxa	*n*	Source
Outgroup taxa		
Alyssum dasycarpum Steph. (Alysseae)	8	USDA No. 304980 (BCN 8252)
Arabis caucasica Schlecht. (Arabideae)	8	CD No. 1143 (BCN 3095)
Barbarea vulgaris R.Br. (Arabideae)	8	BGV No. 1755 (BCN 3511)
Berteroa incana (L.) DC. (Alysseae)	8	USDA No. 337104 (BCN 8253)
Erysimum cheiranthoides L. (Hesperideae)	8	PGRC No. 19703 (BCN 8230)
Isatis tinctoria L. (Lepidieae)	7,14	BER (BCN 3394)
Lepidium sativum L. (Lepidieae)	8,12	BAY No. 128 (BCN 3657)
Lunaria annua L. (Lunarieae)	14	FIR No. 108 (BCN 3459)
Sisymbrium irio L. (Sisymbrieae)	7,14,21,28	BGBe (BCN 8159)
Taucheria lasiocarpa Fisch. (Euclidieae)	7	BGM No. 541 (BCN 3164)
Thlaspi arvense L. (Lepidieae)	7	PRC (BCN 8004)

[a] Tribal classification follows Schulz (1936); nomenclatural authorities follow Warwick (1993).

[b] Haploid chromosome number(s) reported for each species in Warwick and Anderson (1993), Rich (1991) and Manton (1932). Counts conducted on specific accessions used in this study are marked with superscripts and were reported in *Warwick *et al.* (1993, 1994), **Martínez-Laborde (1991), †Gómez-Campo (1978), ‡Sobrino Vesperinas (1978).

[c] Seed sources are represented by the following codes: BAY, Universität Bayreuth, Germany; BER, Berlin, Germany; BGA, Botanic Garden of Technical High School RWTH, Aachen, Germany; BGB, Botanic Garden, Barcelona, Spain; BGBe, Belgian National Botanic Garden; BGC, Botanic Garden, Córdoba, Spain; BGD, Botanic Garden, Dijon, France; BGF, Botanic Garden, Marseille, France; BGG, Botanic Garden Institute "Hanbury", University Degli Studi, Genova, Italy; BGK, Botanic Garden Fominianus, Kiev, Ukraine; BGL, Botanic Garden, University of London, UK; BGM, Botanic Garden of Johannes Gutenberg, University Mainz, Germany; BGN, Botanic Garden, Neubauer Pedagogic High School, Mühlhausen, Germany; BGP, Botanic Garden, University of Palermo, Sicily, Italy; BGPd, Botanic Garden, Padova University, Italy; BGR, Botanic Garden, Reykjavik, Iceland; BGS, Botanic Garden, University Bern, Switzerland; BGU, Botanic Garden, University Udine, Italy; BGV, Botanic Garden, Ecological and Botanical Institute, Vacratot, Hungary; BIC, Botanical Institute, Coimbra University, Portugal; BOR, Botanic Garden, Bordeaux, France; BPS, Botanic Garden, University of Pisa, Italy; CD, Copenhagen, Denmark; EGY, Orman Botanic Garden, Cairo, Egypt; FIR, Firenze, Italy; GAT, Genetic and Breeding Inst., Academy of Sciences, Gatersleben, Germany; GCC, Gómez-Campo Collection, E.T.S.I.A., Madrid, Spain; GUY, National Seed Service Station, Guyancourt, France; HAL, Botanic Garden, Halle University, Germany; ISR, Hebrew University, Jerusalem, Israel; KEW, Royal Botanic Gardens, Kew, UK; MAD, Royal Botanic Garden, C.S.I.C., Madrid, Spain; MAN, Received from Gord Thomas, Agriculture and Agri-Food Canada, Manitoba; MUN, Botanic Gardens of Munich, Germany; NEU, Botanic Garden, University of Neuchâtel, Switzerland; NOV, Novosibirsk, Russia; OCS, Acclimatization Garden Ortova, Santa Cruz de Tenerife, Canary Islands, Spain; OSN, Osnabrück University, Germany; OXF, Oxford University, Oxford, UK; PAR, Natural History Museum, Paris, France; PGRC, Plant Gene Resources Canada, Agriculture and Agri-Food Canada, Ottawa; PRC, Received from Jas Singh, Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa; PRI, Plant Research Institute, Norwich, UK; SAL, Botanic Garden, Salsburg University, Austria; SKO, Skopje, Macedonia; TOR, University of Torino, Italy; USDA, Iowa State University, Ames, Iowa; WGB, Wellesbourne Gene Bank, UK; ZBG, Zahrada Botanic Garden, Tábor, Czech Republic. Collection number on herbarium labels of specimens to be deposited at Herbarium, Agriculture and Agri-Food Canada, Ottawa (DAO) is preceeded by BCN.

remaining accessions were obtained from botanic gardens or other germplasm collections (Table 2). All seeds were stored at 4°C, prior to being sown in a standard germination medium in a greenhouse at 20°C or in a growth cabinet at 18°C. Seedlings were transplanted into a standard soil mixture and were repotted as necessary for good growth until maturity. Leaf samples (1.5 - 3 cm^2) were collected in triplicate from three to six week old seedlings and stored at -80°C to prevent enzyme degradation. Pollen (10 - 20 mg) was also collected from some taxa using bee-sticks (Williams 1980). Plants were grown to maturity and voucher specimens from each accession were made and will be deposited at the herbarium (DAO), Agriculture and Agri-Food Canada, Ottawa. Identifications were confirmed by Dr. S. I. Warwick, Agriculture and Agri-Food Canada.

A single accession was generally used to represent each species although multiple accessions were used to confirm the presence or absence of isozyme duplications in some taxa. The number of seedlings sampled per accession varied depending on germination and seedling survivorship, with eight to twelve seedlings generally being sampled. The number of seedlings assayed electrophoretically ranged from 4 to 33 individuals per accession (mean = 7.4) and is indicated in Table 4. These sample sizes, although insufficient for estimates of genetic variability within

species, are sufficient for evaluation of ploidy level and isozyme number (Gottlieb 1981; Chase and Olmstead 1988).

Electrophoresis

Crude extracts were obtained from the leaf samples (described above) by grinding them with a glass pestle in 1 - 1.5 ml of extraction buffer (0.1M Tris-HCl pH 7.5, containing 10 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.4% Triton X-100 and 14 mM ß-mercaptoethanol (Warwick and Aiken 1986)) in the presence of insoluble polyvinylpolypyrrolidone. Crude extracts were obtained from pollen by soaking pollen-saturated bee-sticks for 16 hours at room temperature in 0.4 ml of the above extraction buffer (altered to exclude Triton X-100) (Weeden and Gottlieb 1979). Pollen-free bee-sticks were also included as controls and showed no isozyme activity. Filter paper wicks (Whatman No. 3) measuring 14 × 4 mm were saturated in the crude extracts and inserted into hydrolyzed potato starch gels (10.5% w/v, 20 × 17.5 × 1 cm). Four gel/electrode buffer systems (pH 5.7 - 8.8) were used (Appendix 1). Wicks were removed after the front had migrated 1 cm as indicated by a bromophenol marker. Gels were run at constant current at 4°C until the marker reached 10 cm (4.5 - 6.5 h). Gels were sliced horizontally into 2 mm-thick slices and assayed histochemically for ten enzyme systems following standard assay procedures (Appendix 2) (Wendel and Weeden 1989). After staining,

gels were rinsed and stored in distilled water at 4°C.

Buffer system A was used for phosphoglucomutase (PGM, EC 5.4.2.2, NADP). Buffer system B was used for isocitrate dehydrogenase (IDH, EC 1.1.1.42, NADP) and shikimate dehydrogenase (SKDH, EC 1.1.1.25, NADP). Buffer system C (pH 8.3) was used for alcohol dehydrogenase (ADH, EC 1.1.1.1, NAD), aminopeptidase (AMP, EC 3.4.11.1), glucose-6-phosphate isomerase (GPI, EC 5.3.1.9, NADP) and malate dehydrogenase (ME, EC 1.1.1.40, NADP). Buffer system D (pH 8.8) was used for fructose-bisphosphate aldolase (FBA, EC 4.1.2.13, NAD), fructose-bisphosphatase (FBP, EC 3.1.3.11, NADP) and triose-phosphate isomerase (TPI, EC 5.3.1.1, NAD). All enzymes migrated toward the anode.

Determination of isozyme number

Concordant with the minimum isozyme number observed in most diploid species including Brassiceae species (Arús *et al.* 1991), certain enzyme systems were expected to display more than one distinct banding region, corresponding to the subcellular compartmentalization of isozymes in the cytosol, plastids and other organelles (Gottlieb 1982). Isozyme number was determined separately for each banding region following standard principles (Weeden and Wendel 1989; Wendel and Weeden 1989; Kephart 1990). By comparing several individuals from each accession, it was possible to

distinguish between multiple bands representing genetic variation occurring at a single locus and multiple bands representing distinct isozymes (Chase and Olmstead 1988). The subunit composition of each isozyme was identified by observing the number of bands in putative single-locus heterozygotes (*i.e.*, two for monomeric enzymes, three for dimeric enzymes, five for tetrameric enzymes). A banding region displaying a single band in all individuals of an accession was interpreted as representing a single isozyme, although the presence of two or more isozymes with co-migrational allozymes was also possible. Isozymes were considered to be duplicated: (1) when the number of bands observed in a banding region exceeded the number of bands expected in a single locus heterozygote; (2) when an accession displayed a "fixed-heterozygous" pattern, *i.e.*, identical heterozygous banding patterns for all individuals of the accession, resulting from the fixation of different alleles at two or more homologous loci; or (3) when relative band intensity in heterozygotes was other than the expected 1:1, 1:2:1 or 1:4:6:4:1 for mono-, di-, and tetrameric enzymes respectively.

Comparisons of leaf and pollen extracts were used to determine which isozymes were located in the cytosol following the method of Weeden and Gottlieb (1979). Isozyme numbers for cytosolic multimeric enzymes were inferred from analysis of pollen extracts of putative

heterozygotes. The absence of heteromeric bands from pollen indicated a single isozyme whereas their presence (*i*.e., interlocus heterodimers) indicated more than one isozyme. In addition, isozyme numbers in *Sinapis arvensis* were confirmed by inheritance studies as discussed below.

Banding regions were numbered beginning with those closest to the anode (*e.g.* FBP-1, FBP-2). Isozymes were given the same number as the banding region in which they occurred, with duplicated isozymes identified by an apostrophe (*e.g.*, *Idh*-2, *Idh*-2′), following the convention of Arus *et al.* (1991).

Inheritance studies

These studies were conducted on seven polymorphic loci (*Fbp*-2, *Gpi*-2, *Idh*-2, *Pgm*-2, *Pgm*-2′, *Tpi*-1, *Tpi*-1′) of *Sinapis arvensis* in order to confirm the number of isozymes within each banding region and to verify the genetic basis of the observed electrophoretic variation. Alleles at each locus were designated alphabetically in order of decreasing anodal mobility. Select individuals identified as putative heterozygotes by electrophoresis were successfully self-fertilized using bud pollination, a method which overcomes self-incompatibility in this species (Williams 1980; Stevens and Kay 1988). Approximately two to three days before anthesis, sepals were removed from buds using forceps. Pollen was transferred to the exposed stigma

using bee-sticks bearing pollen from the same plant. Buds were immediately bagged in dialysis tubing to prevent cross-pollination. Mature seeds were collected and cold treated at 4°C for six weeks before being sown. The numbers of progeny examined for each locus are given in Table 3. Observed segregation ratios for 15 allele pairs were compared to expected Mendelian ratios (1:2:1) using chi-square goodness of fit tests (Sokal and Rohlf 1981). The likelihood of receiving 15 non-significant χ^2 results was assessed using a replicated goodness of fit test with allele pairs as replicates (Sokal and Rohlf 1981). Segregation ratios observed for two loci (*Tpi*-1, *Tpi*-1') heterozygous for the same alleles were compared to a 1:14:1 ratio which is expected under the assumption of Mendelian inheritance at each locus (Weeden and Wendel 1989).

Identification of parentage of allopolyploids *Diplotaxis muralis* and *Erucastrum gallicum*

In order to increase the likelihood of detecting the additive expression of isozymes in studies of allopolyploids, multiple accessions of each allopolyploid and proposed parental species were sampled (Table 2). These included three accessions of *D. muralis*, five accessions of *D. tenuifolia* and two accessions of *D. viminea*, and three accessions of *E. gallicum*, four accessions of *D. erucoides* and three accessions of *E.*

nasturtiifolium. In addition, greater numbers of individuals per accession (8-24) were assayed electrophoretically. Observed banding patterns were interpreted as putative loci and alleles on the basis of the Mendelian segregation observed in *Sinapis arvensis* and the electrophoretic variation observed within each banding region. As described earlier, loci were designated numerically and alleles alphabetically. When duplicated loci were present within a banding region, alleles at both loci were designated in order of decreasing anodal mobility. Isozyme additivity was assessed by comparing the putative genotypes of the allopolyploid species with those of the parental taxa.

RESULTS

Enzyme resolution, subcellular compartmentalization and subunit composition

In the majority of taxa within the tribe, one banding region was observed for ADH, AMP, FBA and SKD and two banding regions were observed for FBP, GPI, IDH, ME, PGM and TPI. All banding regions were well resolved with the exception of IDH-1, ME-2 and TPI-2 which could not be scored consistently and which were excluded from the data analysis.

Within PGM-1 and PGM-2, ghost bands (= shadow bands) were observed for all taxa. Ghost bands are degradation products of primary allozymes and result from technical aspects such as insufficient buffering during extraction, freezing, non-physiologic assay conditions or overheating of gels (Kephart 1990). They are commonly observed for PGM and other enzyme systems (Kephart 1990) and have been previously reported for PGM in *Brassica napus*, *B. oleracea* and *B. rapa* (Coulthart and Denford 1982, Arús and Orton 1983). In the present study, ghost bands were generally present in all samples, although in some samples of weaker banding intensity, they were not observed. They developed more slowly and were fainter than the primary allozyme bands. They were generally slightly more anodal in migration than the primary allozymes, although in some

instances cathodal ghost bands were also observed. As expected (Kephart 1990), the ghost bands were observed to vary in mobility concomitantly with the primary allozymes from sample to sample. Because they are the products of post-translational modification, these ghost bands do not represent the products of the structural genetic loci surveyed. Consequently, these bands were not counted as isozymes in determination of isozyme number.

Certain isozymes were also poorly resolved for some taxa. This resulted from the diversity of species assayed in this study and the need to apply a uniform technique to all taxa. When isozymes could not be scored confidently, they were recorded as missing values. The number of such incidents was minor and was not expected to bias assessments of isozyme number variation or ploidy level.

The lack of interaction between banding regions of multimeric enzymes (FBP, GPI, IDH, ME, TPI) indicated that their isozyme(s) were isolated in different subcellular compartments. Analysis of pollen leachates indicated that isozymes staining within AMP-1, GPI-2, IDH-2 and PGM-2 were located in the cytosol. Based on observations of subcellular compartmentalization in other diploid plant species, isozymes staining within GPI-1 and PGM-1 were likely located in the chloroplasts (Weeden and Wendel 1989). Similarly, the isozyme staining within FBA-1 was likely located in the chloroplasts, the cytosolic form

being very rarely resolved by starch gel electrophoresis (Weeden and Gottlieb 1980). Previous studies conducted on members of subtribe Brassicinae have indicated that isozyme(s) staining within TPI-1 are likely located in the chloroplasts (Quiros 1987). Analysis of pollen extracts revealed the presence of a single well-resolved banding region of ME which differed in mobility from either of the banding regions observed in leaf extracts. This suggested that ME is either encoded by separate loci in leaves and pollen or undergoes post-translational modification in one of these tissues. Determination of the subcellular location of ME in leaves was consequently precluded. However, studies conducted on watermelon and apple have only identified cytosolic forms of this enzyme in leaves (reviewed in Weeden and Wendel 1989). The subcellular locations of isozymes of ADH-1, FBP-1, FBP-2 and SKD-1 could not be determined in this study, since no bands were visible in pollen leachates. In most diploid plants, however, ADH is present as one to three cytosolic isozymes and FBP as two isozymes, one cytosolic, the other plastid (Weeden and Wendel 1989). Isozyme number of SKD varies from one to two in diploid plant species with both cytosolic and plastid isozymes reported (Weeden and Wendel 1989).

Observations of band number in putative heterozygotes of several species indicated that FBP was tetrameric

(five-banded), that GPI, IDH, ME and TPI were dimeric (three-banded), and that AMP, PGM and SKD were monomeric (two-banded). In addition, three-banded putative heterozygotes were also observed at an additional banding region of ADH unique to *Barbarea vulgaris* and *Lunaria annua*, indicating that ADH is also dimeric. Observations of subunit composition were consistent with reports for other plant species, with the exception of ME which has been reported as tetrameric in watermelon and apple (Weeden and Wendel 1989). Lack of allozyme variation within FBA-1 precluded determination of subunit composition for this enzyme.

Inheritance studies

Segregation analyses in progeny of putative heterozygotes of *Sinapis arvensis* confirmed the presence of one isozyme within banding regions FBP-2, GPI-2 and IDH-2 (*Fbp*-2, *Gpi*-2, *Idh*-2) and of two isozymes within PGM-2 (*Pgm*-2, *Pgm*-2′) and TPI-1 (*Tpi*-1, *Tpi*-1′). Statistical analyses of segregation ratios using chi-square goodness of fit tests confirmed Mendelian inheritance at each of these loci (Table 3). No significant departures from Mendelian segregation were observed since the values of χ^2 (range: 0.014 to 5.649) were less than the critical value ($p = 0.05$) of 5.991. A replicated chi-square goodness of fit test determined the probability of receiving 15 non-

Table 3. Percent segregation ratios of selfed progenies of putative heterozygotes of *Sinapis arvensis* and chi-square goodness of fit tests for deviation from expected Mendelian ratio (1:2:1)[a].

Genotype	Family	n	Observed Genotype(%) FF	FS	SS	df	χ^2	p
Fbp-2^{ab}	2657-05-01	38	26.3	60.5	13.2	2	3.000	0.223
	8156-06-22	56	32.1	42.9	25.0	2	1.714	0.424
Gpi-2^{bc}	8156-06-04	68	35.3	48.5	16.2	2	5.029	0.081
	8156-06-03	16	31.3	25.0	43.8	2	4.500	0.105
	8156-06-02	63	30.2	54.0	15.9	2	2.967	0.227
Gpi-2^{cd}	2657-05-01	37	29.7	62.1	8.1	2	5.649	0.059
	8157-34-09	50	18.0	56.0	26.0	2	1.360	0.507
Idh-2^{ab}	2657-04-00	45	22.2	57.8	20.0	2	1.133	0.568
Pgm-2^{cd}	8157-34-01	19	31.2	57.9	10.5	2	2.158	0.340
Pgm-$2'^{de}$	8157-34-27	69	24.6	50.7	24.6	2	0.014	0.993
Tpi-1^{ac}	2657-05-01	39	17.9	56.4	25.6	2	1.103	0.576
Tpi-1^{cd}	8157-34-09	26	15.4	69.2	15.4	2	3.846	0.146
	8157-34-27	52	17.3	51.9	30.7	2	1.962	0.375
	8157-34-32	37	13.5	62.2	24.3	2	4.081	0.130

Table 3 (*cont.*)

Genotype	Family	n	Observed Genotype(%) FF	FS	SS	df	χ^2	p
Tpi-1[ad]	8156-06-22	56	23.2	55.4	21.4	2	0.679	0.712
Total:						30	39.195	0.121

[a] Superscripts designate alleles; n is the number of progeny; FF, SS and FS are homozygous and heterozygous for fast (F) and slow alleles (S); df is the number of degrees of freedom; p is the associated probability; $p > 0.05$ signifies Mendelian segregation.

significant results to be p = 0.121, indicating that the likelihood of committing a type I error during multiple repeats of the same statistical test was non-significant ($p \geq 0.05$) (Sokal and Rohlf 1981). A separate chi-square goodness of fit test compared the segregation ratio observed for *Tpi*-1 and *Tpi*-1′ to the 1:14:1 ratio expected under the assumption of Mendelian inheritance at two loci heterozygous for the same alleles. This test was based on the observation of 23 progeny and indicated that segregation at the two loci (2:20:1) conformed to the expected ratio with $\chi^2 = 3.571$ and p = 0.168.

Isozyme number

Banding regions which did not exhibit electrophoretic variation (ADH-1, FBA-1, GPI-1) were interpreted as representing one isozyme each (*Adh*-1, *Fba*-1, *Gpi*-1). Isozyme number in the ten banding regions exhibiting electrophoretic variation (AMP-1, FBP-1, FBP-2, GPI-2, IDH-2, ME-1, PGM-1, PGM-2, SKD-1, TPI-1) was determined following the criteria described in the Materials and Methods (*cf.* pp. 47-49) and is listed for each taxon in Table 4. ME-1 and PGM-1 each exhibited a single isozyme in all taxa with the exception of *Erucastrum abyssincum* which displayed two isozymes at PGM-1. The remaining eight banding regions exhibited variation in isozyme number. Banding regions displaying isozyme numbers greater than the

Table 4. Isozyme numbers observed at 11 loci and for two enzyme systems in 108 species and 35 genera of tribe Brassiceae and in 11 species from seven other tribes of the Brassicaceae[a].

Taxa	n	*Adh*-1	*Fba*-1	*Fbp*-1	*Fbp*-2	*Gpi*-1	*Gpi*-2	*Idh*-2	*Me*-1	*Pgm*-1	*Pgm*-2	*Tpi*-1	AMP	SKD
Tribe Brassiceae														
Brassica														
B. barrelieri	4	1	1	2	1	1	1	1	1	1	2	2	1	1
B. cretica	5	1	1	1	2	1	1	1	1	1	2	2	1	2
B. deflexa	6	1	1	1	1	1	1	1	1	1	2	1	1	2
B. desnottesii	5	1	1	1	-	1	1	2	1	1	2	2	1	-
B. elongata	18	1	1	1	1	1	1	2	1	1	2	2	1	2
B. fruticulosa														
ssp. *cossoniana*	6	1	1	1	1	1	2	1	1	1	2	2	1	-
ssp. *fruticulosa*	6	1	1	1	1	1	1	1	1	1	2	2	1	-
B. gravinae														
Accession 1	16	1	1	1	2	1	2	2	1	1	2	2	2	3
Accession 2	6	1	1	1	-	1	1	2	1	1	2	2	-	2
Accession 3	6	1	1	1	-	1	-	2	1	1	2	2	-	3
B. hilarionis	6	1	1	1	1	1	1	1	1	1	2	2	1	1
B. incana	4	1	1	1	1	1	1	1	1	1	2	2	1	1
B. insularis	6	1	1	1	2	1	1	1	1	1	2	2	1	1
B. macrocarpa	7	1	1	1	2	1	1	1	1	1	2	2	1	1
B. maurorum	4	1	1	2	1	1	1	1	1	-	-	2	1	-
B. montana	5	1	1	1	1	1	1	1	1	1	2	1	1	1
B. nigra	6	1	1	1	1	1	1	1	1	1	2	2	-	1
B. oleracea														
Accession 1	8	1	1	-	1	1	1	1	1	1	2	2	1	3
Accession 2	19	1	1	1	1	1	1	1	1	1	2	2	1	-
B. oxyrrhina	7	1	1	1	1	1	1	1	1	1	2	2	1	1

Table 4 (*Cont.*)

Taxa	n	*Adh*-1	*Fba*-1	*Fbp*-1	*Fbp*-2	*Gpi*-1	*Gpi*-2	*Idh*-2	*Me*-1	*Pgm*-1	*Pgm*-2	*Tpi*-1	AMP	SKD
B. procumbens	33	1	1	1	1	1	1	1	1	1	2	2	1	1
B. rapa														
Accession 1	6	-	1	1	-	1	1	1	1	1	2	2	1	2
Accession 2	6	-	1	1	2	1	1	1	1	1	2	1	1	2
B. repanda	5	1	1	-	-	1	1	2	1	1	2	2	1	-
B. rupestris	7	1	1	1	1	1	1	1	1	1	2	1	1	1
B. souliei														
ssp. "*dimorpha*"	6	1	1	1	1	1	1	1	1	-	-	3	1	2
ssp. *souliei*	6	1	1	1	1	1	1	1	1	1	2	2	1	1
B. spinescens	6	1	1	1	1	1	1	1	1	1	2	2	1	1
B. tournefortii	6	1	1	2	1	1	1	1	1	-	-	2	4	1
B. villosa														
Accession 1	3	1	1	1	1	1	1	1	1	1	2	1	1	1
Accession 2	6	1	1	1	2	1	1	1	1	1	2	1	1	1
Accession 3	8	1	1	1	1	1	1	1	1	1	2	1	1	1
Cakile														
C. arctica	4	1	1	1	1	1	2	2	1	1	2	2	1	1
C. maritima	6	1	1	1	1	1	2	2	1	1	2	2	2	1
Calepina irregularis														
Accession 1	6	1	1	1	2	1	2	2	1	1	2	1	1	1
Accession 2	5	1	1	1	1	1	1	1	1	1	1	1	1	1
Accession 3	6	1	1	2	2	1	2	2	1	1	2	1	1	-
Carrichtera annua	11	1	1	1	1	1	1	-	1	1	2	1	1	2
Ceratocnemum rapistroides	6	1	1	1	1	1	1	1	1	1	2	2	1	-

Table 4 (*Cont.*)

Taxa	n	*Adh*-1	*Fba*-1	*Fbp*-1	*Fbp*-2	*Gpi*-1	*Gpi*-2	*Idh*-2	*Me*-1	*Pgm*-1	*Pgm*-2	*Tpi*-1	AMP	SKD
Coincya														
C. longirostra	7	1	1	1	1	1	1	2	1	1	2	2	2	1
C. rupestris	6	1	1	1	1	1	1	2	1	1	2	2	1	1
Conringia														
C. orientalis	11	1	1	1	1	1	1	1	1	1	1	1	1	1
C. perfoliata	6	1	1	1	1	1	1	1	1	1	1	1	–	1
Crambe														
C. abyssinica														
Accession 1	6	1	1	1	1	1	1	1	1	1	2	2	3	3
Accession 2	6	1	1	1	1	1	1	1	1	1	2	2	2	3
C. cordifolia	5	1	1	1	1	1	–	1	1	1	2	2	1	–
C. filiformis	6	1	–	1	1	1	–	1	1	–	–	–	1	1
C. hispanica	6	1	1	1	1	1	1	1	1	1	2	2	2	3
C. kralikii	6	–	–	1	1	1	1	1	1	1	2	2	2	–
C. maritima	4	1	–	1	–	1	1	–	1	1	2	2	–	1
C. orientalis	5	1	–	1	1	1	2	2	1	1	2	2	1	–
C. sventenii	6	1	–	1	1	1	–	–	1	1	2	2	–	2
C. tataria	4	1	–	1	1	–	–	1	1	1	2	1	1	2
Diplotaxis														
D. brachycarpa	6	1	1	2	1	1	1	1	1	–	–	1	3	2
D. catholica	6	1	1	1	1	1	1	1	1	1	2	2	–	3
D. cretacea	6	1	1	–	1	–	1	2	1	1	2	2	1	1
D. erucoides														
Accession 1	20	1	1	1	1	1	1	1	1	1	2	2	1	1
Accession 2	10	1	1	1	1	1	1	1	1	1	2	2	1	–
Accession 3	24	1	1	1	1	1	1	1	1	1	2	2	1	1
Accession 4	9	1	1	1	1	1	1	1	1	1	2	2	1	1

Table 4 (*Cont.*)

Taxa	n	*Adh*-1	*Fba*-1	*Fbp*-1	*Fbp*-2	*Gpi*-1	*Gpi*-2	*Idh*-2	*Me*-1	*Pgm*-1	*Pgm*-2	*Tpi*-1	AMP	SKD
D. gomez-campoi	6	1	1	1	1	1	1	1	1	1	2	2	1	-
D. harra														
Accession 1	8	1	1	1	1	1	1	2	1	1	2	2	1	-
Accession 2	6	1	1	1	1	1	1	2	1	1	2	2	-	-
D. muralis														
Accession 1	18	1	1	1	3	1	2	4	1	1	4	4	2	3
Accession 2	18	1	1	1	3	1	2	4	1	1	4	4	2	3
Accession 3	16	1	1	1	3	1	2	4	1	1	4	4	2	3
D. siifolia	5	1	1	1	1	1	1	1	1	1	2	2	1	2
D. simplex	6	1	1	1	1	1	1	2	1	1	2	2	1	1
D. tenuifolia														
Accession 1	13	1	1	1	1	1	1	2	1	1	2	2	1	1
Accession 2	16	1	1	1	1	1	1	2	1	1	2	2	1	1
Accession 3	16	1	1	1	1	1	1	2	1	1	2	2	1	1
Accession 4	24	1	1	1	1	1	1	2	1	1	2	2	1	1
Accession 5	16	1	1	1	1	1	1	2	1	1	2	2	1	1
D. viminea														
Accession 1	11	1	1	1	2	1	1	2	1	1	2	1	1	2
Accession 2	12	1	1	1	2	1	1	2	1	1	2	1	1	2
Enarthrocarpus lyratus	12	1	1	1	1	1	1	1	1	1	2	1	1	1
Eruca vesicaria s. lat.														
ssp. *sativa*														
Accession 1	6	1	1	1	1	1	1	1	1	1	2	2	1	2
Accession 2	6	1	1	1	1	1	1	1	1	1	2	2	1	2
Accession 3	6	1	1	1	1	1	1	1	1	1	2	-	2	2
ssp. *pinnatifida*	6	1	1	-	-	1	1	2	1	1	2	2	1	-
ssp. *vesicaria*	6	1	1	1	1	1	1	2	1	1	2	2	1	2

Table 4 (*Cont.*)

Taxa	n	*Adh*-1	*Fba*-1	*Fbp*-1	*Fbp*-2	*Gpi*-1	*Gpi*-2	*Idh*-2	*Me*-1	*Pgm*-1	*Pgm*-2	*Tpi*-1	AMP	SKD
Erucaria														
E. erucarioides	4	1	1	1	1	–	–	1	1	1	2	1	–	–
E. hispanica	6	1	1	1	1	1	1	1	1	1	2	2	1	–
E. microcarpa	6	1	1	1	1	1	2	2	1	1	2	2	2	2
E. ollivieri	6	1	1	1	1	1	1	1	1	1	2	2	1	–
E. pinnata	4	1	1	1	1	1	1	1	1	1	2	2	–	–
Erucastrum														
E. abyssincum	6	1	1	1	1	1	1	1	1	2	2	3	1	4
E. brevirostre	6	1	1	1	1	1	1	2	1	1	1	2	1	–
E. canariense	5	1	1	1	1	–	1	1	1	1	1	1	–	2
E. gallicum														
Accession 1	8	1	1	1	2	1	2	1	1	2	4	–	2	2
Accession 2	8	1	1	1	2	1	2	1	1	2	4	–	2	2
Accession 3	14	1	1	1	2	1	2	1	1	2	4	–	2	–
E. leucanthum	8	1	1	1	1	1	1	1	1	1	–	2	1	–
E. littoreum														
ssp. *glabrum*	4	1	1	2	1	1	1	1	1	1	2	2	1	2
ssp. *littoreum*	10	1	1	1	1	1	1	1	1	1	2	2	1	1
E. nasturtiifolium														
Accession 1	15	1	1	1	1	1	1	1	1	1	2	2	1	2
Accession 2	24	1	1	1	1	1	1	1	1	1	2	2	1	1
Accession 3	13	1	1	1	1	1	1	1	1	1	1	2	1	–
E. rifanum	6	1	1	2	1	1	1	1	1	1	2	2	1	2
E. strigosum	6	1	1	1	1	1	1	1	1	1	2	2	1	1
E. varium	6	1	1	1	1	1	1	1	1	1	2	2	1	1
E. virgatum														
ssp. *pseudosinapis*	8	1	1	2	1	1	–	1	1	1	2	2	2	–
ssp. *virgatum*	6	1	1	–	–	1	1	1	1	–	2	2	2	–

Table 4 (*Cont.*)

Taxa	n	*Adh*-1	*Fba*-1	*Fbp*-1	*Fbp*-2	*Gpi*-1	*Gpi*-2	*Idh*-2	*Me*-1	*Pgm*-1	*Pgm*-2	*Tpi*-1	AMP	SKD
Euzomodendron bourgaeanum	6	1	1	1	1	1	1	1	1	1	2	1	2	1
Fortuynia bungei	6	1	1	1	-	1	1	1	1	1	2	2	2	-
Guiraoa arvensis	6	-	1	1	1	-	-	1	1	1	2	2	1	-
Henophyton deserti ssp. *zygarrhenum*	6	1	1	1	1	1	2	2	1	1	2	2	-	3
Hirschfeldia incana	8	1	1	1	1	1	1	1	1	-	2	2	2	1
Kremeriella cordylocarpus	6	1	1	1	1	1	1	1	1	1	2	2	1	-
Moricandia														
M. arvensis														
Accession 1	6	1	1	1	1	1	1	1	1	1	2	1	2	1
Accession 2	6	1	1	-	1	1	1	2	1	1	2	1	1	1
Accession 3	6	1	1	-	-	1	1	2	1	1	2	2	-	1
M. foetida	6	1	1	1	1	1	1	-	1	1	2	2	1	2
M. foleyi	6	1	1	1	1	1	1	1	1	-	2	2	1	1
M. moricandioides	6	1	1	1	-	1	1	1	1	1	2	2	1	1
M. nitens	14	1	1	1	1	1	1	2	1	1	2	2	-	2
M. spinosa	6	1	1	1	-	1	1	2	1	1	2	2	-	2
M. suffruticosa														
Accession 1	12	1	1	1	1	1	2	2	1	1	2	2	1	1
Accession 2	6	1	1	-	-	1	2	2	1	1	2	2	1	1
Orychophragmus violaceus	6	1	1	1	1	1	1	1	1	1	2	2	1	1

Table 4 (*Cont.*)

Taxa	n	*Adh*-1	*Fba*-1	*Fbp*-1	*Fbp*-2	*Gpi*-1	*Gpi*-2	*Idh*-2	*Me*-1	*Pgm*-1	*Pgm*-2	*Tpi*-1	AMP	SKD
Pseuderucaria teretifolia	4	1	1	1	1	1	-	2	1	1	2	2	-	2
Pseudofortuynia esfandiarii	6	1	1	1	1	1	1	1	1	1	2	2	1	-
Psychine stylosa	9	1	1	1	1	1	2	1	1	1	2	2	2	-
Raffenaldia primuloides	5	1	1	1	1	1	1	1	1	-	-	2	1	1
Raphanus														
R. raphanistrum	5	1	1	1	1	1	1	1	1	1	2	2	2	1
R. sativus	6	1	1	1	1	1	1	1	1	1	2	1	1	-
Rapistrum														
R. perenne	6	1	1	1	1	1	1	1	1	1	2	1	2	1
R. rugosum	6	1	1	1	1	1	1	2	1	1	2	2	1	2
Rytidocarpus moricandioides														
Accession 1	6	1	1	1	1	1	1	1	1	1	2	2	1	1
Accession 2	6	1	1	-	1	-	-	1	1	-	-	2	1	1
Accession 3	6	1	1	-	1	-	-	1	1	-	-	2	1	1
Accession 4	6	1	1	-	1	-	-	1	1	-	-	2	1	1
Schouwia purpurea	5	1	-	1	1	1	1	1	1	-	-	2	-	-
Sinapidendron														
S. angustifolium	5	1	1	1	1	1	1	2	1	1	2	2	1	2
S. frutescens	6	1	1	1	1	-	1	2	1	1	2	2	1	1

Table 4 (*Cont.*)

Taxa	n	*Adh*-1	*Fba*-1	*Fbp*-1	*Fbp*-2	*Gpi*-1	*Gpi*-2	*Idh*-2	*Me*-1	*Pgm*-1	*Pgm*-2	*Tpi*-1	AMP	SKD
Sinapis														
S. alba	6	1	1	1	1	1	1	1	1	1	2	2	1	-
S. arvensis														
accession 1	8	1	1	1	1	1	1	1	1	1	2	2	1	1
accession 2	15	1	1	1	1	1	1	1	1	1	2	2	1	1
S. aucheri	6	1	1	1	1	1	1	1	1	1	2	2	2	1
S. pubescens s. lat.														
ssp. *pubescens*	6	1	1	1	1	1	-	1	1	-	-	2	4	1
ssp. *virgata*	6	1	1	1	1	1	-	1	1	1	2	2	-	1
S. boivinii	6	1	1	1	1	1	-	1	1	1	3	1	4	1
Succowia balearica	5	1	1	1	1	1	1	1	1	-	2	1	1	1
Trachystoma														
T. aphanoneurum	6	1	1	1	1	1	1	1	1	1	2	2	1	2
T. ballii	6	1	1	1	1	1	1	1	1	1	2	-	1	2
T. labasii	6	1	1	1	1	1	1	1	1	1	2	2	1	2
Vella														
V. anremerica	6	1	1	1	1	1	2	-	1	1	2	2	1	1
V. pseudocytisus	7	1	1	1	1	1	2	1	1	1	2	2	2	-
Zilla spinosa														
ssp. *macroptera*	5	-	-	1	-	-	-	1	1	-	-	2	1	-
ssp. *spinosa*	3	-	-	1	1	1	1	1	1	-	-	2	1	2

Table 4 (*Cont.*)

Taxa	n	*Adh*-1	*Fba*-1	*Fbp*-1	*Fbp*-2	*Gpi*-1	*Gpi*-2	*Idh*-2	*Me*-1	*Pgm*-1	*Pgm*-2	*Tpi*-1	AMP	SKD
Outgroup taxa														
Alyssum dasycarpum	6	1	1	1	1	1	1	1	1	1	2	1	-	1
Arabis caucasica	6	1	1	1	1	-	1	1	1	-	1	1	1	1
Barbarea vulgaris	6	2	1	1	1	1	1	1	1	1	2	1	2	1
Berteroa incana	6	1	1	1	1	1	-	1	1	-	-	1	-	1
Erysimum cheiranthoides	6	1	1	1	1	1	1	1	1	1	2	1	-	1
Isatis tinctoria	6	1	1	1	1	1	1	1	1	1	2	1	1	1
Lepidium sativum	6	1	1	1	1	2	2	1	-	1	2	1	1	2
Lunaria annua	6	2	-	1	1	1	1	1	1	1	2	1	1	3
Sisymbrium irio	6	1	1	1	1	-	1	1	1	1	1	1	1	1
Taucheria lasiocarpa	6	1	1	1	1	1	1	1	1	1	1	1	3	1
Thlaspi arvense	6	1	1	1	1	1	1	1	1	1	1	1	1	1

` A dash indicates that isozyme number could not be determined owing to faintness, poor resolution or uninterpretable banding patterns; n = number of individuals examined.

expected minimum numbers for diploid plants (Table 5) were presumed to possess duplications for these isozymes. Duplications were observed for *Fbp*-1, *Fbp*-2, *Gpi*-2, *Idh*-2, *Pgm*-2 and *Tpi*-1. The criteria used to determine these duplications and the taxa in which they were detected (Table 4) are summarized below in the following order: *Tpi*-1, *Pgm*-2, *Idh*-2, *Fbp*-1, *Fbp*-2 and *Gpi*-2.

The majority of taxa examined displayed one isozyme of AMP (*Amp*-1) and SKD (*Skd*-1). These numbers are lower than the expected minimum numbers generally observed in diploid plants, *i.e.*, two or three isozymes for AMP, one or two isozymes for SKD (Weeden and Wendel 1989; Kephart 1990). When more than one isozyme of AMP or SKD was observed (Table 4), it was not possible to distinguish whether the additional copies resulted from an inability to resolve all isozymes consistently or an from isozyme duplication. Consequently, variation in isozyme number for these enzyme systems could not be used to assess ploidy level or phylogenetic relationships.

Tpi-1: An additional isozyme of *Tpi*-1 (Figure 4a) was observed in all taxa of tribe Brassiceae with the exception of *Brassica deflexa*, *B. montana*, *B. rapa* (accession 2), *B. rupestris*, *B. villosa* (all accessions), *Calepina irregularis* (all accessions), *Carrichtera annua*, *Conringia orientalis*, *C. persica*, *Crambe tataria*, *Diplotaxis brachycarpa*, *D. viminea* (both accessions), *Enarthrocarpus*

Table 5. Comparison of the minimum isozyme number expected for diploid plant species and the isozyme numbers observed for diploid taxa from tribe Brassiceae and from seven other tribes in the Brassicaceae (Outgroup Taxa). Where possible, locus designation is specified along with subcellular localization.

Enzyme	Loci	Subcellular Localization[a]	Expected	Observed	
			Diploid Plants	Brassiceae Taxa	Outgroup Taxa
ADH[b]	*Adh*-1	-	1-3	1	1,2
AMP		c	2-3	1,2,3,4	1,2,3
FBA	*Fba*-1	p	1	1	1
		c	1	not observed	not observed
FBP[c]	*Fbp*-1	-	1	1,2	1
	Fbp-2	-	1	1,2	1
GPI	*Gpi*-1	p	1	1	1
	Gpi-2	c	1	1,2	1
IDH	*Idh*-1	p	1	unresolved	unresolved
	Idh-2	c	1	1,2	1
ME[b]	*Me*-1	-	1	1,2	1
PGM	*Pgm*-1	p	1	1	1
	Pgm-2	c	1	2	1-2
SKD[d]		-	1-2	1,2,3	1,2,3
TPI	*Tpi*-1	p	1	1,2	1
	Tpi-2	c	1	unresolved	unresolved

[a] c = cytosolic; p = plastid; a dash indicates that subcellular localization was not determined in this study.
[b] Only cytosolic isozyme(s) have been reported.
[c] Normally present as two isozymes: 1 cytosolic, 1 plastid.
[d] Both cytosolic and plastid isozymes have been reported.

lyratus, *Erucaria erucarioides*, *Erucastrum canariense*, *Euzomodendron bourgaeanum*, *Moricandia arvensis* (accessions 1, 2), *Raphanus sativus*, *Rapistrum perenne* and *Succowia balearica*. The duplication was not observed in any outgroup taxa. The isozyme duplication was identified by the presence of three-banded, fixed-heterozygous patterns in some taxa, by the presence of five-banded heterozygous patterns in other taxa, or by the relative staining intensities 1:2:2 and 2:2:1 in putative heterozygotes of other taxa. The genetic basis of the duplication was determined by inheritance studies in *Sinapis arvensis* (*cf.* page 55).

Pgm-2: An additional isozyme of *Pgm*-2 (Figure 4b) was observed in all taxa of tribe Brassiceae with the exception of *Calepina irregularis*, *Conringia orientalis* and *Conringia persica*. The duplication was also present in some outgroup taxa (*Alyssum dasycarpum*, *Barbarea vulgaris*, *Erysimum cheiranthoides*, *Isatis tinctoria* and *Lunaria annua*) but absent from others (*Arabis caucasica*, *Sisymbrium irio*, *Taucheria lasiocarpa* and *Thlaspi arvense*). The duplication was identified by the presence of two-banded, fixed-heterozygous patterns in leaf and pollen extracts of some taxa or by the presence of three- and four-banded heterozygous patterns. The genetic basis of the duplication has been confirmed by inheritance studies conducted on *Sinapis arvensis* (*cf.* page 55), *Brassica*

oleracea (Arús and Orton 1983; Gotoh and Ikehashi 1992), *B. rapa* (McGrath and Quiros 1991b) and *Raphanus sativus* (Ellstrand and Devlin 1989).

Idh-2: A three-banded, fixed-heterozygous pattern indicated the presence of an isozyme duplication for *Idh*-2 (Figure 4c) in: *Brassica elongata*, *B. gravinae* (all accessions), *B. repanda*, *Cakile arctica*, *Cakile maritima*, *Coincya longirostra*, *Coincya rupestris*, *Diplotaxis cretacea*, *D. harra* (accession 2), *D. simplex*, *D. tenuifolia* (all accessions), *D. viminea* (both accessions), *Erucastrum brevirostre*, *Pseuderucaria teretifolia* and *Sinapidendron frutescens*. Five-banded heterozygous patterns, also indicative of an isozyme duplication, were observed in *Brassica desnottesii*, *Diplotaxis harra* (accession 1), *Moricandia arvensis* (accessions 2, 3) and *M. nitens*. In addition, putative heterozygotes displaying relative band intensities 1:2:2 and 2:2:1 were observed for *Diplotaxis siifolia*, *Eruca vesicaria* ssp. *pinnatifida* and ssp. *vesicaria*, *Erucaria microcarpa*, *Rapistrum rugosum* and *Sinapidendron angustifolium*, suggesting the presence of an isozyme duplication in these taxa. The duplication of *Idh*-2 was confirmed by the presence of interlocus heterodimers in pollen extracts of *Cakile maritima*, *Diplotaxis cretacea*, *D. simplex*, *D. tenuifolia*, *Moricandia arvensis* and *Pseuderucaria teretifolia*.

Fbp-1: A fixed-heterozygous pattern was observed for

Fbp-1 (Figure 4d) in: *Brassica barrelieri*, *B. maurorum*, *B. tournefortii*, *Diplotaxis brachycarpa*, *Erucastrum littoreum* ssp. *glabrum*, *E. rifanum* and *E. virgatum* ssp. *pseudosinapis*. The alleles encoding this pattern were of relatively similar electrophoretic mobilities such that five distinct bands could not be distinguished. In contrast, the thickness of the fixed-heterozygous pattern clearly distinguished it from the homozygous patterns observed in other taxa.

Fbp-2: A fixed-heterozygous pattern was also observed at *Fbp*-2 (Figure 4e) in a separate group of diploid taxa: *Brassica cretica*, *B. insularis*, *B. macrocarpa*, *B. rapa* (accession 2), *B. villosa* (accession 2) and *Diplotaxis viminea* (both accessions). The five bands of the fixed-heterozygous pattern were clearly visible in each of these taxa.

Gpi-2: A three-banded, fixed-heterozygous pattern was observed at *Gpi*-2 (Figure 4f) in *Cakile arctica* and *C. maritima*. The duplicated nature of *Gpi*-2 in *C. martima* was also confirmed by the observation of interlocus heterodimers in pollen extracts. In addition, putative heterozygotes with band intensities 1:2:2 or 2:2:1 were observed in *Erucaria microcarpa*, *Psychine stylosa*, *Vella anremerica* and *V. pseudocytisus*, suggesting an isozyme duplication for *Gpi*-2 in these taxa.

Figure 4a. Photograph and line drawings of a gel, illustrating two banding regions for TPI. Lanes 1,2 - *Coincya rupestris*; 3,4 - *Zilla spinosa*; 5,6 - *Cakile maritima*; 7,8 - *Crambe hispanica*; 9,10 - *Orychophragmus violaceus*; 11,12 - *Conringia orientalis*; 13,14 - *Calepina irregularis*; 15,16 - *Sisymbrium irio*. Banding region TPI-1 corresponds to the loci *Tpi*-1 and *Tpi*-1′ which cannot be clearly distinguished owing to comigrational allozymes. Banding region TPI-2, corresponding to locus *Tpi*-2, appears in lanes 3 and 4 only. This locus was not clearly resolved for all taxa and was excluded from the analysis. Five-banded heterozygous and three-banded fixed heterozygous patterns provided evidence for the duplication of *Tpi*-1 in lanes 2,5,6 and 1,3,4,7-10, respectively. Single-banded homozygous patterns suggested the presence of a single isozyme in lanes 11-16. The symbol – indicates bands which are faint in the photograph but visible to the naked eye.

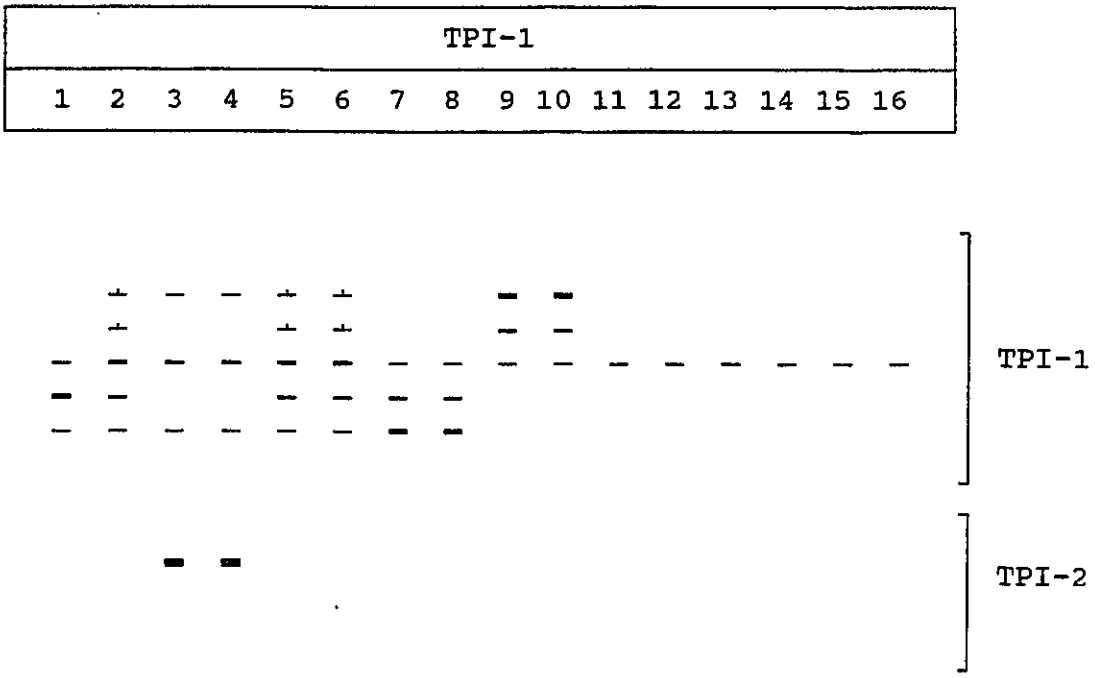
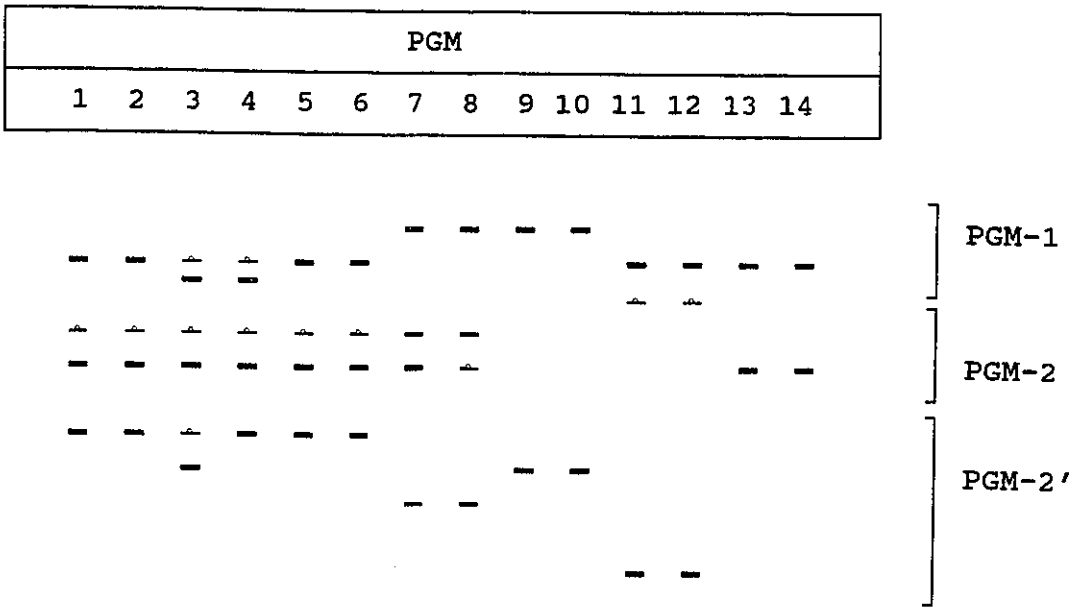

TPI-1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
TPI-1
TPI-2

Figure 4b. Photograph (spliced for aesthetic purposes) and line drawings of a gel, illustrating three distinct banding regions for PGM. Lanes 1,2 - *Diplotaxis cretacea*; 3,4 - *Erucastrum rifanum*; 5,6 - *Cakile maritima*; 7,8 - *Orychophragmus violaceus*; 9,10 - *Sisymbrium irio*; 11,12 - *Conringia orientalis*; 13,14 - *Calepina irregularis*. Banding region PGM-1, corresponding to locus *Pgm*-1, is present in all lanes. Banding regions PGM-2 and PGM-2′ are present in lanes 1-8 providing evidence for the duplication of locus *Pgm*-2 in these taxa. Only one banding region (PGM-2 or PGM-2′) is present in lanes 9-14, indicating a single copy of *Pgm*-2 in these taxa. The symbol ÷ denotes ghost bands which are degradation products of primary allozymes (explained in detail in the text).


PGM
1 2 3 4 5 6 7 8 9 10 11 12 13 14
PGM-1
PGM-2
PGM-2′

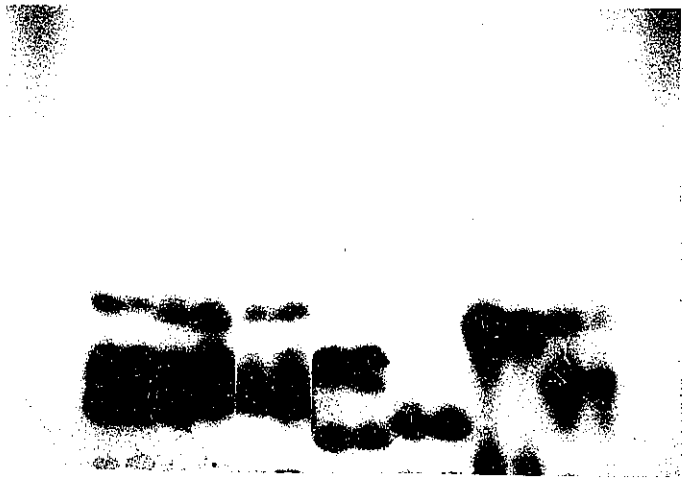


Figure 4c. Photograph (spliced for aesthetic purposes) and line drawings of a gel, illustrating banding region IDH-2. Lane(s) 1 - *Diplotaxis harra*; 2,3 - *Diplotaxis cretacea*; 4 - *Moricandia nitens*; 5 - *Diplotaxis viminea*; 6,7 - *Sinapidendron frutescens*; 8,9 - *Coincya rupestris*; 10,11 - *Cakile maritima*. Banding region IDH-2 corresponds to the duplication of locus *Idh*-2. Three-banded, fixed heterozygous patterns in all lanes provided evidence for the duplication of *Idh*-2. Three distinct bands cannot be distinguished in lanes 6 and 7; these were observed before overstaining occurred.

IDH-2										
1	2	3	4	5	6	7	8	9	10	11

Figure 4d. Photograph (spliced for aesthetic purposes) and line drawings of a gel, illustrating banding region FBP-1. Lane(s) 1,2 - *Brassica tournefortii*; 3 - *Brassica cretica*; 4 - *Brassica maurorum*; 5 - *Brassica rapa*; 6,7 - *Erucastrum rifanum*. Banding region FBP-1 corresponds to locus *Fbp*-1. Fixed heterozygous patterns in lanes 1, 2, 4, 6 and 7 provided evidence for the duplication of *Fbp*-1. Single-banded homozygous patterns in lanes 3 and 5 suggested the presence of a single copy of *Fbp*-1. Separate bands within the fixed heterozygous pattern cannot be distinguished owing to the similar electrophoretic mobilities of the fast and slow alleles. These patterns were presumed to be five-banded on the basis of the tetrameric structure of the enzyme and the observation of five distinct bands in other taxa of the tribe.

FBP-1
1 2 3 4 5 6 7

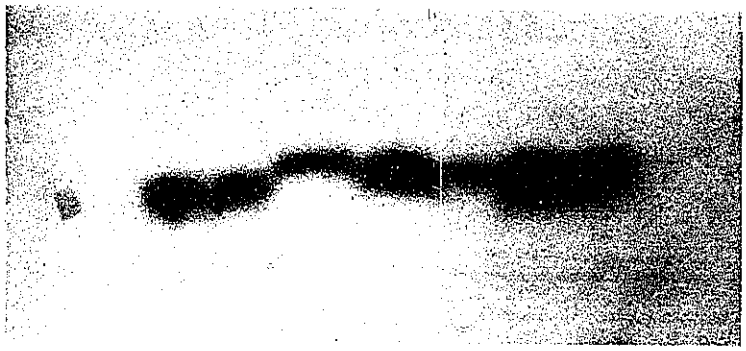


Figure 4e. Photograph (spliced for aesthetic purposes) and line drawings of a gel, illustrating banding region FBP-2. Lanes 1,2 - *Brassica rapa*; 3,5 - *Brassica tournefortii*; 4 - *Diplotaxis viminea*; 6,7 - *Brassica cretica*. The banding region FBP-2 corresponds to locus *Fbp*-2. The five-banded, fixed heterozygous patterns observed in lanes 1, 2, 4, 6 and 7 provided evidence for the duplication of *Fbp*-2. The single-banded homozygous pattern observed in lanes 3 and 5 suggested the presence of a single copy of *Fbp*-2. The symbol - denotes bands which are not visible on the photograph but discernible by the naked eye.

FBP-2						
1	2	3	4	5	6	7

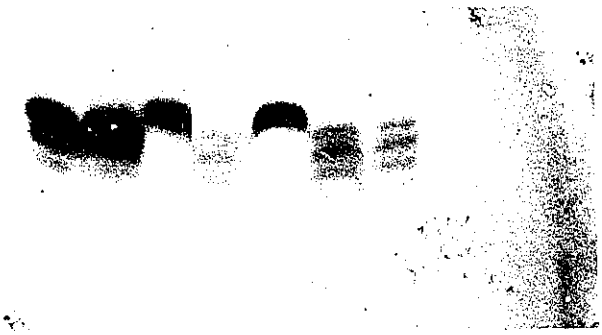
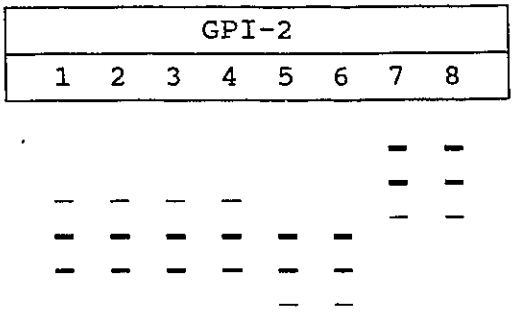


Figure 4f. Photograph (spliced for aesthetic purposes) and line drawings of a gel, illustrating banding region GPI-2. Lanes 1-4 - *Cakile maritima*; 5,6 - *Cakile arctica*; 7,8 - *Lepidium sativum*. The banding region GPI-2 corresponds to the duplicated locus *Gpi*-2. The three-banded, fixed heterozygous patterns observed in all lanes provided evidence for the duplication of *Gpi*-2.


GPI-2
1 2 3 4 5 6 7 8

Ploidy level

Within tribe Brassiceae, isozyme numbers in taxa with $n \leq 13$ and $n = 14\text{-}18$ were similar. Both groups exhibited the minimum isozyme number expected for diploid plants for the majority of isozymes surveyed (Table 5) (Weeden and Wendel 1989; Kephart 1990), indicating that they are diploid. The similarity in isozyme numbers between taxa with $n \leq 13$ and taxa with $n = 14\text{-}18$ refuted the hypothesis that taxa with $n = 14\text{-}18$ are polyploid, having arisen by entire genome duplication from taxa with lower base chromosome numbers (Gómez-Campo 1980; Al-Shehbaz 1985). Rather, these data suggested that aneuploidy has played a more significant role than polyploidy in the evolution of base chromosome numbers among taxa in the tribe. The absence of heteromeric bands from pollen extracts of *Crambe sventenii* ($n = 15$), *Moricandia arvensis* ($n = 14$) and *M. nitens* ($n = 14$) confirmed the presence of a single copy of *Gpi*-2 in these taxa.

As detailed above, four isozymes (*Fbp*-1, *Fbp*-2, *Gpi*-2, *Idh*-2) were observed to be duplicated in certain groups of taxa within the tribe, with only two isozymes (*Pgm*-2, *Tpi*-1) duplicated in the majority of taxa examined. Because polyploid species are expected to display isozyme duplications for the majority of enzyme systems the observed partial genome duplication was insufficient to suggest widespread polyploidy within the tribe. Rather, these duplications suggested a widespread occurrence in the

tribe of a secondarily-balanced polyploid genome structure consistent with that originally proposed for the three diploid *Brassica* crop species (Prakash and Hinata 1980).

Isozyme numbers greater than those expected for diploid plants were observed for: *Amp*-1 and *Pgm*-2 in *Sinapis boivinii* (n = 18); for *Tpi*-1 in *Brassica souliei* ssp. *dimorpha* (n = 22); and for *Gpi*-2 in *B. fruticulosa* ssp. *cossoniana* (n = 16) and *Moricandia suffruticosa* (n = 28). The duplicated nature of *Gpi*-2 in *M. suffruticosa* was confirmed by the presence of interlocus heterodimers in pollen extracts. These observations are consistent with the autotetraploid origins proposed for these taxa. Similarly, complex banding patterns were observed for a number of isozymes in *Brassica gravinae* (2 of 3 accessions), *Calepina irregularis* (2 of 3 accessions), *Crambe orientalis*, *Erucastrum abyssinicum*, and *Lepidium sativum*, suggesting that they were of polyploid origin. These accessions likely represent autopolyploids, consistent with the euploid series reported for these species: *viz.*, n = 10, 20; n = 7, 14, 21; n = 15, 30; n = 8, 16; and n = 8, 12[d], respectively. In addition the isozyme duplications observed for *Gpi*-2 and *Idh*-2 in *Henophyton deserti* (n = 42) were consistent with its obvious polyploid origin. Although other species with

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[d] The base chromosome number of genus Lepidium *is controversial with both* x=4 *(Vaarma 1951) and* x=8 *(Manton 1932) proposed.*

reported euploid series (*viz.*: *Crambe hispanica*, n = 15, 30; *C. kralikii*, n = 15, 30, 45; *C. tataria*, n = 15, 30, 60; *Vella pseudocytisus*, n = 17, 34; *Isatis tinctoria*, n = 7, 14; *Sisymbrium irio*, n = 7, 14, 21, 28) or with polyploid origins (*viz.*: *Crambe abyssinca*, $n = 3x = 45$; *C. cordifolia*, $n = 4x = 60$; *Moricandia spinosa*, $n = 3x = 42$) were included in this study, their low levels of genetic variation precluded the detection of isozyme duplications and assessment of ploidy level. In contrast, the relative band intensity (1:4:6:4:1) of putative heterozygotes at *Fbp*-2 in *Carrichtera annua* (n = 8, 16) suggested that the accession examined in this study was diploid. Similarly, *Erucastrum leucanthum* (n = 8, 16) and *E. nasturtiifolium* accessions 1 and 2 (n = 8, 16) appeared to be diploid based on the allelic segregation observed at *Gpi*-2, and on the relative band intensity 1:2:1 observed for putative heterozygotes at this locus. In contrast to other purported autopolyploids, the accession of *Erucastrum littoreum* ssp. *brachycarpum* examined in this study appeared to be diploid. Although this accession was reported as being hexaploid ($n = 3x = 24$) (Gómez-Campo 1978), it displayed the same numbers of isozymes as diploid taxa in the tribe. Putative heterozygotes of *E. littoreum* ssp. *brachycarpum* displayed simple banding patterns at *Amp*-1, *Fbp*-2, *Gpi*-2, *Pgm*-2′ and *Skd*-1, comparable to those observed in diploid species and unlike the complicated

patterns observed in polyploid taxa. Furthermore, the relative banding intensities observed in these heterozygotes conformed to the banding intensities expected in diploid taxa, 1:1, 1:2:1 and 1:4:6:4:1 for mono-, di- and tetrameric enzymes, respectively; banding intensities in heterozygotes of polyploid taxa are far more variable depending on the exact genotype of the heterozygote (Weeden and Wendel 1990).

Allopolyploid species *Diplotaxis muralis* (n = 10+11) and *Erucastrum gallicum* (n = 7+8) exhibited multi-banded isozyme patterns for the majority of enzyme systems surveyed. These patterns will be discussed in detail below with respect to those observed in the proposed parental species (see below).

Parentage of the allopolyploid *Diplotaxis muralis*

In *Diplotaxis tenuifolia* and *D. viminea*, ten enzyme systems were encoded by 16 and 18 loci, respectively (Table 6). Both species displayed the same number of loci for each enzyme except for FBP and SKD for which *D. viminea* possessed an isozyme duplication (*Fbp*-2′) and an additional locus (*Skd*-2), respectively. All loci were used for determination of allopolyploid parentage with exception of *Adh*-1 which was too faint to be scored consistently.

Diplotaxis tenuifolia (87 individuals) was polymorphic at eight loci (*Amp*-1, *Fbp*-2, *Gpi*-2, *Pgm*-1, *Pgm*-2, *Pgm*-2′,

Table 6. Alleles observed at 17 loci in the allopolyploid species *Diplotaxis muralis* and its proposed parental taxa *D. tenuifolia* and *D. viminea*.

	D. tenuifolia	*D. muralis*	*D. viminea*
Amp-1	a,b	b,c	c
Fba-1	a	a	a
Fbp-1	a	a	a
Fbp-2	a,b,c	b	b
Fbp-2′	not observed	d	d
Gpi-1	a	a	a
Gpi-2	a,b,c,d	a,c,d	a
Idh-2	a	a,b	b
Idh-2′	c	c,d	d
Me -1	a	a	a
Pgm-1	a,b	b	b
Pgm-2,-2′	c,d,e,f	d,f	d,e
Pgm-2′	e,f,h,i	f,h	h
Skd-1	a,b,c	b,c	c
Skd-2	not observed	d	d
Tpi-1	a	a,c	c
Tpi-1′	c,d	c,d	c

Skd-1, *Tpi*-1′), displaying numerous multilocus genotypes. In contrast, *D. viminea* (23 individuals) was monomorphic at all loci except *Pgm*-2 and exhibited only two multilocus genotypes. Of the 52 individuals of *D. muralis* examined, 48 displayed the same multilocus genotype. Only *Gpi*-2 varied with three different banding patterns observed. All banding patterns for *D. muralis* could be explained by combining alleles from each of the proposed parental taxa (Table 6). All three taxa were monomorphic for the same allele for four loci (*Fba*-1, *Fbp*-1, *Gpi*-1, *Me*-1). Allozyme patterns at each of the remaining loci are summarized below:

Amp-1: *D. tenuifolia* was polymorphic for alleles a and b, and *D. viminea* monomorphic for allele c; *D. muralis* displayed a two-banded, fixed-heterozygous pattern for alleles b and c.

Fbp-2, *Fbp*-2′: *D. tenuifolia* was polymorphic for alleles a, b and c at *Fbp*-2 and did not exhibit *Fbp*-2′. *D. viminea* was monomorphic for *Fbp*-2^{b} and *Fbp*-$2'^{d}$ which interacted to produce a five-banded, fixed-heterozygous pattern of relative banding intensity 1:4:6:4:1 which was interpreted as two homotetramers and three interlocus heterotetramers. A five-banded, fixed-heterozygous pattern, but of different relative banding intensity (≈ 9:4:6:4:1), was also observed in *D. muralis*. This pattern was interpreted as the presence of two copies of *Fbp*-2^{b}

(one inherited from each proposed parent) and one copy of *Fbp*-2′^{d} (inherited from *D. viminea*).

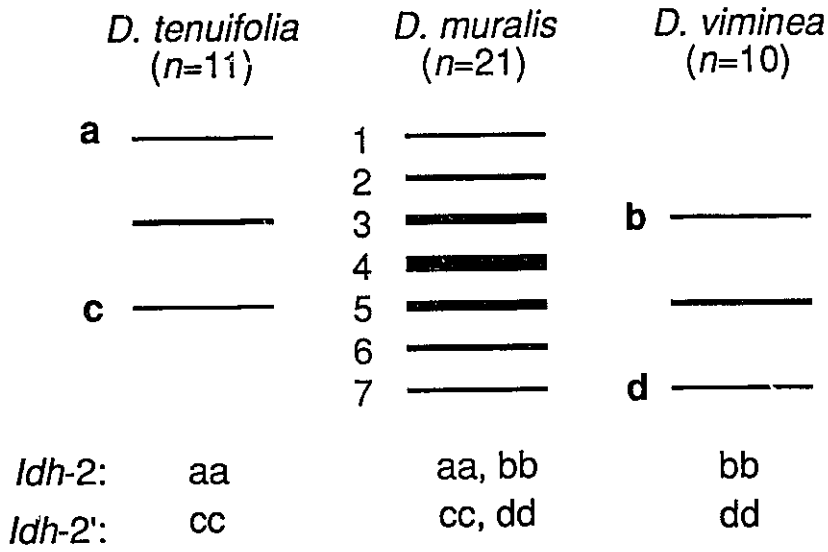
Gpi-2: *D. tenuifolia* was polymorphic for alleles a, b, c and d, while *D. viminea* was monomorphic for a. The majority of *D. muralis* (48 of 52 individuals) displayed a three-banded, fixed-heterozygous pattern (1:2:1) for alleles a and c. Two were similarly fixed for a and d, and two were single-banded and presumably homozygous for a.

Idh-2, *Idh*-2′: *D. tenuifolia* and *D. viminea* both displayed three-banded, fixed-heterozygous patterns consisting of two homodimers encoded by *Idh*-2 and *Idh*-2′ and an interlocus heterodimer. *D. tenuifolia* was monomorphic for *Idh*-2^{a} and *Idh*-2′^{c}, and *D. viminea* was monomorphic for *Idh*-2^{b} and *Idh*-2′^{d}. *D. muralis* displayed a seven-banded, fixed-heterozygous pattern consistent with the inheritance of these four loci. Bands 1, 3, 5 and 7 were interpreted as homodimers encoded by each locus, and bands 2, 3, 4, 5, and 6 as interlocus heterodimers (Figure 5).

Pgm-1: *D. tenuifolia* was polymorphic for alleles a and b, while *D. viminea* and *D. muralis* were monomorphic for b.

Pgm-2, *Pgm*-2′: *D. tenuifolia* was polymorphic for four alleles at both *Pgm*-2 (c, d, e, f) and *Pgm*-2′ (e, f, h, i). *D. viminea* was monomorphic for allele h at *Pgm*-2′ and was polymorphic for *Pgm*-2, with accessions 1 and 2 monomorphic

Figure 5. Banding patterns observed at *Idh*-2 and *Idh*-2′ in the allopolyploid *Diplotaxis muralis* and its proposed diploid progenitors *D. tenuifolia* and *D. viminea*. Putative genotypes are indicated below each banding pattern. The banding pattern of *D. muralis* is consistent with the inheritance of *Idh*-2^{aa} and *Idh*-$2'^{cc}$ from *D. tenuifolia*, and *Idh*-2^{bb} and *Idh*-$2'^{dd}$ from *D. viminea*. The seven-banded pattern consists of four homodimers (corresponding to bands 1, 3, 5 and 7) and six interlocus heterodimers (corresponding to bands 2, 3, 4, 4, 5, and 6) resulting from the interaction of allele pairs a/b, a/c, a/d, b/c, b/d and c/d, repectively.


D. tenuifolia
(n=11)
D. muralis
(n=21)
D. viminea
(n=10)
a
c
1
2
3
4
5
6
7
b
d
Idh-2: aa aa, bb bb
Idh-2': cc cc, dd dd

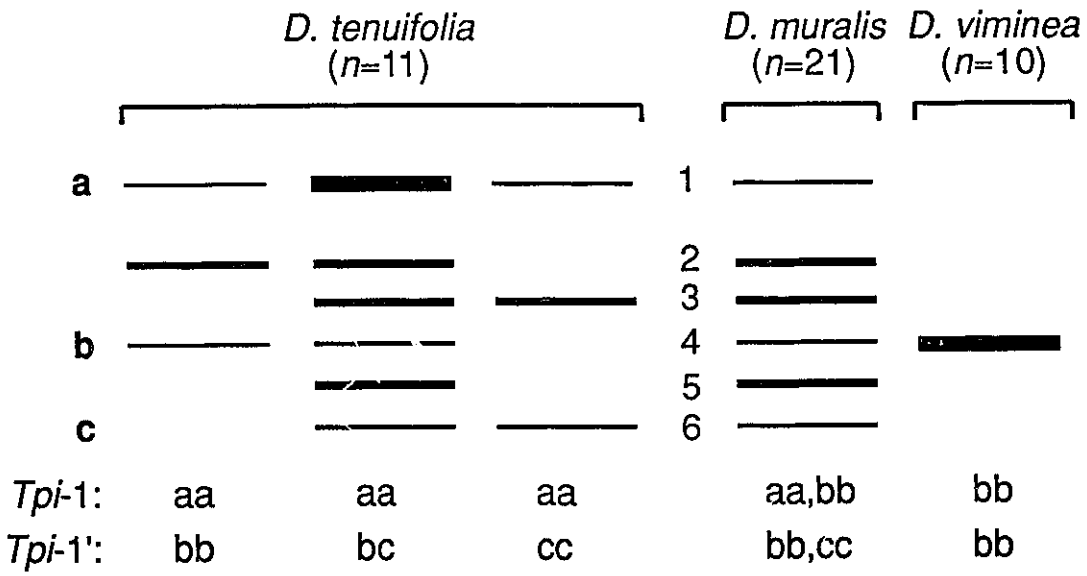
for alleles d and e, respectively. *D. muralis* exhibited a three-banded pattern corresponding to alleles d, f and h which could not be assigned to loci because of the shared status of alleles between parents.

Skd-1, *Skd*-2: *D. tenuifolia* was polymorphic for alleles a, b and c at *Skd*-1, and did not exhibit *Skd*-2. *D. viminea* was monomorphic for alleles c and d at *Skd*-1 and *Skd*-2, respectively. *D. muralis* displayed a three-banded pattern consistent with a two-banded, fixed-heterozygous pattern for alleles b and c at *Skd*-1 and a single homozygous band for allele d at *Skd*-2.

Tpi-1, *Tpi*-1′: In total, six bands and three banding patterns were observed in *Diplotaxis tenuifolia*. These patterns were interpreted as being encoded by two loci, as in other Brassiceae species (Figure 6). In contrast to the complicated banding patterns of *D. tenuifolia*, *D. viminea* displayed a single intense band, suggesting that the two loci (*Tpi*-1, *Tpi*-1′) were fixed for the same allele, b. *D. muralis* also displayed a six-banded pattern, but this differed in banding intensity (≈ 1:2:2:1:2:1) from that of *D. tenuifolia* (≈ 4:2:2:1:2:1). This banding pattern was interpreted as four monomorphic loci (*Tpi*-1^{aa}, *Tpi*-1^{bb}, *Tpi*-$1'^{bb}$, *Tpi*-$1'^{cc}$) consisting of three homodimers (bands 1, 4, 6) and three interlocus heterodimers (bands 2, 3, 5) (Figure 6).

The additive isozyme profiles observed for all loci

Figure 6. Banding patterns observed at *Tpi*-1 and *Tpi*-1′ in the allopolyploid *Diplotaxis muralis* and its proposed diploid progenitors *D. tenuifolia* and *D. viminea*. Putative genotypes are indicated below each banding pattern. The banding pattern of *D. muralis* is consistent with the inheritance of *Tpi*-1^{aa} and *Tpi*-$1'^{cc}$ from *D. tenuifolia*, and *Tpi*-1^{bb} and *Tpi*-$1'^{bb}$ from *D. viminea*. The six-banded patterns of *D. tenuifolia* and *D. muralis* consist of three homodimers (bands 1, 4, 6) and three interlocus heterodimers (bands 2, 3, 5), the latter resulting from the interaction of allele pairs a/b, a/c and b/c, respectively.


D. tenuifolia
(n=11)
D. muralis
(n=21)
D. viminea
(n=10)
a
b
c
1
2
3
4
5
6
Tpi-1:
aa
aa
aa
aa,bb
bb
Tpi-1':
bb
bc
cc
bb,cc
bb

examined are consistent with the hypothesis that *D. tenuifolia* and *D. viminea* are the parental taxa of *D. muralis*.

Parentage of the allopolyploid *Erucastrum gallicum*

In *Diplotaxis erucoides* and *Erucastrum nasturtiifolium*, nine enzyme systems (SKD was excluded as it was not resolved for all accessions) were encoded by 14 loci (Table 7).

Diplotaxis erucoides (63 individuals) was polymorphic at eight loci (*Amp*-1, *Fbp*-2, *Gpi*-2, *Idh*-2, *Pgm*-1, *Pgm*-2, *Tpi*-1, *Tpi*-1′), while *Erucastrum nasturtiifolium* (54 individuals) was polymorphic at five loci (*Fbp*-2, *Gpi*-2, *Pgm*-2′, *Tpi*-1 and *Tpi*-1′). Both exhibited several multi-locus genotypes. *E. gallicum* (30 individuals), on the other hand, was monomorphic at all loci and exhibited a single multi-locus genotype. The majority of banding patterns recorded for *E. gallicum* could be explained by combining alleles from each of the proposed parental taxa (Table 7); however, unique bands were observed for two loci in *E. gallicum*, *Fbp*-2 and *Tpi*-1. All three taxa were monomorphic for the same alleles for five loci (*Adh*-1, *Fba*-1, *Fbp*-1, *Gpi*-1, *Me*-1). Allozyme patterns at each of the remaining loci are summarized below:

Amp-1: *D. erucoides* was polymorphic for alleles b, c and d, and *E. nasturtiifolium* monomorphic for allele a. *E.*

Table 7. Alleles observed at 14 loci in the allopolyploid species *Erucastrum gallicum* and its proposed parental taxa *Diplotaxis erucoides* and *E. nasturtiifolium*.

	D. erucoides	*E. gallicum*	*E. nasturtiifolium*
Adh-1	a	a	a
Amp-1	b,c,d	a,c	a
Fba-1	a	a	a
Fbp-1	a	a	a
Fbp-2	b,c	a,b	b,c
Gpi-1	a	a	a
Gpi-2	b,c,d,e	b,d	a,b,c,d
Idh-2	a,b	a	a
Me -1	a	a	a
Pgm-1	a,b,c	b,d	d
Pgm-2	d,e,f	e,g	f,g
Pgm-2'	g	g,h	g,h
Tpi-1	a	?	a,b,c
Tpi-1'	a,b,c	?	a,b,c

Nota: In *E. gallicum*, the banding pattern of *Tpi*-1 and *Tpi*-1' could not be interpreted in genetic terms (see text for explanation).

gallicum displayed a two-banded, fixed-heterozygous pattern for alleles a and c.

Fbp-2: *D. erucoides* and *E. nasturtiifolium* were both polymorphic for alleles b and c. The banding pattern for *E. gallicum* was not additive, consisting of a five-banded, fixed-heterozygous pattern for allele b and a unique allele a. The pattern was of relative banding intensity 1:4:6:4:1 and was interpreted as two homotetramers and three interlocus heterotetramers.

Gpi-2: *D. erucoides* was polymorphic for alleles b, c, d, and e, while *E. nasturtiifolium* was polymorphic for alleles a, b, c, and d. *E. gallicum* displayed a three-banded, fixed-heterozygous pattern for alleles b and d. This banding pattern, of relative intensity 1:2:1, was interpreted as representing two homodimers and an interlocus heterodimer.

Idh-2: *D. erucoides* was polymorphic for alleles a and b, while *E. nasturtiifolium* and *E. gallicum* were monomorphic for allele a.

Pgm-1: *D. erucoides* was polymorphic for alleles a, b and c, while *E. nasturtiifolium* was monomorphic for allele d. *E. gallicum* displayed a two-banded, fixed-heterozygous pattern corresponding to alleles b and d.

Pgm-2, *Pgm*-2′: At *Pgm*-2, *D. erucoides* was polymorphic for alleles d, e and f, while *E. nasturtiifolium* was polymorphic for f and g. At *Pgm*-2′, *D. erucoides* was

monomorphic for allele g, while *E. nasturtiifolium* was polymorphic for g and h. All individuals of *E. gallicum* displayed three bands of relative intensity 1:2:1, corresponding to the inheritance of *Pgm*-2^{c} and *Pgm*-2′^{g} from *D. erucoides* and *Pgm*-2^{g} and *Pgm*-2′^{h} from *E. nasturtiifolium*.

Tpi-1, *Tpi*-1′: At *Tpi*-1, *D. erucoides* was monomorphic for allele a, while *E. nasturtiifolium* was polymorphic for alleles a, b and c. Both species were polymorphic for alleles a, b and c at *Tpi*-1′. All individuals of *E. gallicum* displayed a four-banded pattern which could not be interpreted in genetic terms. While bands 2 and 4 corresponded in electrophoretic mobility to alleles b and c, bands 1 and 3 were never observed in either proposed parental taxon.

Based on the overall additive isozyme profiles observed for the majority of loci examined, it can be concluded that *D. erucoides* and *E. nasturtiifolium* are the parental taxa of *E. gallicum*. The presence of unique alleles at *Fbp*-2 and *Tpi*-1/1′ suggest that either these alleles were present in the populations of *D. erucoides* and *E. nasturtiifolium* which gave rise to *E. gallicum* and lacking in the ones included in this study, or have arisen since the time of origin.

DISCUSSION

Inheritance studies

Inheritance studies in *Sinapis arvensis* established Mendelian inheritance at seven loci, *Fbp*-2, *Gpi*-2, *Idh*-2, *Pgm*-2, *Pgm*-2′, *Tpi*-1 and *Tpi*-1′. This is believed to be the first report of the mode of inheritance at *Fbp*-2, *Tpi*-1 and *Tpi*-1′ in any species of the tribe. Mendelian inheritance has been established previously for *Adh*-1, *Amp*-1, *Pgm*-2 and *Pgm*-2′ in *Brassica oleracea* (Arús and Orton 1983; Gotoh and Ikehashi 1992), *Amp*-2, *Gpi*-2, *Pgm*-2′, *Skd*-1 and *Skd*-2 in *B. rapa* (Chen *et al.* 1990; McGrath and Quiros 1991) and *Pgm*-1, *Pgm*-2 and *Pgm*-2′ in *Raphanus sativus* (Ellstrand and Devlin 1989). Because the isozyme patterns observed in this study for other species of the tribe were similar to those for which the genetic basis has been established, it is reasonable to assume that they are encoded by homologous loci. The mode of inheritance at *Fba*-1, *Fbp*-1, *Gpi*-1 and *Me*-1 has not been documented for any species of tribe Brassiceae. However, allelic segregation was observed at *Fbp*-1 in *Coincya longirostra* and *Moricandia arvensis* (accession 1) and at *Me*-1 in *Coincya longirostra*, *Crambe tataria*, *Diplotaxis harra* and *Guiraoa arvensis*, consistent with reports of Mendelian inheritance at these loci in other angiosperms (Weeden and Wendel 1989). Because *Fba*-1 and *Gpi*-1 were monomorphic in

all taxa examined in this study, determination of their mode of inheritance was not possible; however, their genetic basis has been documented in other angiosperms (Weeden and Wendel 1989).

Phylogenetic interpretation of isozyme number variation

Isozyme number variation proved to be phylogenetically informative at various taxonomic levels, as discussed below for each duplication.

Duplication of *Tpi*-1 - The *Tpi*-1 duplication was detected in 83 of the 101 diploid species examined in tribe Brassiceae. These species constitute a diverse assemblage of taxa, representing 28 genera, and six of the seven subtribes (Table 8). The duplication was not observed in any of the outgroup taxa, and appears to be diagnostic for tribe Brassiceae. The duplication supports the inclusion of *Orychophragmus* in the tribe and rejects the proposals of Gómez-Campo (1980) and Al-Shehbaz (1985) to exclude it on the basis of its atypical geographical distribution (China) and lack of the typical morphological features. The suggestion by Botschantez (1966, cited in Gómez-Campo 1977) that *Orychophragmus* is more closely aligned with the tribe Hesperideae was not substantiated since the *Tpi*-1 duplication was not observed in *Erysimum cheiranthoides*, the representative of tribe Hesperideae examined in this study.

Table 8. Genera[a] of tribe Brassiceae exhibiting the *Tpi*-1 duplication; presence of duplication denoted thus •.

Brassicinae:	Raphaninae:
Brassica•	*Calepina*
Coincya•	*Ceratocnemum*•
Diplotaxis•	*Crambe*•
Eruca•	*Didesmus*•
Erucastrum•	*Enarthrocarpus*
Hirschfeldia•	*Guiraoa*•
Sinapidendron•	*Kremeriella*•
Sinapis•	*Raffenaldia*•
Trachystoma•	*Raphanus*•
	Rapistrum•
Cakilinae:	
Cakile•	Vellinae:
Erucaria•	*Carrichtera*
	Psychine•
Moricandiinae:	*Rytidocarpus*•
Conringia	*Schouwia*•
Moricandia•	*Succowia*
Orychophragmus•	*Vella*•
Pseuderucaria•	
	Zillinae:
Savignyinae:	*Fortuynia*•
Euzomodendron	*Zilla*•

[a] Only genera with diploid taxa are included; *Henophyton* excluded accordingly.

The lack of detection of the duplication in four genera (*Carrichtera*, *Enarthrocarpus*, *Euzomodendron*, *Succowia*) was likely due to fixation of the same allele at both loci as there was evidence of the duplication in closely allied taxa. More specifically, *Vella* is considered to be closely related to *Carrichtera*, *Euzomodendron* and *Succowia* on the basis of: (1) their similar chloroplast genomes, (2) their indented, conduplicate cotyledons, (3) their non-heterocarpic, reduced fruits, and (4) their endemism to the western Mediterranean region (except *Carrichtera* which is more widespread) (Gómez-Campo 1980; Warwick and Black 1994). Similarly, hybridization studies have shown *Enarthrocarpus* to be closely related to *Brassica* and *Erucastrum* (reviewed in Warwick and Black 1993a), both of which exhibited the *Tpi*-1 duplication. Fixation of the same allele at both loci may also explain inconsistencies in detection of the duplication among species of *Brassica*, *Crambe*, *Diplotaxis*, *Erucaria*, *Erucastrum*, *Raphanus* and *Rapistrum*, and among accessions of *Brassica rapa* and *Moricandia arvensis* (Table 4).

The duplication was also undetected in two genera (*Calepina* and *Conringia*) which lack close relatives in the tribe and whose membership in the tribe is debatable (Botschantzev 1966 in Al-Shehbaz 1985; Gómez-Campo 1980). Because of their uncertain taxonomic affinities, it was not

possible to assume the presence of two isozymes in these taxa. Rather, the observation of a single band at *Tpi*-1 in *Calepina* and *Conringia* may suggest that they possess a single isozyme, providing support for their exclusion from the tribe. Further evidence for the exclusion of these taxa is discussed below in conjunction with the duplication for *Pgm*-2.

Duplication of *Pgm*-2 - The *Pgm*-2 duplication was observed in all diploid taxa within tribe Brassiceae with the exception of *Calepina irregularis* (accession 2)[c], *Conringia orientalis* and *Conringia persica*, suggesting that it was present in the common ancestor which gave rise to the tribe. The absence of both the *Pgm*-2 and the *Tpi*-1 duplications from *Calepina* and *Conringia* clearly supports their exclusion from the tribe. These data support the proposal of Gómez-Campo (1980) to exclude *Calepina* from the tribe on the basis of its non-heterocarpic fruit and only "somewhat conduplicate" cotyledons. Although Al-Shehbaz (1985) supported retaining the genus in the tribe because it had no apparent close relatives outside the tribe, the isozyme data clearly support its exclusion. Similarly, the isozyme data support the proposal of Botschantzev (1966,

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[c] Although two isozymes of Pgm-2 *were observed in accessions 1 and 3 of* Calepina irregularis, *these accessions displayed greater than expected numbers of isozymes for* Fbp-*1,* Fbp-*2,* Gpi-*2 and* Idh-*2, suggesting that they were autopolyploid, consistent with the euploid series proposed for this species.*

cited in Hedge 1976) to exclude *Conringia* from the tribe based on its lack of the typical features. Al-Shehbaz (1985) believed the exclusion of *Conringia* to be unfounded, and cited the nearly conduplicate cotyledons of *C. planisiliqua* as evidence to support its retention in the tribe. The isozyme data, however, clearly advocate the exclusion of *Conringia*, suggesting that the "nearly conduplicate" cotyledons of *C. planisiliqua* may have arisen by convergent evolution.

The *Pgm*-2 duplication cannot be considered diagnostic for tribe Brassiceae since it was also detected in five taxa from tribes Alysseae, Arabideae, Hesperideae, Lepidieae and Lunarieae. The lack of detection of the duplication in four taxa from tribes Arabideae, Euclideae, Lepidieae and Sisymbrieae suggests that the *Pgm*-2 duplication may also represent a useful taxonomic tool for determining phylogenetic relationships among tribes.

Duplication of *Idh*-2 - The *Idh*-2 duplication was unique to Tribe Brassiceae and was detected in 23 diploid species representing a diverse assemblage of eleven genera and four subtribes (Table 9). The taxa displaying the duplication do not appear to conform to any of Schulz's subtribes, with exception of the Cakilinae as discussed below. However, the duplication corresponds well to phylogenies derived from restriction site analysis of chloroplast DNA (cpDNA).

Table 9. Genera[a] of tribe Brassiceae exhibiting the *Idh*-2 duplication; presence of duplication denoted thus *.

Brassicinae:	Raphaninae:
*Brassica**	*Calepina*
*Coincya**	*Ceratocnemum*
*Diplotaxis**	*Crambe*
*Eruca**	*Didesmus*
*Erucastrum**	*Enarthrocarpus*
Hirschfeldia	*Guiraoa*
*Sinapidendron**	*Kremeriella*
Sinapis	*Raffenaldia*
Trachystoma	*Raphanus*
	*Rapistrum**
Cakilinae:	
*Cakile**	Vellinae:
*Erucaria**	*Carrichtera*
	Psychine
Moricandiinae:	*Rytidocarpus*
Conringia	*Schouwia*
*Moricandia**	*Succowia*
Orychophragmus	*Vella*
*Pseuderucaria**	
	Zillinae:
Savignyinae:	*Fortuynia*
Euzomodendron	*Zilla*

[a] Only genera with diploid taxa are included; *Henophyton* excluded accordingly.

The presence of the duplication in *Moricandia*, *Pseuderucaria* and ten species of the Rapa/Oleracea lineage supports the findings of Warwick and Black (1994) that some members of subtribe Moricandiinae are artificially separated from the Rapa/Oleracea lineage of subtribe Brassicinae (Figure 7). The lines separating subtribes Brassicinae and Moricandiinae are also regarded as artificial on the basis of morphology (Al-Shehbaz 1985) and hybridization studies (reviewed in Takahata *et al.* 1993). Within the Rapa/Oleracea lineage, the *Idh*-2 duplication was detected among the majority of the most primitive taxa (Warwick and Black 1993b, 1994), suggesting that they shared a common ancestor. One may have expected the *Idh*-2 duplication to be detected in the other primitive members of the lineage, namely, *Moricandia moricandioides* and *Rytidocarpus moricandioides*. However, lack of genetic variation within the IDH-2 banding region precluded the detection of isozyme duplications in these two species, despite the fact that four accessions of *R. moricandioides* were examined.

The *Idh*-2 duplication was also detected in six taxa of the Nigra lineage, falling within two of the cpDNA groups (groups I and II) recognized by Warwick and Black (1993b) (Figure 8). The *Idh*-2 duplication suggests a common ancestor for these two groups, a result which is consistent with their similar geographical distributions in the

Figure 7. Relationship of taxa exhibiting the *Fbp*-2 and *Idh*-2 duplications to the phylogeny of the Rapa/Oleracea lineage of subtribe Brassicinae as determined by Warwick and Black (1994). Their phylogeny is based on the "strict consensus of 64 equally most-parsimonious Wagner trees generated from chloroplast DNA restriction site mutations (175 phylogenetically informative characters ...)" and includes "the percentage of Wagner bootstrap replicates supporting each clade ... indicated along the internode for that clade" (Warwick and Black 1994). Species complexes described in detail by Warwick and Black (1994) are marked *, whereas species not surveyed in the present study are marked †.

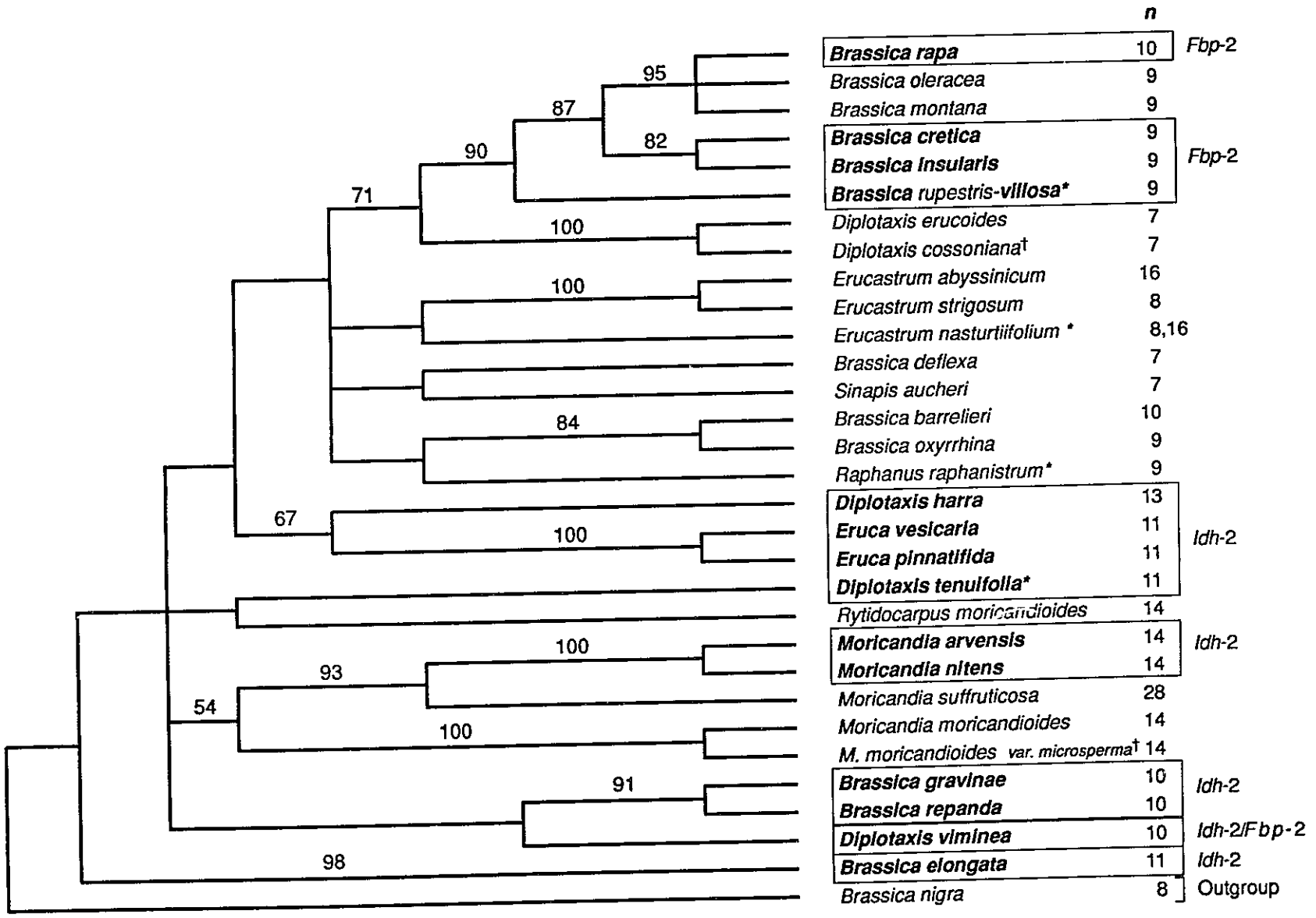
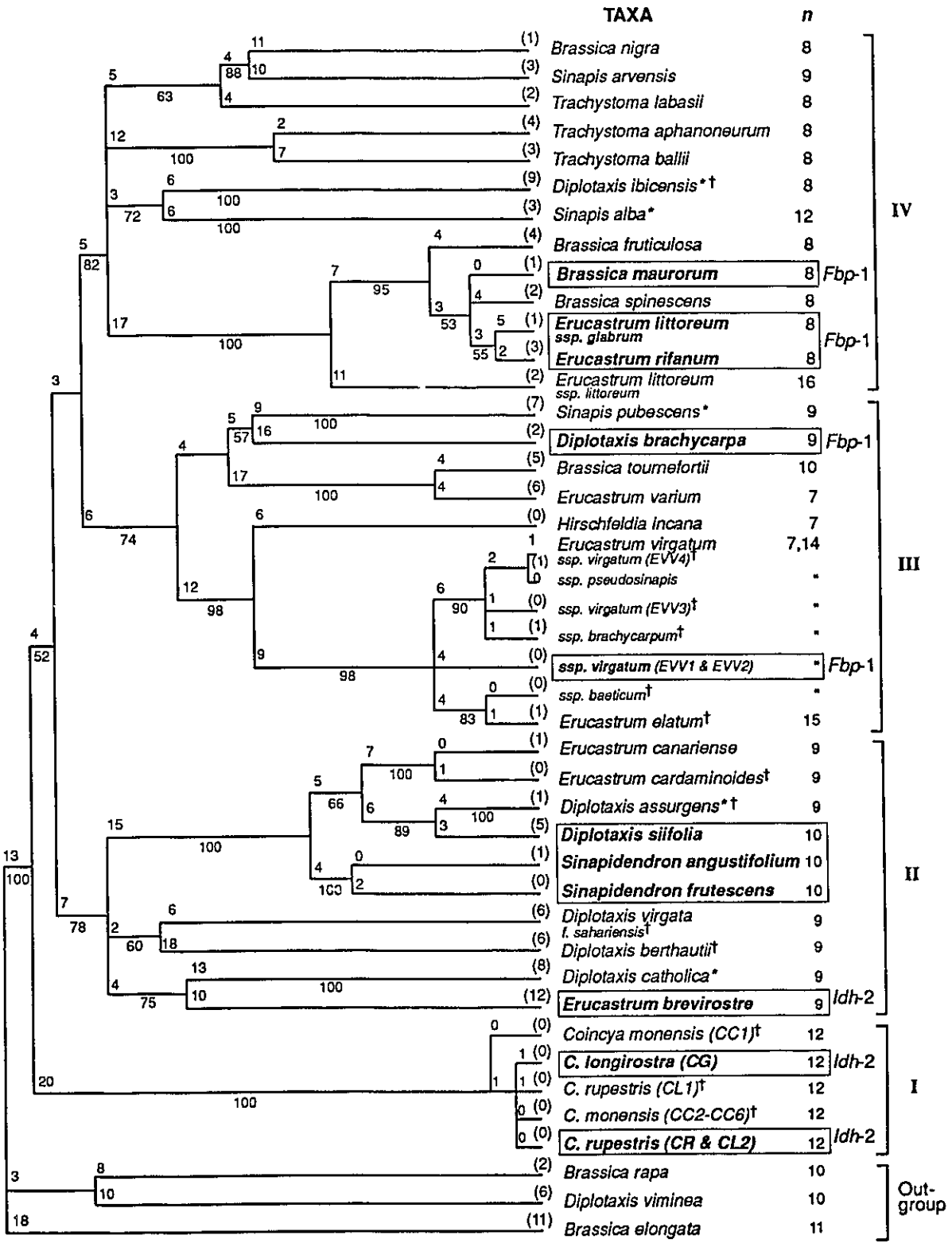

n
Brassica rapa 10 Fbp-2
Brassica oleracea 9
Brassica montana 9
Brassica cretica 9
Brassica insularis 9 Fbp-2
Brassica rupestris-villosa* 9
Diplotaxis erucoides 7
Diplotaxis cossoniana† 7
Erucastrum abyssinicum 16
Erucastrum strigosum 8
Erucastrum nasturtiifolium * 8,16
Brassica deflexa 7
Sinapis aucheri 7
Brassica barrelieri 10
Brassica oxyrrhina 9
Raphanus raphanistrum* 9
Diplotaxis harra 13
Eruca vesicaria 11
Eruca pinnatifida 11 Idh-2
Diplotaxis tenuifolia* 11
Rytidocarpus moricandioides 14
Moricandia arvensis 14 Idh-2
Moricandia nitens 14
Moricandia suffruticosa 28
Moricandia moricandioides 14
M. moricandioides var. microsperma† 14
Brassica gravinae 10 Idh-2
Brassica repanda 10
Diplotaxis viminea 10 Idh-2/Fbp-2
Brassica elongata 11 Idh-2
Brassica nigra 8 Outgroup
95
87
82
90
71
100
100
84
67
100
100
93
54
100
91
98

Figure 8. Relationship of taxa exhibiting the *Fbp*-1 and *Idh*-2 duplications to the phylogeny of the Nigra lineage of subtribe Brassicinae as determined by Warwick and Black (1993b). Their phylogeny is represented by "a majority-rule (50%) consensus tree based on PAUP analysis of 204 synapomorphic chloroplast DNA restriction site mutations" with autapomorphies indicated in parentheses at end of each branch, branch length indicated above each branch, percent probability values obtained in bootstrap analyses indicated below branch and major groups denoted by Roman numerals (Warwick and Black 1993b). Species complexes described in detail by Warwick and Black (1993b) are marked *, whereas species not surveyed in the present study are marked †.


TAXA
n
Brassica nigra 8
Sinapis arvensis 9
Trachystoma labasii 8
Trachystoma aphanoneurum 8
Trachystoma ballii 8
Diplotaxis ibicensis*† 8
Sinapis alba* 12
Brassica fruticulosa 8
Brassica maurorum 8 Fbp-1
Brassica spinescens 8
Erucastrum littoreum ssp. glabrum 8
Erucastrum rifanum 8 Fbp-1
Erucastrum littoreum ssp. littoreum 16
IV
Sinapis pubescens* 9
Diplotaxis brachycarpa 9 Fbp-1
Brassica tournefortii 10
Erucastrum varium 7
Hirschfeldia incana 7
Erucastrum virgatum 7,14
ssp. virgatum (EVV4)†
ssp. pseudosinapis *
ssp. virgatum (EVV3)† *
ssp. brachycarpum† *
ssp. virgatum (EVV1 & EVV2) * Fbp-1
ssp. baeticum† *
Erucastrum elatum† 15
III
Erucastrum canariense 9
Erucastrum cardaminoides† 9
Diplotaxis assurgens*† 9
Diplotaxis siifolia 10
Sinapidendron angustifolium 10
Sinapidendron frutescens 10
Diplotaxis virgata f. sahariensis† 9
Diplotaxis berthautii† 9
Diplotaxis catholica* 9
Erucastrum brevirostre 9 Idh-2
II
Coincya monensis (CC1)† 12
C. longirostra (CG) 12 Idh-2
C. rupestris (CL1)† 12
C. monensis (CC2-CC6)† 12
C. rupestris (CR & CL2) 12 Idh-2
I
Brassica rapa 10
Diplotaxis viminea 10
Brassica elongata 11
Out-group

western Mediterranean region (Warwick and Francis 1994). Results from the present study parallel those from studies of juvenile characteristics and morphometric analyses, in suggesting a close relationship for the western Mediterranean members of *Erucastrum*, *Diplotaxis* and *Sinapidendron* (Gómez-Campo and Tortosa 1974; Takahata and Hinata 1986).

Duplications for *Idh*-2 and *Gpi*-2 were observed in *Cakile* (*C. arctica*, *C. maritima*) and *Erucaria* (*E. microcarpa*), clearly defining them as a monophyletic group and supporting their classification in the subtribe Cakilinae (Schulz 1923). Owing to a lack of genetic variation, it was not possible to distinguish between the presence of one or two isozymes at IDH-2 in *E. erucarioides*, *E. hispanica*, *E. ollivieri* and *E. pinnata*, or at GPI-2 in *E. hispanica* and *E. pinnata*. Consequently, isozyme number variation for these isozymes could not be used to assess species relationships within the Cakilinae. It should be noted, however, that isozyme number variation at *Gpi*-2 may be phylogenetically informative within genus *Erucaria*. More specifically, in contrast to the *Gpi*-2 duplication observed in *E. microcarpa*, allelic segregation confirmed the presence of a single *Gpi*-2 isozyme in *E. ollivieri*.

Duplication of *Fbp*-1 - The *Fbp*-1 duplication was detected in one species of *Diplotaxis*, three species of

Brassica and three species of *Erucastrum*, all in subtribe Brassicinae. Because the duplication was observed in so few taxa within each genus, it cannot be considered as diagnostic for any of the three genera or for the subtribe. However, six of the taxa fall within two of the cpDNA groups (groups III and IV of the Nigra lineage) recognized by Warwick and Black (1993b), supporting their close phylogenetic association and suggesting that the duplication was likely present in their common ancestor (Figure 8). Three of the taxa (*B. maurorum*, *E. littoreum* ssp. *glabrum*, *E. rifanum*) constitute a monophyletic group within group III (Warwick and Black 1993b) and two of these (*B. maurorum*, *E. littoreum* ssp. *glabrum*) have been placed in the same cytodeme (Takahata and Hinata 1983). The detection of the duplication in only a single species of the Rapa/Oleracea lineage (*B. barrelieri*) suggests that the duplication arose more than once within the subtribe.

Duplication of *Fbp-2* - An additional isozyme of *Fbp-2* was detected in five species of *Brassica* and one species of *Diplotaxis*. All six taxa are classified in the subtribe Brassicinae and have been identified as members of the Rapa/Oleracea cpDNA lineage (Figure 7) (Warwick and Black 1993b). While several lines of evidence have established the close association of the five *Brassica* species (see below), *D. viminea* is quite distinct as evidenced by its separate cytodeme status (Harberd 1972) and its placement

in a separate cpDNA group within the Rapa/Oleracea lineage (Warwick and Black 1993b). Based on the distinctness of these two groups of taxa and the observation of a single isozyme in many intermediate taxa (*e.g.*, *D. erucoides*, *D. tenuifolia*, *Erucastrum nasturtiifolium*) (*sensu* Warwick and Black 1993b), it is likely that the *Fbp*-2 duplication evolved separately in *Brassica* and *Diplotaxis*.

The presence of the duplication in *Brassica cretica*, *B. insularis*, *B. macrocarpa* and *B. villosa* is consistent with their classification in Section *Brassica* (Snogerup *et al.* 1990), their common cytodeme status (Harberd 1972) and their similar chloroplast genomes (Figure 7) (Warwick and Black 1993b). The presence of the duplication in *B. rapa* (Section *Rapa* (Miller) Salmeen) as well suggests that all five species share a monophyletic origin, thus confirming previous studies which have identified the close association of *B. rapa*, to members of Section *Brassica* (Attia and Röbbelen 1986; Pradhan *et al.* 1992; Song *et al.* 1990; Song and Osborn 1992; Takahata and Hinata 1992; Warwick and Black 1991, 1993b). Further investigation is required in order to determine if the single band observed at FBP-2 in other members of Section *Brassica* (*B. hilarionis*, *B. incana*, *B. montana*, *B. oleracea*, *B. rupestris*) represented two isozymes fixed for the same allele or a single monomorphic isozyme. However, observation of allelic segregation clearly established the

presence of a single isozyme in *B. oleracea* var. *alboglabra*, indicating that variation in isozyme number occurs within the section.

Duplication of *Gpi-2* - In addition to the species of *Cakile* and *Erucaria* discussed above, *Psychine stylosa*, *Vella anremerica* and *V. pseudocytisus* also displayed a duplication for *Gpi*-2. The duplication suggests a monophyletic origin for *Psychine* and *Vella*, consistent with their classification in subtribe Vellinae (Schulz 1923) and their placement in the Vellinae cpDNA lineage (Warwick and Black 1994).

Ploidy level

Isozyme numbers in taxa with $n \leq 13$ and $n = 14$-18 were similar. Both groups exhibited the minimum number of isozymes expected for diploid plants at a majority of the loci surveyed, indicating that they are diploid. The expected minimum isozyme number was observed in taxa with $n = 14$ (*Moricandia*, *Pseuderucaria*, *Rytidocarpus*), $n = 15$ (*Crambe*, *Psychine*), $n = 16$ (*Fortuynia*, *Zilla*), $n = 17$ (*Euzomodendron*, *Vella*) and $n = 18$ (*Schouwia*, *Succowia*), refuting the widely accepted hypothesis that taxa in the tribe with $n \geq 14$ arose by complete genome duplication of lower base chromosome numbers (Harberd 1976; Gómez-Campo and Hinata 1980; Al-Shehbaz 1985). These observations further suggest that aneuploidy has played a more

significant role in the evolution of base chromosome numbers in the tribe than total genome duplication.

The duplications observed for six of the eleven isozymes surveyed are consistent with the extensive gene duplication detected at the cDNA level in *Brassica oleracea* and *B. rapa* (McGrath *et al.* 1990; McGrath and Quiros 1991) and suggest that gene duplication is widespread within the tribe. Similarly, the observed isozyme duplications, a minimum of two in the majority of taxa in the tribe, indicate that secondary polyploidy, originally proposed for genus *Brassica* (reviewed in Prakash and Hinata 1980), is a feature of the tribe as a whole. The duplication of *Pgm*-2 observed in some outgroup taxa provides preliminary evidence of secondary polyploidy in other tribes (Alysseae, Arabideae, Hesperideae, Lepidieae, Lunarieae). Gene duplication has also been reported for *Arabidopsis thaliana* (Sisymbrieae), in which the proportion of duplicated loci was determined to be 14-17% (McGrath *et al.* 1993; Kowalski *et al.* 1994).

Chromosome evolution

In the present series of experiments, no attempt was made to identify the cytological process which gave rise to the observed isozyme duplications. Consequently, it was not possible to distinguish between isozyme duplications resulting from amplication of entire genomes and those

resulting from partial genome duplication. The isozyme duplications observed for tribe Brassiceae are consistent with the extensive gene duplication proposed for genus *Brassica* by Catcheside (1934) and Sikka (1940) (described in detail in introduction) but cannot differentiate between base numbers $x = 5$ or $x = 6$ or between the cytological processes associated with change in chromosome number. Since no isozyme duplications were found to be present as three copies in any species of *Brassica*, the present data are not consistent with hypotheses of genome triplication (Chen and Heneen 1991; Lagercrantz *et al.* 1994; Kowalski *et al.* 1994). It is important to note, however, that isozyme electrophoresis is limited in its ability to detect gene duplications; that is, only isozymes of distinct electrophoretic mobilities can be distinguished; that is, without quantatative analysis of enzyme staining intensity, duplicated loci with the same alleles would be scored as a single locus. Consequently, although the isozyme data support extensive gene duplication, the level of this duplication is likely to have been underestimated. Therefore, the present data cannot reject models ($x = 3$, $x = 15$) proposing triplication of the *Brassica* genome.

Although the present study did not attempt to identify the mechanism responsible for the observed duplications, it is possible to use isozyme electrophoresis to differentiate between tandem and non-tandem duplications. This is

achieved by analyzing linkage relationships in lines which are heterozygous for both copies of the duplicated gene (Pichersky and Gottlieb 1983). Consequently, the isozyme duplications detected in this study provide a useful model for determining the nature of gene duplication in the Brassiceae. Past observations of translocations in *Brassica* spp. (Gustafsson *et al.* 1976) and *Hirschfeldia incana* have suggested that "this type of aberration was one of the mechanisms originating duplicated loci in *Brassica*" (Quiros *et al.* 1988). Consistent with this hypothesis, studies of the *Arabidopsis thaliana*, *Brassica oleracea* and *B. rapa* genomes using cDNA probes have shown few duplicate genes to occur as tandem copies (McGrath and Quiros 1991; McGrath *et al.* 1990, 1993; Kowalski *et al.* 1994). Based on this evidence, it is a strong possibility that the isozyme duplications detected in this study will also be found to be non-tandem (unlinked).

The presence of two copies of *Gpi*-2 in *Brassica fruticulosa* ssp. *cossoniana* ($n = 2x = 16$) and *Moricandia suffruticosa* ($n = 2x = 28$), of three copies of *Tpi*-1 in *B. souliei* ssp. *dimorpha* ($n = 2x = 22$) and of three copies of *Pgm*-2 and four copies of *Amp*-1 in *Sinapis boivinii* ($n = 2x = 18$) supported the purported autotetraploid origins of these taxa (Sobrino Vesperinas 1978; Al-Shehbaz 1985; Baillargeon 1986). Isozyme duplications also strongly suggested polyploidy in certain accessions of

species reported to have euploid series (*Brassica gravinae*, *Calepina irregularis*, *Crambe orientalis*, *Erucastrum abyssinicum*, *Lepidium sativum*) (Vaarama 1951; Gómez-Campo and Hinata 1980; Al-Shehbaz 1985). In contrast to other polyploid taxa, the purported hexaploid *Erucastrum littoreum* ssp. *brachycarpum* ($n = 3x = 24$) (Gómez-Campo 1978) exhibited the same numbers of isozymes as diploid taxa in the tribe. In addition, putative heterozygotes of this species displayed banding patterns and relative banding intensities more resemblant of diploid taxa than of polyploid taxa. These observations are inconsistent with the reported hexaploid count for this taxon and suggest that additional chromosome counts are warranted.

Determination of parentage in the allopolyploids *Diplotaxis muralis* and *Erucastrum gallicum*

The multi-banded, fixed-heterozygous patterns observed at the majority of loci in *Diplotaxis muralis* and *Erucastrum gallicum* provided strong evidence for an allopolyploid origin of these species, with parental taxa contributing distinct alleles (Weeden and Wendel 1989; Kephart 1990). Comparisons of allozyme patterns between allopolyploids and proposed parental taxa confirmed *D. tenuifolia* and *D. viminea*, and *D. erucoides* and *E. nasturtiifolium* as respective diploid progenitors of *D. muralis* and *E. gallicum*. Consequently, the present data

confirm the hypotheses of Harberd and McArthur (1972) and Warwick and Black (1993b) regarding the parentage of these allopolyploid species. It should be noted, however, that the hypothesis proposing *D. cossoniana* as maternal parent of *E. gallicum* (Warwick and Black 1993b) cannot be discounted as viable seed of this species was not available for study. Additivity of allozyme patterns has also been used to determine the diploid progenitors of numerous other allotetraploid species (Roose and Gottlieb 1976; Weeden and Wendel 1989), including *Brassica napus* (Coulthart and Denford 1982, Truco and Arûs 1987).

Polyploidization has been considered a rare process such that "each polyploid species typically was thought to have had a single origin, initially resulting in genetic uniformity across all individuals of the species" (Soltis and Soltis 1993). In their recent review, however, Soltis and Soltis (1993) cite over 40 examples of polyploid species which have been shown to have multiple origins, including *Brassica napus* (Song and Osborn 1992). Based on this evidence, they argue that "multiple origins of polyploids are the rule and not the exception". In contrast, the present data may indicate a limited number of origins for *D. muralis* and *E. gallicum*, since the numbers of multilocus genotypes observed within each species was so low. In order to accurately assess the number of origins of these allopolyploids, however, it would be necessary to

examine a greater number of accessions, representing a broader geographic range.

The duplicate isozymes observed at the majority of loci in *Diplotaxis muralis* and *Erucastrum gallicum* may indicate that they are of relatively recent origin since loss of duplicate isozyme expression over time has been documented in several allopolyploid species of plants and fish (reviewed in Soltis and Soltis 1993).

CONCLUSION

Isozyme duplications were phylogenetically informative at various taxonomic levels in the tribe. Isozyme duplications for *Pgm*-2 and *Tpi*-1 revealed that tribe Brassiceae is monophyletic with inclusion of *Orychophragmus* and exclusion of *Calepina* and *Conringia*, resolving the taxonomic position of these previously highly disputed genera with respect to the tribe (Gómez-Campo 1980; Al-Shehbaz 1985). Duplications for *Gpi*-2 and *Idh*-2 clearly supported subtribe Cakilinae as monophyletic. In contrast, the duplication for *Idh*-2 provided evidence for the artificial separation of subtribes Moricandiinae and Brassicinae, and for inclusion of *Moricandia* and *Pseuderucaria* in the Rapa/Oleracea lineage as defined by Warwick and Black (1993b). These results parallel those of previous morphological, molecular and hybridization studies (Al-Shebaz 1985; Takahata *et al.* 1993; Warwick and Black 1994). Duplication of *Idh*-2 in several species of *Diplotaxis*, *Erucastrum* and *Sinapidendron* supported a common ancestor for groups I and II of the Nigra lineage as defined by Warwick and Black (1993b). Similarly, duplication of *Fbp*-1 in several species of *Brassica*, *Diplotaxis* and *Erucastrum* supported a common ancestor for groups III and IV of the same lineage (Warwick and Black 1993b). Duplication of *Fbp*-2 confirmed the close

phylogenetic association of several members of *Brassica* Section *Brassica* and of these taxa to *B. rapa*, as suggested by other lines of evidence (reviewed in Warwick and Black 1991, 1993b). Duplication of *Gpi*-2 suggested a common ancestor for *Psychine* and *Vella* and supported their common placement in the Vellinae.

Isozyme numbers observed in tribe Brassiceae were similar to the expected minimum number for diploid plants, indicating that taxa with n = 7-18 are basically diploid and rejecting the hypothesis that all taxa with n = 14-18 arose by entire genome duplication from taxa with lower base chromosome numbers (Gómez-Campo 1980; Al-Shehbaz 1985). These data suggested that aneuploidy has played a more significant role in the evolution of base numbers among taxa in the tribe than has entire genome duplication. The observed isozyme duplications supported the hypothesis of secondary polyploidy for genus *Brassica* (Prakash and Hinata 1980) and suggested the widespread occurrence in the tribe of a secondarily-balanced polyploid genome. The observed isozyme duplications were also consistent with the extensive gene duplication detected in *Brassica* by other molecular approaches (McGrath *et al.* 1991, 1993; Kowalski *et al.* 1994; Lagercrantz *et al.* 1994) and suggested that gene duplication may also be a feature of other tribes in the family. The present study left unresolved the base number of the tribe.

Inheritance studies conducted on *Sinapis arvensis* showed that segregation ratios at seven loci (*Fbp*-2, *Gpi*-2, *Idh*-2, *Pgm*-2, *Pgm*-2′, *Tpi*-1, *Tpi*-1′) conformed to those expected under Mendelian inheritance. These studies also established the genetic basis of the isozyme duplications observed for *Pgm*-2 and *Tpi*-1.

Comparison of allozyme patterns between allopolyploids and proposed parental taxa, confirmed *Diplotaxis tenuifolia* (n = 11) and *D. viminea* (n = 10), and *D. erucoides* (n = 7) and *Erucastrum nasturtiifolium* (n = 8) as progenitors of *D. muralis* (n = 10+11) and *E. gallicum* (n = 7+8), respectively.

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Appendix 1. Electrode and gel buffer systems used in this study.

System	Buffers		Current (mA)	Running Time (h)
	Gel	Electrode		
A	0.065M L-histidine (free base), pH 5.7	14% electrode buffer, pH 5.7	25	5.0
B	0.065M L-histidine (free base), pH 6.5	25% electrode buffer, pH 6.5	25	6.5
C	0.067M lithium hydroxide 0.184M boric acid, pH 8.3	10% electrode buffer, 90% 0.051M tris, 0.006M citric acid, pH 8.3	60	5.5
D	0.3M boric acid, pH 8.2	0.076M tris, 0.005M citric acid, pH 8.8	55	4.5

Appendix 2. Procedures for stain preparation. All reagents were obtained from Sigma Chemical Company with the exception of ethanol, obtained from Consolidated Alcohols Ltd., HCl from ACP Chemicals Inc., $MgCl_2$ from Fisher Scientific Company and NaOH from BDH Chemicals.

<u>Alcohol dehydrogenase (ADH) 1.1.1.1</u>	
0.1M Tris (pH 8.0)	75 ml
95% Ethanol	21 ml
NAD	60 mg
MTT (100 mg/10 ml formamide)	750 µl
PMS (3 mg/1 ml H_2O)	750 µl
<u>Aminopeptidase (AMP) 3.4.11.1</u>	
Presoak 20 minutes in 0.25M Boric acid solution. Pour off and add stain:	
L-Leucyl-ß-naphthylamide HCl	53 mg*
Black K salt	23 mg*
* dissolve in 1 ml dimethyl formamide	
Solution A (0.125M NaOH and 0.176 maleic anhydride)	50 ml
Solution B (0.325M NaOH)	10 ml
dH_2O	40 ml
<u>Fructose-bisphosphate aldolase (FBA) 4.1.2.13</u>	
0.1M Tris (pH 8.0)	75 ml
Fructose-1,6-diphosphate	190 mg
NAD	30 mg
Sodium arsenate	225 mg
Glyceraldehyde-3-phosphate dehydrogenase (3500 units/ml)	30 µl
MTT (100 mg/10 ml formamide)	750 µl
PMS (3 mg/1 ml dH_2O)	750 µl
<u>Fructose-1,6-diphosphatase (FBP) 3.1.3.11</u>	
0.1M Tris (pH 8.0)	75 ml
Fructose 1-6 diphosphate	75 mg
NADP	7.5 mg
Phosphoglucose isomerase	20 mg
Glucose-6-phosphate dehydrogenase (80 units/ml)	400 µl
0.1M $MgCl_2$	4 ml
MTT (100 mg/10 ml formamide)	750 µl
PMS (3 mg/1 ml dH_2O)	750 µl
<u>Glucose-6-phosphate isomerase (GPI) 5.3.1.9</u>	
0.1M Tris (pH 8.3)	75 ml
Fructose-6-phosphate	35 mg
NADP	4 mg
Glucose-6-phosphate dehydrogenase (80 units/ml)	400 µl
0.1M $MgCl_2$	4 ml
MTT (100 mg/10 ml formamide)	750 µl
PMS (3 mg/1 ml dH_2O)	750 µl

Isocitrate dehydrogenase (IDH) 1.1.1.42

0.1M Tris (pH 8.0)	75 ml
DL-Isocitric Acid	150 mg
NADP	8 mg
0.1M $MgCl_2$	4 ml
MTT (100 mg/10 ml formamide)	750 µl
PMS (3 mg/1 ml dH_2O)	750 µl

Malate dehydrogenase (ME) 1.1.1.40

0.1M Tris (unadjusted)	75 ml
DL-malic acid	315 mg
NADP	15 mg
Stir above, titrate with HCl to pH 7.2	
0.1M $MgCl_2$	4 ml
MTT (100 mg/10 ml formamide)	750 µl
PMS (3 mg/1 ml dH_2O)	750 µl

Phosphoglucomutase (PGM) 5.4.2.2

0.1M Tris (pH 8.0)	75 ml
Glucose-1-phosphate	57 mg
NADP	4 mg
Glucose-6-phosphate dehydrogenase (80 units/ml)	400 µl
Glucose-1,6-diphosphate (5 mg/33 ml dH_2O)	4 ml
0.1M $MgCl_2$	4 ml
MTT (100 mg/10 ml formamide)	750 µl
PMS (3 mg/1 ml dH_2O)	750 µl

Shikimate dehydrogenase (SKD) 1.1.1.25

0.1M Tris (pH 8.5)	75 ml
Shikimic acid	48 mg
NADP	8 mg
MTT (100 mg/10 ml formamide)	750 µl
PMS (3 mg/1 ml dH_2O)	750 µl

Triose-phosphate isomerase (TPI) 5.3.1.1

0.1M Tris (pH 8.0)	75 ml
Dihydroxyacetone phosphate	10 mg
Ethylenediaminetetraacetic acid	30 mg
NAD	23 mg
Sodium arsenate	345 mg
Glyceraldehyde-3-phosphate dehydrogenase (3500 units/ml)	100 µl
MTT (100 mg/10 ml formamide)	750 µl
PMS (3 mg/1 ml dH_2O)	750 µl

[a]Abbreviations:
MTT: tetrazolium thiazolyl blue
NAD: ß-nicotinamide adenine dinucleotide
NADP: ß-nicotinamide adenine dinucleotide phosphate
PMS: phenazine methosulfate