Surinder Kumar Gupta *Editor*

Biotechnology of Crucifers

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Preface

Despite the recent advances made in the improvement of crucifer crops using conventional breeding techniques, the yield levels and the oil and meal quality that were expected could not be achieved. The understanding of genetic material (DNA/RNA) and its manipulation by scientists have provided the opportunity to improve crucifers by increasing their diversity beyond conventional genetic limitations. The application of biotechnological techniques will have two major benefits: first, it allows to choose from a number of techniques/methods for appropriate selection of favorable variants, and second, it gives an opportunity to utilize alien variation available in the crucifers to develop high-yielding varieties with good nutritional quality and resistance to insects, pests, and diseases.

Realizing the importance of biotechnology, there is an urgent need to update current techniques for enhancing crucifer crop production at the global level. The editor approached the leading scientists of the world for write-ups on the advances made in the area of crucifer biotechnology to be packaged into one volume for the benefit of students, nutritionists, and biotechnologists as well as researchers engaged in the improvement of Brassicas. The book consists of 12 chapters. Chapter 1 deals with the importance, origin, and evolution of Brassicas, while Chaps. 2 and 3 describe the major advances made in cytogenetics at the molecular level and the introgression of genes from wild species. Chapter 4 deals with microspore culture and double haploid technology, while Chap. 5 describes phytoremediation in crucifers. These are followed by chapters on genome analysis (Chap. 6) and genetic engineering of lipid biosynthesis in seeds (Chap. 7). Metabolism and detoxification of crucifer phytoalexins, the molecular basis of cytoplasmic male sterility, and self-incompatibility have been discussed in detail in Chaps. 8, 9, and 10. Chapters 11 and 12 provide brief accounts of the molecular basis of hybrid technology and genetic modifications for pest resistance.

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Chatha, India Surinder Kumar Gupta

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Chapter 1
The Importance, Origin, and Evolution

Surinder Kumar Gupta

Abstract The family Brassicaceae constitutes one of the world's most economically group of plants which includes important vegetable oilseeds and condiment crops. Amongst the crucifer crops, rapeseed is the main source of fats and oil and shown an upward trend during the past 25years (Kalia and Gupta 1997). Besides improvement in the nutritional profile of the Brassica oil and its meal, the conventional breeding as well modern biotechnological tools have led to the improvement of various agronomically important quantitative and qualitative characters. The nuclear restriction fragment length polymorphism technology has greatly aided in determining the degree of genetic variability among various Brassicas as well in studying their evolution pattern. The oldest references regarding origin and cultivation of rapeseed come from Asia, though the evolution of this crop took place in many countries throughout the globe. Lack of consistency in names, inclusion of too many forms in one species, and the entirely different forms of present day Brassicas from their ancestors make this genus a complex member of Brassicaceae and poses several taxonomic and classification problems. Still many attempts have been made to establish the origin of various Brassica species and their interrelationships through cytogenetic, chemotaxonomic, and molecular studies. The present chapter focuses on the importance origin and evolutionary developments in crucifers.

Keywords *Brassicaceae* • Rapeseed • *B. rapa* • *B. juncea* • *B. carinata* • Origin • Evolution • RFLP • Oilcrops

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1.1 Introduction

Brassicas are the world's third important source of vegetable oils after palm and soya bean (Beckman 2005) and contribute 14 % to the world's total vegetable oil pool. The production has shown a steady upward trend during the past 25 years. Brassica oilseed crops grow at relatively low temperature. In temperate regions of the world, oilseed rape *(B. napus*) and toria /turnip rape/Indian mustard are grown in subtropical parts of the Asia and is the main source of oil. The mode of reproduction varies from species to species. *B. napus, B. juncea and B. carinata* are predominately self- pollinated, although they show some degree of cross- pollination ranging from 5 to 30 %, whereas, *B. rapa, B. oleracea and B. nigra* show cross- pollination due to sporophytic self- incompatibility. All the cultivated Brassica species are highly polymorphic including oilseed crops, root crops, and vegetables such as Chinese cabbage, Broccoli, and Brussels sprouts. These Brassica vegetables are dietary staple food in various parts of the world. However, our discussion in this chapter shall concentrate on the importance and origin of major species of Brassicas.

B. napus and *B. campestris* with both spring and winter type are grown in Canada and Europe. However, in countries like India and China, the production is also shared by other species, viz, *B. oleracea* and *B. juncea*. Rapeseed oil has gradually become important domestic and industrial oil in the western nations as a result of breeding for improved oil and meal quality and better processing techniques.

1.2 History

The family *Brassicaceae* contains over 338 genera and 3,709 species (Al-Shehbaz et al. 2006). The crop Brassicas have been very important as food crops in the form of vegetables, oilseeds, feed and fodder, green manure, and condiments and have played a great role in the human history by contributing a good share of food in one form or another. The Greek, Roman, and Chinese writings of 500–200 BC refer to *rapiferous* forms of *B. rapa* and also described their medicinal values (Downey and Robellen 1989).

Species grown as oilseed crops are *B. napus, B. junca, B. rapa* and *B. carinata*. The vegetable Brassica include *B. napus, B. rapa* (Chinese Cabbage, pak-choi, Chinese mustard, broccoli and kale); *B. oleracea* (cabbage, broccoli, cauliflower, Brussels sprouts, kale, etc.,) *Raphanus sativus* and *Lepidum sativum*, *B. nigra* (black mustard), *B. juncea*, (brown mustard) and *Sinapis alba* are the main condiment of crops.

Early records indicate that Brassicas cultivated for several years in Asia. Seeds *of B. juncea* have been excavated from Chanhundaro, a site of Indus Valley civilization that existed in the plains of Punjab along the river of Indus ca 2300–1750 (Piggot 1950). Species from the genus Brassica were in use and also in Gallia (Fussel 1955) and the seeds of the species had also been found in old German graves and Swiss constructions from the Bronze Age (Neuweiller 1905; Schiemann 1932;

Witmack 1904). In Dodoneus's "Herbalist" (1578), a mention has been made regarding the growing of *B. rapa var. rapifera* in 1470 as a winter crop. In his "Herball," Gerarde (1597) had very clearly differentiated between turnips *(B. rapa)* and navews *(B. napus*). Rape has been recorded as an oilseed crop in Europe at least since the Middle Ages., but it is still uncertain which species was cultivated (Appelquist and Ohlson 1972).

Domestication of rapeseed in Europe appears to have started in the early Middle Ages, although the true turnip was probably introduced by Romans since many other oil-yielding plants, particularly olive tree, were available in Southern Europe, *B. rapa* initially spread mainly as turnip rape crop within Europe. *B. rapa* had a wide distribution before the recorded history. Indian Sanskrit literature first mentions the plants about 1599 BC as Siddharth (Prakash 1961). Seed of both *B. rapa* and *juncea* were found in the archaeological excavation of ancient village Banpo, China, that existed in Neolithic times 6,000–7,000 years ago (Liu 1985). *B. nigra* (black mustard) is mentioned in Greek literature for its medicinal value. Ancient records indicated the cultivation rape seed was predominant during the thirteenth century. The rapeseed oil is used as major source of lamp oil and it was replaced by petroleum by the end of nineteenth century

A high quality of rapeseed named as Canola developed through genetic modification following the conventional plant breeding. Canola emerged in the 1970s as a viable oilseed, with high quality oil and meal for both human as well as livestock consumption (Shahidi 1990). Today, the fatty acid profile of Canola is considered as the most desirable, of all vegetable oil profiles by nutritionists (Stringam et al. 2003). The occurrence of two important components, glucosinolates and erucic acid were considered antinutritional for animal and humans, respectively. The high amount of glucosinolates in the meal still remained a major concern in the expansion of market of the vegetable oil derived from rapeseed. Prior to 1960, the erucic acid (a long chain fatty acid) content of rapeseed oil was not of particular interest while evaluating the oil use for edible purposes. The concern was felt by the European Economic Community (EEC) in 1960 with France. West Germany, Italy, the Netherland, Belgium, and Luxemburg as the founder members, for the development of low erucic acid varieties (less than 5 %). As a result, the traditional rapeseed oil started being considered as unsafe for human health. This led to the concentration of rapeseed breeding efforts toward the development of such varieties in late 1960s and early 1970. The application of gas liquid chromatography (Craig and Murphy 1959) led to the identification of low erucic acid plants in *B. napus* and *B. campestris* with the first low erucic acid plants in them identified in 1968 and the first *B. campestris* variety in 1971. In 1977, the cultivation of such varieties was made mandatory.

The oilseed Brassica has another important byproduct known as meal/cake. It is an excellent source of protein with a favourable balance of amino acids. However, its use was limited by its high glucosinolate content, which is a constituent of most of the plants of Brassicas. Traditional rapeseed varieties contained high levels of glucosinolates in the meal which when fed to livestock in sufficient quantities led to the problems related with nutrition, digestion, and thyroid. The development of fast

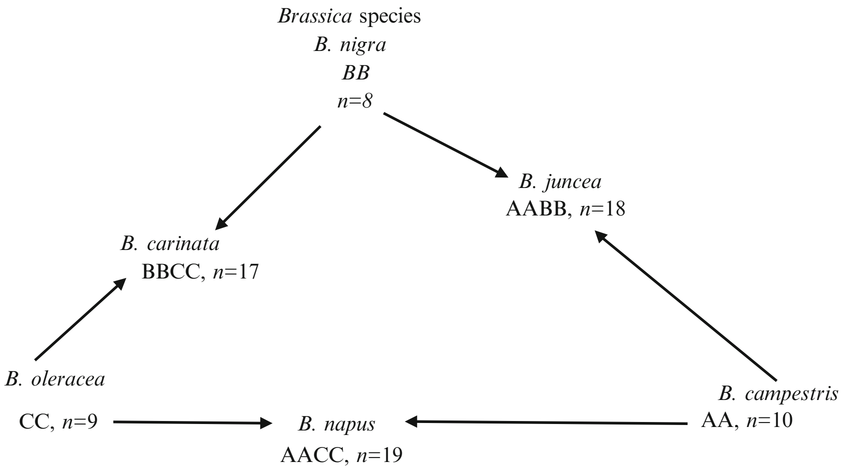

Fig. 1.1 Evolution of cultivated Brassica species and its relatives (Nagaharu 1935)

and accurate chemical methods led to the identification of plants of the *B. napus* cultivar Bronowski from Poland, which was essentially free of the harmful glucosinolates normally found in rapeseed. The low glucosinolate genes were then incorporated in the well adapted and high yielding cultivars of *B. napus* and subsequently transferred to *B. campestris*.

The Brassicaceae family comprise of 25 tribes (Al-Shehbaz et al. 2006). The tribe Brassiceae contains genus Brassica and its wild relatives. It comprised of 48 genera and approximate 240 species (Warwick and Hall 2009). Schulz (1919, 1936) established the basic taxonomic classification and he recognized ten sub-tribes whereas Gómez-Campo (1999) recommended 9 subtribes. The subtribes Brassicinae, Moricandiinae and Raphaninae are of the great relevance to the scientists who are working on the Brassica species. The relationship among the species viz., *Brassica*, *Sinapis, Diplotaxis*, *Erucastrum, Herschfeldia, Eruca* and *Raphanus*. These sub tribes have been studied by Prakash and Hinata (1980) and Takahata and Hinata (1983, 1986).

Further more during the domestication, man has modified the entire plant and the present day Brassicas are entirely different from their ancestors. Also the occurrence of similar plant forms in more than one Brassica species resulted in considerable confusion and misclassification by early botanists (Downey and Robellen 1989). The cytogenetic relationships between the Brassica species as well as their closest allies were first explained systematically by Nagaharu (1935) about 70 years ago (Fig. 1.1) These relationships show that *B. campestris* (2n = 20, AA), *B. nigra* (2n = 16,BB) and *B. oleracea* (2n = 18,CC) are the primary species and *B. napus* (2n = 38, AACC), *B. carinata* (2n = 34,BBCC) and *B. juncea* (2n = 36, AABB) are the amphidiploids resulting from paired crossings between the primary species. Morinaga (1928, 1929a, b, 1934a, b) discussed that crop Brassicas include six cytodemes, three elementary ones with 16, 18 and 20 chromosomes as diploid and three with higher chromosomes number of 34, 36 and 38 as tetraploid, the latter having evolved through interspecific hybridization in nature between any two of the elementary taxa. Herberd (1972, 1976) defined coeno species and on the basis of their chromosome number, they have been classified into 43 diploid and 13 tetra ploid cytodemes (Warwick and Black 1993).

Morinaga and his associates carried extensive cytogenetic studies in oilseed Brassicas and clarified the relationships between them (Prakash and Hinata 1980). According to the hypothesis of Morinaga and his student Nagaharu (1935), the three species with the higher chromosome number, *B. napus* L, *B juncea* L, Czern and Coss, and *B. carinata* A. Braun, are amphidiploids combining in pairs the chromosome sets of the low chromosome number species *B. nigra*, *B. oleracea* and *B. rapa.* Nagaharu (1935) verified the hypothesis with successful resynthesis of *B. napus*. Resynthesis of *B. juncea* and *B. carinata* was accomplished by Frandsen (1943, 1947). Further verification of these species relationships were obtained from the studies on phenolic compound (Dass and Nybom 1967), protein pattern (Vaughan 1977), isozymes (Coulthart and Denford 1982; Chen et al. 1989), nuclear DNA and RFLP (Song et al. 1988a, b), molecular analysis of nuclear and chloroplast DNA and fluorescence in situ hybridization (Snowdon 2007; Warwick and Sauder 2005; Lysak et al. 2005). Robellen (1960) suggested that the low chromosome number species might have developed from the ancestral species, which could have even lower chromosome numbers. Also the chromosome analysis of the monogenomic species revealed that only six chromosomes were distinctly different, the remaining being homologous with one or another of the basic set of six.

Olsson (1954) suggested that all the 20 chromosome forms of leafy, *oleiferous*, and *rapiferous* Brassicas should be grouped into one species *B. campestris*. This was in support of the views of Howard (1940) that the name *B. campestris* should be reserved for the forms with $2n = 20$ and *napus* for the forms with $2n = 38$. He proposed that the name sarson and toria should be *B. campestris* L. var. sarson and B. *campestris* L. var. toria, respectively. Singh (1958) considered yellow and brown sarson as varieties whereas Prakash (1973) considered them as the form of subspp. *oleifera*. Toxopens et al. (1984) suggested a classification and nomenclature of *B. campestris* should be changed to *B. rapa.*

1.2.1 B. rapa

The name *B. rapa* was mentioned as annual weed by Linnaeus (1973) in "Species Planatarum". It was described as a plant with rough, stiff hairs when young, and just like *B. rapa* by DeCandolle et al. (1824). However, when it was realized that *B. campestris* and the turnip rape *B. rapa* have been classified as same species, a confusion was created in nomenclature and the wild type was often subordinated under *B. rapa* (Reiner et al. 1995). *B. rapa* subspp *campestris* (formerly subspp. *sylvestris*), the wild form of *oleifera* is morphologically indistinguishable from the cultivated spring oilseed rapa. *B rapa* subspp. *dichotoma* commonly referred as toria, is an oilseed crop grown in Indian subcontinent. The yellow sarson and brown sarson (*B. rapa* subspp. *trilocularis*) are also grown in this continent.

B. rapa is thought to have originated in the mountainous areas near the Mediterranen sea (Tsunoda 1980). The orginal progenitor of the Indian and European forms was the same and that the Indian brown sarson evolved in the northwest of the Indian subcontinent from the original stock as suggested by the Russian workers, (Sinskaia 1928; Vavilov and Bukinich 1929), who regarded India as one of the independent centers of origin. The species appears to have attained a wide distribution throughout the Europe, parts of Africa, Asia and the Indian subcontinent before the recorded history. As B. rapa was most intensively grown at that time, it can be concluded that this crop was the major source of producing large quantities of vegetable oils. Seeds of B. rapa were first recorded in Europe in 1620 by the Swiss botanist Casper Banhin. However, Boswell (1949) was of the view that these existed much earlier than this. As per some anonymous authors, rapeseed was grown in Europe as early as in the thirteenth century. Prakash and Hinata (1980) also suggested that oleiferous *B. campestris* subspecies developed in two places giving rise to two different races, one European and other Asian. There is a lot of evidence that European oilseed type *B. rapa* must be very close to the turnip type *B. rapa* genetically because it was produced out of it only some 100 years ago. On other hand in China Lintao Caizi very well known to the world as *B. chinensis* (leafy type B. rapa n = 10) is used as oilseed crop. This can interpreted as parallel to the evolution of the oilseed type of the turnip type *B. rapa* in Europe (Sun et al. 1991). Alam (1945) concluded that sarson and toria types of *B. rapa* grown as oilseed crops in India and Pakistan evolved in Afghan that Persian area and migrated South India and further East. Song et al. (1988a, b) studied the phylogenetic analysis of 17 cultivated and 5 wild population of *B. rapa*. All the 17 cultivated forms were designated into two distinct groups as European and East Asia group. The phylogenetic grouping seems to correspond with the respective geographic distribution of the cultivated and wild forms of Brassica.

1.2.2 B. napus

B. napus is an amphidiploid resulting from the cross between the plants of B. *oleracea* and *B. rapa* and is comparatively of recent origin (Olsson 1960). It is uncertain to maintain if *B. napus* is found wild or not, since wild forms of this crop are difficult to find (Hinata and Prakash 1984). However, if wild *napus* exists, it must be a European- Mediterranean species that originated in the area of overlap between *B. oleracea* and *B. campestris*. Olsson (1960) suggested that *B. napus* could have arisen several times by spontaneous hybridization between the different forms of *B. rapa* and *B. oleracea*. Song and Osborn (1992) on the basis of chloroplast and mitochondrial DNA analysis suggested that *B. montana* (n = 9) might be closely related ancestral species of *B. rapa* and *B. montana* was the maternal donor. The parental origins of *B. napus* were also investigated using six microsatellite markers located in the chloroplast genome by Allender et al. (2005). Allender and king (2010) using chloroplast and nuclear markers concluded that it is highly unlikely that *B. oleracea* or any of the C- genome species are closely related to the maternal progenitor of most *B napus* accession. They also suggested that either of *B. rapa* and *montana* or a common ancestor could have been the maternal parent of *B. napus*. Though, they suggested that *B. oleracea* was not the parent of most of *B. napus* accessions, a small number of accessions shared *B. oleracea* haplotype. Similarly, the phylogenetic analysis based on nuclear RFLP data also suggests that *B. napus* has multiple origins (Song et al. 1990, 1993). The various cytoplasm types found in *B. napus* accessions correspond to the progenitor diploid species which provide a strong evidence for the multiple origins of this crop (Song et al. 1997).

In *B. napus* as well as *B. campestris*, a range of morphological forms are found both having annual and biennial types. Keeping this in view, Olsson (1960) suggested that B. *napus* could have arisen several times by spontaneous hybridization of different forms of *B. campestris* and *B. oleracea*. The majority of the cultivated *B. napus* accessions appear to have arisen by an interspecific cross in which a wild nine or ten chromosome species having the B. montana cytoplasm type.

Mizushima and Tsunoda (1967) inferred that *B. napus* was found in the coast of northern Europe because *B. oleracea* extended its territory up to northern Europe from the Irano Turanean regions with its high adaptability to low temperature. Sinskaia (1928) and Schiemann (1932) were also of the view that it might have originated in the Mediterranean region or in the western or northern Europe. In Europe, production of oleiferous, *B. napus* might have started during the middle Ages. In Asia, it was introduced during the nineteenth century. The Chinese and Japanese germplasm was developed by crossing European *B. napus* cultivars with indigenous *B. rapa* cultivars (Shiga 1970). Today most of the oilseed rape produced in China, Korea and Japan is harvested from *B. napus* cultivars. It is less adapted to the Indian sub continent due to the short days and warm growing conditions.

1.2.3 B. juncea

B. juncea is an amphidiploid and results from an interspecific cross between the plants of *B. rapa* and *B. nigra* and it has longer history than *B napus.* A number of workers have suggested that China as the centre of origin where the maximum diversity is found (Prain 1898; Sinskaia 1928; Vavilov 1949). It came to India from China through a North Eastern route and its immigration to India has been independent of an Aryan incursion (Prain 1898). According to Sun (1970), *B. juncea* originated in Middle East from where it spread to Asia. Afghanistan is thought to be as secondary centre of origin (Olsson 1960; Mizushima and Tsunoda 1967; Tsunoda and Nishi 1968) from where it spread to secondary centre on the Indian sub continent as an major oilseed crops (Hemingway 1995; Prakash and Hinata 1980). The analysis of Fraction I protein data (Uchimiya and Wildman 1978) and chloroplast DNA established the fact that *B. rapa* served as female parent in the formation of the species (Erickson et al. 1983; Palmer et al. 1983; Palmer 1988; Song et al. 1988a, b; Warwick and Black 1991; Yang et al. 2002). Qi et al. (2007) reported that some phenotypes may have evolved with *B. nigr*a as maternal parent as evidenced from the investigation on nuclear Internal Transcribe Spacer (ITS) regions of ribosomal DNA from 15 different Chinese vegetables and one oilseed form. Wu et al. (2009) studied the relationship among 95 *B. juncea* accessions originated from China, India, Pakistan and Japan following the sequenced Related Amplified Polymorphisms (SRAPs). Although winter sown accessions exhibited more genetic diversity than the spring sown accessions yet, SRAP markers did not provide clear cut separation between Indian/Pak and China winter sown mustard. Data supporting the polyphyletic origin are parallel variation observed a nuclear RFLP pattern of *B. campestris* and *B. juncea* (Song et al. 1988a, b). Wu et al. (2009) and Qi et al. (2007) also supported the idea that vegetables and oilseed forms have polyphyletic origin and evolved separately during the course of evolution.

1.2.4 B. carinata

B. carinata is commonly known as Abyssinian or Ethiopian mustard and it is indigenous Brassica oilseed and vegetable crop in Ethiopia. It is also an amphidiploid species derived from two parental species *B. nigra* as a female and *B. oleracea* a male parent (Uchimiya and Wildman 1978; Palmer et al. 1983; Song et al. 1988b; Erickson et al. 1983). Quiros et al. (1988) suggested on the basis DNA analysis that *B carinata* is an amphidiploid of the recent origin and may have the multiple origin. Song et al. (1988a, b) also confirmed on the basis of RFLP study that *B. carinata* came from *B. nigra* and *B. oleracea*. This species is a new introduction to India however it is being bred for potential commercial production in Spain, Canada, India and Australia.

1.2.5 B. nigra

B. nigra is an ancient crop which finds mention in the Sanskrit Upnisdas as a Sarshap (Prakash 1961). Hemingway (1995) placed it in Irano-Turanian, Saharo-Sindian region This species became wide spread in old world probably having its origin in Asia minor. The distribution in Europe, Mediterranan and Ethopian plateau (Bailey 1930; Schulz 1919; Mizushima and Tsunoda 1967) suggest that *B. nigra* originated in central and Southern Europe.

1.3 Conclusion

Brassica have a range of morphotypes and accordingly vary in their origin, cultivation, use, and history. The evolution of each species of Brassica has witnessed a shift in their morphophysiological traits from their original form to present day cultivars. Canola is one of the examples in rapeseed. In *B. oleracea* present day cultivars have resulted from mutation followed by adaptation and selection.

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Chapter 2
Molecular Cytogenetics

Annaliese S. Mason

Abstract Cytogenetics has played a key role in the history of scientific research in the Brassicaceae since the start of the last century. The discovery of the *Brassica* "U's Triangle" species, elucidation of phylogenetic relationships and investigations of chromosome evolution all contributed to building up the basic genomic understanding of the Brassicaceae we have today. The advent of molecular cytogenetics in this family in the last 20 years has led to a progressively greater understanding of the factors underlying chromosome dynamics and organisation, meiotic and mitotic mechanisms and cell division processes. In addition, linking molecular cytogenetics with other molecular techniques, such as marker studies, DNA sequencing and protein expression analysis, has bridged the gap between chromosomes and linkage groups, resulting in a wealth of new information in this family. Future prospects for molecular cytogenetics in the Brassicaceae are bright. The recent and imminent release of additional Brassicaceae genomes will greatly facilitate development of probes for fluorescent in situ hybridisation as well as a comprehensive understanding of gene expression and protein interactions during cell division.

Keywords Cytogenetics • Fluorescent in situ hybridisation • Chromosomes • *Brassica* • *Arabidopsis*

2.1 Introduction

Cytogenetics, literally "cell genetics", traditionally refers to the study of chromosomes. Cytogenetics conventionally encompasses studies of chromosome number, structure and organisation, chromosomal aberrations and chromosome behaviour

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