

## Use of Biosystematic Data, Including Molecular Phylogenies, for Biosafety Evaluation

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### Abstract

Biosystematics, the scientific study of the kinds and diversity of organisms and relationships among them, serves many social and economic needs, particularly for biosafety evaluation in agriculture. The first use is the correct classification and nomenclature of species, which ensures retrieval of relevant information about them. Molecular techniques are used to clarify genetic affinities (phylogenies) and to determine the closest wild relatives of crop plants. Systematic studies reveal data on the breeding system and sexual compatibility of related species, likelihood of such crosses and the factors affecting the successful production and survival of hybrid progeny. They provide information on the distribution of crop and wild relatives worldwide, their ecological requirements, status in natural environments and if weedy, the areas of infestation and patterns of spread. This information is needed on a case by case basis for evaluation of both the likelihood of escape of a transgenic trait and the environmental impact if such an escape occurred. The production of transgenic crops has increased our need for such information. Examples will be drawn primarily from rapeseed and its wild relatives, and will include examples of confusion caused by the existence of Latin synonyms for the same species and their preferential use, and problems associated with the taxonomic assignment of species to separate or even "wrong" genera.

### Introduction

Biosystematics, the scientific study of the kinds and diversity of organisms and relationships among them, serves many social and economic needs, particularly to agriculture (Small, 1993). Biosystematic knowledge is critical for certification of the absence or presence of botanical materials in commodities regulated by trade barriers and for prohibitions to the movement of plant material, food quality control, and customs.

This paper will review the direct and indirect uses of biosystematics in biosafety evaluation. New biotechnology methods for integrating genes and genomes and the production of transgenic crops have increased our need for information about the wild relatives of our crop plants. Such information is needed on a case by case basis for evaluation of both the likelihood of escape of a transgenic trait through cross pollination with a related species (Table 1), as well as the environmental impact if such an escape occurred (Raybould and Gray, 1993; Dale, 1994). Examples will be drawn primarily from *Brassica* (rapeseed) and its wild relatives.

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The most obvious use of biosystematics is the correct classification and nomenclature of species, which ensures ready access to and retrieval of relevant information about them. Systematic expertise is essential for the proper interpretation and processing of such information. Plant biosystematists analyze plant variation, delimit groups, name them according to a set of rules [International Codes of Nomenclature], describe their attributes, and provide identification systems for retrieval of this information. These may be in the forms of floras (regional, national, and worldwide basis) or computerized information systems, such as databases, checklists, and maps. For the purpose of assessing whether or not there is appreciable risk of transfer from genetically modified plants to a related taxon, one should obtain advice from taxonomists specializing in that particular crop group.

Molecular techniques, now an integral part of biosystematic studies, are used to clarify genetic affinities (phylogenies) among related species and are specifically applied in biosafety evaluation to determine the closest wild relatives of crop plants and validate traditional taxonomic circumscriptions. Systematic studies also generate data on the breeding system and sexual compatibility of related crop, wild and weedy species, the likelihood of such crosses, and the factors affecting the successful production and survival of hybrid progeny and subsequent backcross generations. They also provide information on the distribution of crop and wild relatives worldwide, as well as their ecological requirements, status in natural environments, and if weedy the areas of infestation and patterns of spread.

## *Brassica* crop species and their relatives

### Taxonomic classification

The Mustard family, one of the ten most economically important plant families, consists of several crop species (Warwick, 1993). These are grown as sources of edible oil (*Brassica napus*, *B. rapa*, and *B. juncea*), industrial oil (*B. rapa*, *Crambe abyssinica* Hochst. ex O.E. Schulz), vegetables (*B. oleracea*, *Raphanus sativus*), and mustard condiments (*B. juncea*, *B. nigra*, and *Sinapis alba*) [Scientific authorities for species names not specified in the text are given in Table 4]. The hierarchy inherent in the taxonomic classification system of the family provides the framework for identifying relatives and predicting relationships for *Brassica* crop species:

Family: Mustard [Cruciferae or Brassicaceae]

Tribe: Brassiceae (54 genera, 221 species: Table 2)

Subtribe: Brassicinae (9 genera; asterisks Table 2)

Genus: *Brassica* (35 species)

Cytodeme: eg. *Brassica rapa* and related n=10 species

(includes *B. campestris*, *B. chinensis*, *B. pekinensis*)

The family with approximately 350 genera and 3,500 species, is subdivided into groups known as tribes. Although there is some controversy over the exact number and delimitation of tribes, the Brassiceae tribe which contains the genus *Brassica*, is considered a natural group, having originated from a common ancestor. The tribe is

distinguished by its two-segmented fruit and unique arrangement of the cotyledons or first leaves in the seed. The tribe is further divided on the basis of fruit length into six subtribes: Brassicinae, Moricandiinae, Raphaninae, Cakilinae, Vellinae, and Zillinae. The morphological distinction of subtribe Brassicinae from Raphaninae and Moricandiinae is not well founded (Warwick and Black, 1994) and hybridization data have indicated the possibility of genetic exchange among these three subtribes (reviewed in Warwick and Black, 1993b). Subtribe Brassicinae has nine genera and *Brassica*, 35 species. The species are further grouped into cytodemes (see hybridization section below). It is reasonable to predict from the taxonomic framework that wild *Brassica* species are the closest relatives of the *Brassica* crop species, followed by species in the related genera of subtribe Brassicinae.

The recognition of wild relatives in the literature can, however, be obscured by the use of taxonomic synonyms (either Latin or common names) for the same species. One such example is the confusion which exists over the use of *Brassica rapa* versus *Brassica campestris* (reviewed in Toxopeus *et al.*, 1984). Both species were described by Linnaeus in 1753 to indicate turnip and wild-weedy plants, respectively. The two taxa are interfertile and were first combined in 1833 by Metzger under the name *B. rapa* which, according to the International Rules of Nomenclature, makes this the correct name for the species. Other interfertile  $n=10$  taxa, such as *B. chinensis* L. and *B. pekinsis* (Lour.) Rupr. have also been placed in synonymy under *B. rapa*.

Confusion may also arise from differential preferred taxonomic treatment in different parts of the world. For example, North Americans have tended not to recognize the genera *Sinapis* and *Hirschfeldia* as distinct from *Brassica* and therefore usually refer to *Sinapis arvensis*, *S. alba* and *Hirschfeldia incana* (L.) Lagr.-Foss. under their synonyms *Brassica kaber* (DC.) Wheeler, *B. hirta* Moench and *B. adpressa* Boiss. Proposed taxonomic treatments for the new Flora of North America will recognize the three genera *Brassica*, *Sinapis* and *Hirschfeldia* (Warwick, in prep.). Common names are especially confusing and indeed the names "rape" or "rapeseed" in Europe and "canola" in Canada, are applied to two different species: *B. rapa* or *B. napus*, and may soon be applied to a third species *B. juncea*.

## Origins of the crop

Knowledge of the origin of the crop species is of major importance in biosafety evaluation studies. If the crop is of polyploid origin, it is important to identify the parental genomes and understand the range of variation and distribution associated with each of the purported parents. The main *Brassica* crop species are *B. rapa* ( $n=10$ , genome AA), *B. oleracea* ( $n=9$ , genome CC) and *B. nigra* ( $n=8$ , genome BB). These genomes have combined (likely more than once) to give the amphidiploid crop species *Brassica napus* ( $n=19$ ; AACC), *B. carinata* ( $n=17$ , BBCC) and *B. juncea* ( $n=18$ , AABB). For example, the CC genome (= *B. oleracea* cytodeme) includes the species: *B. alboglabra* L.H. Bailey, *B. bourgeauii* Webb) Kuntze, *B. cretica* Lam., *B. hilarionis* Post, *B.*

*incana* Ten., *B. insularis* Moris, *B. macrocarpa* Guss., *B. montana* Pourret, *B. oleracea*, *B. rupestris* Raf., and *B. villosa* Biv., and recent molecular studies by Song *et al.* (1992) have indicated multiple origins for *Brassica napus* involving the more primitive members of the CC genome complex.

## Molecular-based phylogenies

In a series of molecular studies (Warwick and Black, 1993a, 1994), phylogenetic relationships or genetic relatedness in the tribe Brassiceae were examined at all taxonomic levels from cytodeme to tribal rank in order: 1) to test the validity of the traditional taxonomic classification scheme, and 2) to produce a natural and biologically informative classification of the tribe. The molecular studies were based on the chloroplast genome, which is a highly conserved DNA molecule in terms of its structure, number of genes and their arrangement. Restriction site variation of the chloroplast DNA is therefore most suitable for the intergeneric and interspecific comparisons required in this study. Radioactive probes from the large single copy region of the chloroplast genome were hybridized with southern blots of 17 restriction endonucleases digests and the data visualized from the autoradiographs obtained. In the phylogenetic analyses of the data, the 870 restriction sites were treated as a two-state character (presence or absence) and analyzed with the computer program Phylogenetic Analysis Using Parsimony (PAUP).

The data revealed somewhat unexpected results on species relatedness that have important implications for biosafety evaluation studies of *Brassica* and *Sinapis* crops, as follows:

1. The genus *Brassica* is split into two main groups, which are designated as Rapa-Oleracea (Rapeseed/Cabbage) and Nigra (Mustard) lineages (Fig. 1). These two lineages have also been observed in DNA studies by Pradhan *et al.* (1992) and designated as "*Brassica*" and "*Sinapis*" lineages, respectively;
2. *Brassica rapa* and *B. oleracea* (parents of *B. napus*) share a common origin, both derived from primitive members of the *B. oleracea* cytodeme, whereas *B. nigra* and *Sinapis arvensis* share a common origin in a separate lineage (Fig. 1);
3. *Brassica* is artificially separated taxonomically from several related genera in the subtribe Brassicinae and even from two of the other subtribes (Table 3, Fig. 1). Certain species in these related genera are, therefore, closer to the cabbage and oilseed crop species than the latter are to the mustard crop species.
4. The position in the phylogenetic tree could be used to predict which weedy relatives might hybridize with the *Brassica* crops. In some cases this was not taxonomically evident, particularly if the species was in a different genus, eg. *Erucastrum gallicum*. Subsequent studies based on this prediction have shown that *E. gallicum* is sexually compatible with *Brassica napus* (G. Séquin-Swartz, unpubl. observ.);

5. The species base for potential genetic exchange is, therefore, much broader than originally predicted from traditional taxonomic circumscriptions and likely to be different for crops in the two lineages. Biosystematic information on these closely related genera is therefore equally relevant and we have summarized such information [species checklist, cytodeme status, chromosome number, hybridization data, life cycle, growth form, ecology, and geographical distribution] in a five part guide to the wild germplasm of the tribe (references cited in Warwick and Francis, 1994);
6. The molecular phylogeny agrees with hybridization data (discussed below) as regards the close genetic relatedness of genera in these lineages.

### Hybridization studies

Extensive hybridization studies have been conducted among the *Brassica* crop species and their wild relatives, through natural, hand-pollinated and artificial crosses (summarized in Warwick and Black, 1993b; Scheffler and Dale, 1994). On the basis of chromosome number and crossing ability, Harberd in 1972 defined the *Brassica* coenospecies as the "group of wild species sufficiently related to the six cultivated species of *Brassica* to be potentially capable of experimental hybridization with them." The coenospecies corresponds closely to the taxonomic subtribe Brassicinae, with the inclusion of the genera *Raphanus*, *Enarthrocarpus* and *Moricandia*. A total of 45 diploid cytodesmes or crossing groups and six amphidiploid taxa are described for the coenospecies (Warwick and Black, 1993b). The cytodeme is characterized by a single diploid chromosome number and can be defined as a group of interfertile taxa capable of genetic exchange, usually isolated geographically and often given specific rank in different parts of the range. A given cytodeme is usually cross-sterile with other cytodesmes through ordinary sexual means. However, the existence of six naturally occurring inter-cytodeme hybrids (*B. carinata* A. Braun, *B. juncea*, *B. napus* described above, *Diploaxis muralis* ( $n=10+11$ ), *Erucastrum gallicum* ( $n=7+8$ ) and *Erucastrum elatum* (Ball) O.E. Schulz ( $n=7+8$ )), suggests that further possibilities for intercytodeme exchange may exist.

Knowledge of intra-cytodeme membership may be critical since one can predict that taxa included in a single cytodeme would have similar potential for intergenomic crossing and must also be considered in biosafety evaluation studies. The levels of sexual compatibility of the two parental genomes, mitotic and gametic stability, variation due to specific parental genotype and direction of the cross may also have an effect on intergenomic hybridization (Baranger *et al.*, 1995, Dale, 1994). For example, the use of a polyploid female parent such as *Brassica napus* is usually associated with greater hybridization success. One might predict that the polyploid *Brassica juncea* (which is being developed as a new oilseed crop in Canada) or the polyploid weedy relatives, such as *Erucastrum gallicum*, would also have an increased tolerance for intergenomic hybridization. Risk evaluation requires information not only on F1 hybrid production

but on introgression of genes in subsequent backcross generations as studied for example in transgenic *Brassica napus* and weedy *B. rapa* by Mikkelsen *et al.* (1996).

Recently, several studies have documented the natural crossing of related species with *Brassica napus* under field conditions. These include wild populations of *Brassica rapa* (Jorgensen and Andersen, 1994); *B. juncea* (Frello *et al.*, 1995); *Hirschfeldia incana* (= *Brassica adpressa*) (Eber *et al.*, 1994; Lefol *et al.*, 1995), and *Raphanus raphanistrum* (Eber *et al.*, 1994; Baranger *et al.*, 1995).

## Reproductive biology

Data on breeding systems and sexual compatibility of related crop, wild and weedy species form part of traditional biosystematic studies. The three diploid species of *Brassica* (*B. oleracea*, *B. rapa*, and *B. nigra*) are insect-pollinated and strongly outbreeding with sporophytic self-incompatibility controlled by a multiple allelic series of genes at the S-locus. This increases the probability risk for transfer of transgenic traits as compared to a primarily selfing crop species. The majority of taxa in the tribe are also self-incompatible and controlled by similar self-incompatibility systems (Seguin-Swartz, Scarth and Warwick, in prep.). Self-compatibility in the tribe has multiple origins. Self-compatibility and predominantly autogamous breeding systems are usually associated with annual habit in each case and/or with amphidiploid status, for example *Brassica napus*, *Brassica juncea*, *Erucastrum gallicum*, and *Diptotaxis muralis*.

## Distribution of crop and wild relatives worldwide

The biosystematic literature, particularly floras, are primary sources of information on species distribution. In areas where transgenic crop plants are to be released, it is important to be fully informed about the geographic distribution of crops, weeds and ruderal populations, including the extent of overlapping ranges and relative abundance. In the case of *Brassica* crops, the native tribal range primarily extends from the Atlantic Ocean to the Himalayan region, occupying Mediterranean, Irano-Turanian and Saharo-Sindian phytogeographic zones, extending southward into East and South Africa, with a limited representation of species in North America (Warwick and Francis, 1994). The occurrence of species of tribe Brassiceae in Canada is summarized in Table 4. Base line data on current ecological requirements, as well as status in natural environments and weedy habitats, degree of invasiveness, are needed prior to the release of transgenic crops in order to monitor any possible environmental changes. This has been summarized for the *Brassica* wild relatives in Warwick and Francis (1994).

## Conclusion

Biosystematic data play a key role in identifying and assessing the potential candi-

dates for intergenomic hybridization, one of the key steps in risk assessment of transgenic releases. Traditional biosystematic data (classical taxonomy, hybridization, cytology, phytogeography, and ecology) provide the essential information for biosafety evaluation. Molecular data are used to clarify the genetic affinities of a crop and related taxa. A molecular-based phylogenetic tree tests the naturalness and validity of classical taxonomy for a particular group and may be of additional predictive value in determining candidates for gene transfer, where inconsistencies are observed, as shown here for *Brassica* and related genera. Even after the the potential for gene transfer has been documented, it is important to realize that many additional factors will influence the rate of successful hybridization under field conditions, i.e. the level of "risk". These include distance from the parents, synchrony of flowering, abundance and method of pollen spread (insect or wind-pollinated), distance of pollen movement and environmental conditions permitting cross-pollination (Scheffer and Dale, 1994).

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**Table 1** Examples of gene movement between crop plants and related taxa (adapted from Small, 1984; Klinger *et al.* 1992; Raybould and Gray, 1993; Dale 1994)

Crop species	Related taxa
<i>Beta vulgaris</i> L. subsp. <i>vulgaris</i> (sugar beet)	<i>B. vulgaris</i> subsp. <i>maritima</i> (L.) Arcangeli (wild beet)
<i>Curcubita pepo</i> L. (squash or gourd)	<i>C. texacana</i> (Scheele) Gray
<i>Daucus carota</i> L. subsp. <i>sativus</i> (carrot)	<i>D. carota</i> L. subsp. <i>carota</i> (wild carrot)
<i>Medicago sativa</i> L. subsp. <i>sativa</i> (alfalfa)	<i>M. falcata</i> L. and <i>M. sativa</i> complex
<i>Oryza sativa</i> L. (rice)	<i>O. perennis</i> Moench (perennial rice)
<i>Pennisetum americanum</i> subsp. <i>americanum</i> (L.) Lecke (pearl millet)	<i>P. americanum</i> subsp. <i>stenostachyum</i> (Klotzskh) Brunken (shibra)
<i>Raphanus sativus</i> L. (radish)	<i>R. raphanistrum</i> L. (wild radish)
<i>Setaria italica</i> (L.) Beauv. (foxtail millet)	<i>S. viridis</i> (L.) Beauv. (green foxtail)
<i>Sorghum bicolor</i> (L.) Moench (sorghum)	<i>S. halepense</i> (L.) Pers. (johnsongrass)
<i>Zea mays</i> L. (maize)	<i>Zea mexicana</i> (Schrad.) Kuntze (teosinte)

**Table 2.** Genera of the tribe Brassiceae (number of species in brackets)

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<i>Ammosperma</i> (2)	<i>Hemicrambe</i> (2)
<i>Boleum</i> (1)	<i>Henophyton</i> (1)
<i>Brassica</i> * (35)	<i>Hirschfeldia</i> * (2)
<i>Cakile</i> (7)	<i>Kremeriella</i> (1)
<i>Calepina</i> (1)	<i>Moricandia</i> (9)
<i>Carrichtera</i> (1)	<i>Morisia</i> (1)
<i>Ceratocnemum</i> (1)	<i>Muricaria</i> (1)
<i>Chalcanthus</i> (2)	<i>Orychophragmus</i> (2)
<i>Coincya</i> * (6)	<i>Otocarpus</i> (1)
<i>Conringia</i> (6)	<i>Physorhynchus</i> (2)
<i>Cordylocarpus</i> (1)	<i>Pseuderucaria</i> (2)
<i>Crambe</i> (25)	<i>Pseudofortuynia</i> (1)
<i>Crambella</i> (1)	<i>Psychine</i> (1)
<i>Didesmus</i> (2)	<i>Quezeliantha</i> (1)
<i>Diplotaxis</i> * (28)	<i>Quidproquo</i> (1)
<i>Dolichorhynchus</i> (1)	<i>Raffenaldia</i> (2)
<i>Douepia</i> (1)	<i>Raphanus</i> (2)
<i>Enarthrocarpus</i> (5)	<i>Rapistrum</i> (2)
<i>Eremophyton</i> (1)	<i>Rytidocarpus</i> (1)
<i>Eruca</i> * (3)	<i>Savignya</i> (1)
<i>Erucaria</i> (9)	<i>Schouwia</i> (1)
<i>Erucastrum</i> * (20)	<i>Sinapidendron</i> * (4)
<i>Euzomodendron</i> (1)	<i>Sinapis</i> * (5)
<i>Fezia</i> (1)	<i>Succowia</i> (1)
<i>Foleyola</i> (1)	<i>Trachystoma</i> * (3)
<i>Fortuynia</i> (2)	<i>Vella</i> (5)
<i>Guiraoa</i> (1)	<i>Zilla</i> (1)

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**Table 3** Division of genera and species into the two molecular lineages: Rapa-Oleracea (Rapeseed-Cabbage) and Nigra (Mustard)

Genus	No. species	
	Rapa-Oleracea Lineage	Nigra Lineage
<i>Brassica</i>	19	5
<i>Diplotaxis</i>	7	12
<i>Erucastrum</i>	6	8
<i>Sinapis</i>	1	3
<i>Eruca</i>	2	-
<i>Moricandia</i>	6	-
<i>Raphanus</i>	2	-
<i>Enarthrocarpus</i>	2	-
<i>Coincya</i>	-	6
<i>Hirschfeldia</i>	-	1
<i>Sinapidendron</i>	-	2
<i>Trachystoma</i>	-	3

**Table 4** Occurrence of weed and crop species of tribe Brassiceae in Canada (revised from Warwick 1993).

Species	Common name	Distribution <sup>1</sup>
<i>Brassica juncea</i> (L.) Czern	Indian mustard	NT-M NF NS PE NB PQ ON MB SK AB BC
<i>Brassica napus</i> L.	rapeseed, canola	NT-M NF NS PE NB PQ ON MB SK AB BC
<i>Brassica nigra</i> (L.) Koch	black mustard	NF NS NB PQ ON SK BC
<i>Brassica oleracea</i> L.	cabbage	[rare escape not naturalized] NF PE PQ ON AB BC
<i>Brassica rapa</i> L.	bird rape	NT-M YT NF NS PE NB PQ ON MB SK AB BC
<i>Cakile edentula</i> (Bigelow) Hook.	sea-rocket	NF NS PE NB PQ ON BC
<i>Cakile maritima</i> Scop.		BC
<i>Conringia orientalis</i> (L.) Dumort.	hare's-ear mustard	NF NS PE NB PQ ON MB SK AB BC
<i>Diplotaxis muralis</i> (L.) DC.	sand-rocket	NS PE NB PQ ON MB SK AB (BC?)
<i>Diplotaxis tenuifolia</i> (L.) DC.	wall-rocket	NS NB PQ ON (AB?) (BC?)
<i>Eruca vesicaria</i> (L.) Cav. subsp. <i>sativa</i> (Mill.) Thellung (= <i>E. sativa</i> Mill.)	garden rocket	[rare escape, not naturalized] PQ ON SK AB
<i>Erucastrum gallicum</i> (Willd.) O.E. Schulz	dog mustard	NT-M NF NS PE NB PQ ON MB SK AB BC
<i>Raphanus raphanistrum</i> L.	wild radish	NF NS PE NB PQ ON (MB?) SK AB BC
<i>Raphanus sativus</i> L.	radish	NT-M NF NS PE NB PQ ON MB SK AB BC
<b>Table 4 Continued</b>		
<i>Rapistrum rugosum</i> (L.) All.		[rare escape, not naturalized] PQ ON
<i>Sinapis alba</i> L.	white mustard	YT NS PE NB PQ ON MB SK AB BC
<i>Sinapis arvensis</i> L.	wild mustard	NT-M YT NF NS PE NB PQ ON MB SK AB BC

<sup>1</sup> Northwest Territories, Dist. Mackenzie, NT-M; Yukon Territory, YT; Newfoundland and Labrador, NF; Prince Edward Island, PE; Nova Scotia, NS; New Brunswick, NB; Quebec, PQ; Ontario, ON; Manitoba, MB; Saskatchewan, SK; Alberta, AB; British Columbia, BC.

Figure Caption

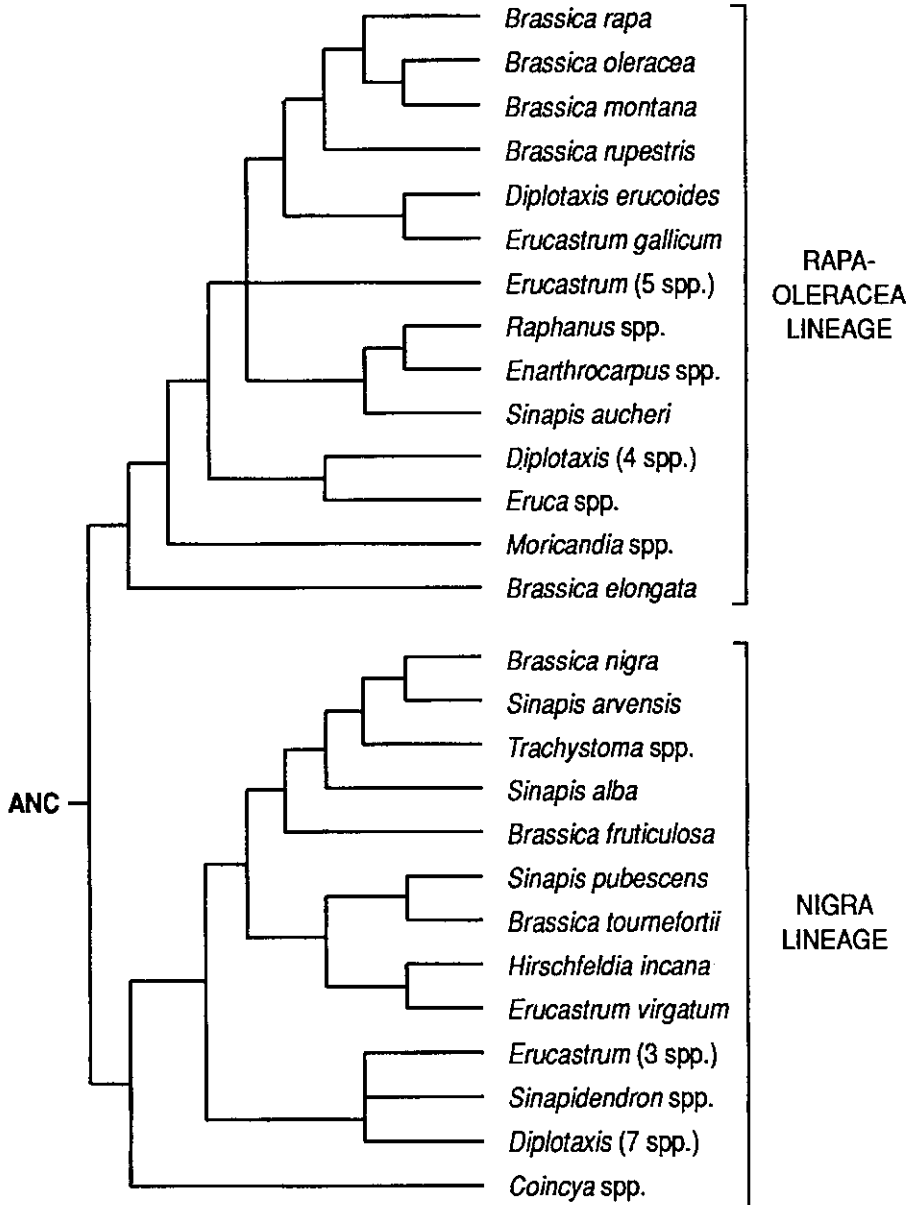


Fig.1 Phylogenetic tree based on chloroplast DNA restriction site data

for *Brassica* and related genera, showing the two molecular lineages: Rapa-Oleracea (Rapeseed-Cabbage) and Nigra (Mustard). ANC shows the common hypothetical common ancestor.

