

Genetic diversity and evolution of *Brassica* genetic resources: from morphology to novel genomic technologies – a review

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Abstract

Brassica species have an economic and medicinal importance. Estimation of the amount and distribution of genetic diversity within *Brassica* species is essential for establishing efficient management, conservation and breeding practices. This review discusses the taxonomy, gene pool, and *Brassica*-derived phytochemicals and their nutraceutical importance. It also surveys the recently advanced studies of the genetic diversity and phylogenetic studies of *Brassica* species at the level of morphological, cytological, biochemical and molecular markers that have proven to be useful for evaluating the genetic variation, taxonomic relationships and species identity, and could be useful for improving *Brassica* crops through future promising breeding programmes.

Keywords: biochemical and molecular markers, *Brassica*, genetic diversity, karyotype, morphological traits, relationships

Introduction

The plant genus *Brassica* L. belongs to the tribe Brassiceae, which in turn belongs to the largest family Brassicaceae (Rakow, 2004; El-Esawi, 2012). Brassicaceae includes 380 genera and 3000 species (Heywood, 1993), whereas Mabberley (1997) recorded 365 genera and 3250 species. Judd *et al.* (1999) also recorded 419 genera and 4130 species, while Warwick *et al.* (2006) recorded 338 genera and 3709 species belonging to this family. *Brassica* species play an essential role in horticulture and agriculture, as well as contributing to the health of populations around the world (Rakow, 2004; El-Esawi, 2012; El-Esawi *et al.*, 2012a). They are important sources of vegetable oil, vegetables and condiments (Zhao, 2007). *Brassica juncea*, *Brassica napus*, *Brassica rapa* and *Brassica carinata* provide about 12% of the worldwide vegetable oil supply (Labana and Gupta, 1993). The oil is either utilized for human consumption or as a biofuel or renewable resource in the petrochemical industry. Moreover, *Brassica* species are

valuable sources of potassium, dietary fibre, phenolics, vitamins A, C and E, and various health-enhancing factors such as anticancer compounds (Fahey and Talalay, 1995; Zhao, 2007). Brassicaceae produce glucosinolates that are broken down to isothiocyanates that limit tumour development and provide protection against human cancers and heart diseases (King, 2005; El-Esawi, 2012). Several biotic and abiotic factors affect Brassicaceae growth (Relf and McDaniel, 2009; Consentino *et al.*, 2015; El-Esawi *et al.*, 2015; Jourdan *et al.*, 2015). Moreover, disease resistant *Brassica* varieties are required in future breeding programmes in order to improve their agricultural production and conservation strategies. Knowledge of the amount and distribution of genetic variability within a species is vital for establishing efficient conservation and breeding practices (Avisé, 1994; Chaveerach *et al.*, 2007; El-Esawi, 2008, 2012). It helps plant breeders develop, through selection and breeding, new or more productive crops, that are resistant to pests and diseases and highly adapted to changing environments. It also provides information for domestication and designing sampling protocols (Bretting and Widrlechner, 1995; Yu *et al.*, 2001). Therefore, this review discusses the taxonomy, gene pool and *Brassica*-derived phytochemicals and their nutraceutical importance.

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It also highlights the recent and current knowledge of the application of morphological, cytological, biochemical and molecular markers in the genus *Brassica* L. that help understand its genetic variability, phylogeny, conservation and breeding system as a basis for further research to improve the crop and develop new varieties.

Brassica-derived phytochemicals and their nutraceutical importance

Brassica vegetables are valuable sources of nutrients and health-promoting phytochemicals possessing nutraceutical and antioxidant activity such as vitamins, carotenoids, dietary fibre, phenolics, soluble sugars, minerals and glucosinolates (Wagner *et al.*, 2013). Brassicaceae produces glucosinolates which are broken down to isothiocyanates that limit tumour development and provide protection against a range of human cancers and heart diseases (King, 2005; El-Esawi, 2012). Glucosinolates and other sulphur-containing metabolites act as anti-cancer agents due to their ability to induce detoxification enzymes in mammalian cells and to decrease the rate of tumour development. Isothiocyanates are modulators of Phases 1 and 2 enzyme activity and neutralize cancer-causing chemicals that damage cells by interfering with tumour growth (King, 2005). *Brassica* secondary products have antioxidant, antiviral and antibacterial effects as well as inducing the immune system and modulating steroid metabolism (King, 2005). Moreover, *Brassica*-derived phytochemicals may counteract different genetic pathways and exhibit chemopreventive activity (Wagner *et al.*, 2013).

Anti-inflammatory properties of *Brassica*-derived phytochemicals have been reported (Juge *et al.*, 2007). These useful effects may be mediated through the stimulation of antioxidants and the prohibition of proinflammatory signalling pathways via regulation of different transcription factors that may be further controlled by miRNAs and epigenetic modifications (Wang *et al.*, 2009a, b; Wagner *et al.*, 2013). Moreover, *Brassica*-derived phytochemicals show antiviral and anti-infective activity (Yanaka *et al.*, 2009). The kappa B (nuclear factor κ B) transcription factor plays an important role in inflammatory processes, and is an attractive target to treat inflammation-related diseases (Wagner *et al.*, 2013). *Brassica*-derived phytochemicals may also mediate anti-inflammatory effects through an interaction with reduced redox regulators such as thioredoxin, glutathione, or redox factor 1, resulting in changes of the reducing milieu needed for the correct DNA binding (Heiss and Gerhäuser, 2005; Wagner *et al.*, 2013).

Nrf2 is a transcription factor playing an essential role in regulating inflammation and chemoprevention (Wagner *et al.*, 2013). Under basal conditions, Nrf2 is bound to its cytosolic inhibitor (Keap1) (Surh, 2003). In the presence

of isothiocyanates, Nrf2 could be activated through two distinct cellular signalling pathways, leading to the liberation of Nrf2 from its inhibitor Keap1. The liberated Nrf2 is transferred to the nucleus and binds together with many cofactors, including small Maf proteins (MafF, MafG and MafK). *Brassica*-derived phytochemicals also influence epigenetic mechanisms. Epigenetic aberrations, occurring in the early stages of carcinogenesis, represent an initial process of cancer development. Phytochemicals may intervene in this process to prevent cancer (Gerhäuser, 2013; Wagner *et al.*, 2013).

Taxonomy and gene pool of Brassica species and their wild relatives

The genus *Brassica* L. is one of the most economically important genera in the tribe Brassiceae, which in turn belongs to the family Brassicaceae (Cruciferae) (Rakow, 2004). There are several different opinions regarding the number of genera and species included in the Brassicaceae. Heywood (1993) recorded about 380 genera and 3000 species in this family, whereas Mabberley (1997) recorded 365 genera and 3250 species. Judd *et al.* (1999) also recorded 419 genera and 4130 species, while Warwick *et al.* (2006) recorded 338 genera and 3709 species belonging to this family. The genus *Brassica* L. comprises a diverse group of species including major vegetable and oilseed crops with a wide range of agronomic traits (Rich, 1991; Christopher *et al.*, 2005). The cytogenetic relationship of the six main economically important species of the *Brassica* genus was depicted in the U triangle (UN, 1935). Three of these species are diploid (*B. oleracea*, $2n = 18$; *B. rapa*, $2n = 20$; *B. nigra*, $2n = 16$), and three are amphidiploid (*B. napus*, $2n = 38$; *B. juncea*, $2n = 36$; *B. carinata*, $2n = 34$). *Brassica* species are characterized by a wide range of adaptations and abilities to adapt to a wide range of habitats and growing environments (King, 2005; Hong *et al.*, 2008).

Brassica oleracea is an important vegetable crop species which includes many vegetable cultivars called cole crops (Katz, 2003). These cole crops comprise cabbage (*B. oleracea* subspecies *capitata*), cauliflower (*B. oleracea* subspecies *botrytis*), brussels sprout (*B. oleracea* subspecies *gemmifera*), broccoli (*B. oleracea* subspecies *italica*), Kale and collards (*B. oleracea* subspecies *acephala*) and kohlrabi (*B. oleracea* subspecies *gongylodes*). The cole crops have extreme morphological characteristics. Examples of such morphologies include the enlarged inflorescences of cauliflower and broccoli; the enlarged stems of kohlrabi and marrowstem kale; the enlarged single apical bud of cabbage; and the several axillary buds of brussels sprout (Hong *et al.*, 2008). *Brassica oleracea* generally grows slowly and has a large storage capacity for

nutrients, which accounts for its adaptation to diverse natural habitats. It has a recent history of cultivation (Gomez-Campo and Prakash, 1999; Navabi, 2009).

Brassica rapa, commonly known as field mustard or turnip mustard, is a crop species widely cultivated as a leaf vegetable, a root vegetable and an oilseed. There are three well-defined groups of *B. rapa*, based on their morphological characteristics (CFIA, 1999): (1) the oleiferous or oil-type rape, often referred to Polish rape or summer turnip rape, of which canola is a specific form containing low erucic acid in its oil and low glucosinolate content in its meal protein; (2) the leafy type *B. rapa*, comprising the chinensis group (pak-choi, celery mustard), the pekinensis group (Chinese cabbage) and the perviridis group (tender-green); and (3) the rapiferous type *B. rapa*, including the rapifera group (turnip, rapini) and the ruvo group (turnip broccoli, Italian turnip) (CFIA, 1999). Rakow (2004) also stated that seven varieties of vegetable *B. rapa* types are known, and these are: var. *campestris*, var. *pekinensis*, var. *chinensis*, var. *para-chinensis*, var. *narinosa*, var. *japonica* and var. *rapa*. The var. *pekinensis* is adapted to a cooler climate. The var. *chinensis* is a leaf vegetable which differentiated from oilseed rape types of middle China, var. *para-chinensis* is a derivative of the var. *chinensis*, and var. *campestris* is the most primitive leaf vegetable. The var. *narinosa* has a high cold tolerance and is similar to var. *chinensis* in its adaptation. The var. *japonica* is a leaf vegetable of Japan. The var. *rapa* (turnip) is cultivated all over the world as a vegetable and as fodder for animals (Rakow, 2004).

Brassica napus L., commonly known as canola or oilseed rape, is the amphidiploid (allotetraploid) of *B. rapa* and *B. oleracea* (Tsunoda, 1980; Rakow, 2004). It is the most productive oilseed species under cultivation. Both winter and summer annual forms of *B. napus* are grown as oilseeds in many countries of the world. Its high-yield potential might be related to the high photosynthetic rate per unit leaf area which is positively related to chloroplast number per unit leaf area and to chloroplast volume. There are also root-forming *B. napus* types, known as tuber-bearing swede or rutabaga, grown as vegetables and fodder for animals (Rakow, 2004).

Brassica nigra, commonly known as black mustard, is an annual weedy plant cultivated for its seeds. It is found growing wild as a weed in the cultivated fields in the Mediterranean region (Tsunoda, 1980). Plants of *B. nigra* can reach a height of up to 2 m and do not require vernalization for flower induction (Rakow, 2004). *Brassica juncea* L. is an amphidiploid species originated from crosses between *B. rapa* and *B. nigra* (Rakow, 2004). It has a great seed yield potential for semi-arid conditions, and is known to be more drought tolerant than rapeseed species (Rabhani *et al.*, 1999). It is grown as an oilseed and leafy vegetable. *Brassica carinata*, or Ethiopian mustard, is an

amphidiploid species originated from crosses between *B. nigra* and *B. oleracea*, and contains mustard oil (Rakow, 2004).

The genetic resources available for the breeding of *Brassica* crops are regulated by the boundaries of their primary, secondary and tertiary gene pools (Harlan, 1975; Branca and Cartea, 2011). *Brassica oleracea* represents the primary gene pool, but many studies have been achieved to assess the other gene pools and their important use (Branca and Cartea, 2011). Studies on pachytene chromosome morphology helped in investigating the secondary gene pool, and identified the basic genomes of *Brassica* crops (Branca and Cartea, 2011): AA ($2n = 20$) is for *B. rapa*, BB ($2n = 16$) for *B. nigra* and CC ($2n = 18$) for *B. oleracea*. Investigations on genomic libraries of *B. napus* and *B. oleracea* revealed shared fragments among A, B and C-genomes, suggesting their partial homology and the origin of the amphidiploid species *B. napus*, *B. carinata* and *B. juncea* from the parental diploid ones (Hosaka *et al.*, 1990; Branca and Cartea, 2011). The phylogenetic studies explain the evolution of *Brassica* and allied genera from a common ancestor with $n = 6$ through increase in the number of chromosomes and partial homology of A, B and C genomes (Song *et al.*, 1990; Branca and Cartea, 2011). Finally, the tertiary gene pool involves species and genera related to *Brassica* crops in 36 cytodesmes such as *Diplotaxis*, *Enarthrocarpus*, *Eruca*, *Erucastrum*, *Hirschfeldia*, *Rhynchosinapis*, *Sinapis*, *Sinapodendron* and *Trachystoma* genera (Harbered, 1976; Branca and Cartea, 2011). These gene pools can confer favourable alleles and useful traits using special methods. Tissue culture techniques and protoplast culture helped in the introgression of beneficial genes overcoming genetic boundaries (Branca and Cartea, 2011).

Different wild *B. oleracea* species with a chromosome number of $n = 9$ (including Atlantic *B. oleracea*) were collected. Four wild *B. oleracea*-related species were found in Sicily (*B. rupestris*, *B. incana*, *B. villosa* and *B. macrocarpa*) (Branca and Cartea, 2011). Each accession was divided into three parts, stored at the UPM (Spain), the University of Tohoku (Sendai, Japan) and also at seed banks of those countries, where the collection was done (Izmir, Thessaloniki, Greece, Bari, Italy, France, Kew, UK) (Branca and Cartea, 2011). In Europe, and under the aegis of the European Cooperative Program for Crop Genetic Resources Networks (ECPGR), a working group on Brassicas was established in 1991. One of the major efforts of this group has been to set up a European *Brassica* database (Bras-EDB), which was developed by the Center for Genetic Resources, Netherlands (Boukema and van Hintum, 1998; Branca and Cartea, 2011). This database involves cultivated plant materials as well as wild ones and comprises 36 collections from 22 countries and more than 19,600 accessions (Branca and Cartea, 2011).

Brassica collections were characterized for their broadening agricultural use, including assessing and utilizing genetic variation in *B. carinata* for its utilization as an oil-seed crop (Branca and Cartea, 2011). The main aim was to create a core collection of four important *Brassica* species (*B. oleracea*, *B. rapa*, *B. napus* and *B. carinata*). This project was an important attempt to unify efforts on *Brassica* germplasm within the EU and it was complementary to the activities of the ECPGR Working Group on *Brassica* who also evaluated different wild species based on DNA analysis, morphological traits and quality aspects focused on oils and nutraceutical compounds. The role of the Working Group is to highlight the usefulness of the wild germplasm for breeding practices and their importance for improving *Brassica* crops, as well as to select the most appropriate accessions of the future European Genebank Integrated System (Astley *et al.*, 2007; Branca and Cartea, 2011).

Genetic diversity of *Brassica* species

Genetic diversity is the variation of individual genotypes within and among species. It permits species to adjust to a changing environment, whether these changes are due to human or natural factors (Chaveerach *et al.*, 2007; El-Esawi, 2008, 2012). The genetic composition of whole populations varies from place to place across a species range. These differences might emerge as a result of chance occurrences, such as the genetic composition of outspreading individuals which form a new population (founder effect), or changes in allele frequencies which result from chance crossings in very small populations (genetic drift) (Meffe and Carroll, 1994; Husband and Schemske, 1996; Falk *et al.*, 2001). Differences among populations may also arise when the environment in different locations exposes the individuals to different conditions and optima for survival and reproduction (fitness). For these and other reasons, populations can diverge from one to another in their genetic profile. This divergence is especially strong and quick when there is a little gene flow among populations (e.g., limited dispersal of pollen or seeds or limited movement of animals across physiographic barriers) (Falk *et al.*, 2001). Over evolutionary time, such among-population genetic differences could accumulate, resulting in the development of a new species (allopatric speciation).

Effective conservation of *Brassica* genetic resources requires a complementary approach that makes use of both *ex situ* and *in situ* conservation methods to maximize the genetic diversity available for use (Karp *et al.*, 1997). *In situ* conservation is a method for conserving forest species and wild crop relatives and is based on the maintenance of the whole ecosystem in which the target taxa are present with other species (Hodgkin *et al.*, 1995). *In situ*

conservation helps evolution to continue, increases the amount of diversity which could be conserved, and strengthens the links between conservation workers and the communities who have maintained and used the resources (Karp *et al.*, 1997).

Ex situ conservation aims at maintaining the accessions without change in their genetic constitution (Frankel *et al.*, 1995). The methodologies used are designed to minimize the possibility of selection, mutation, random genetic drift or contamination. For many species, long-term *ex situ* conservation can be undertaken by storing seeds for long periods at low humidities and temperatures (Karp *et al.*, 1997). The major world germplasm collections of *Brassica* are located in Centre for Genetic Resources (CGN, The Netherlands), Institute for Horticultural Plant Breeding (IVT, The Netherlands), Horticultural Research Institute (HRI, UK) and Gene Bank of Crop Research Institute (UK) (van der Meer *et al.*, 1984). Genetic diversity can be estimated based on morphological, cytological, biochemical and molecular markers.

Morphological traits

Morphological variation in plant species has been described for traits which are controlled by a single or multiple gene systems. The greater the number of gene loci determining the trait, the more continuous the variability will be (Ayala, 1982; El-Esawi *et al.*, 2012a). The expression of morphological traits is affected by the environment. Thus, the variation patterns in these morphological traits are considered to be the result of both environmental and genetic attributes. The differentiation of populations in a species is carried out by coding the measurements of quantitative traits and their evaluation based on different biological and mathematical assumptions using a number of computer softwares (Rohlf, 2000). Morphological traits have been used by different evolutionists to assess the genetic variation and phylogenetic relationships among populations of the plant species, for example, soybean (Iqbal *et al.*, 2008), rice (Rashid *et al.*, 2008; Bibi *et al.*, 2009), *Lactuca* (El-Esawi, 2008) and *Brassica* (Rabbani *et al.*, 1999; Kop *et al.*, 2003; Balkaya *et al.*, 2005).

Rabbani *et al.* (1999) assessed the morphological variation in oilseed mustard. The results showed a considerable level of variation among all accessions for various traits. Seedling characteristics exhibited less variation, while the largest variation was recorded for flowering and maturity stage characters. Some of the related traits were significantly correlated with each other. Furthermore, Balkaya *et al.* (2005) evaluated the morphological variation of white head cabbage (*B. oleracea* var. *capitata* subvar. *alba*). Cluster analysis based on 12 quantitative and ten qualitative

variables identified ten groups. Morphological variability was high among the genotypes studied.

Cytological markers and karyotyping

Karyotype study is a beneficial tool in taxonomy either to characterize taxa or to reconstruct their phylogeny (Koopman and De Jong, 1996; El-Esawi, 2008). Its value for phylogeny reconstruction in *Brassica* species has been confirmed and demonstrated (Allam *et al.*, 1985). The cytological markers have been used by taxonomists to assess the relationships and genetic variability in plants, for example, barley (Jahan and Vahidy, 2008), *Lactuca* (Sammour *et al.*, 2013; El-Esawi and Sammour, 2014) and *Brassica* (Allam *et al.*, 1985; Hasterok and Maluszynska, 2000; Kulak *et al.*, 2002).

Allam *et al.* (1985) studied the karyotype and meiotic behaviour of *B. oleracea*. The results revealed that the two karyotypes appeared to be similar in their gross morphology though they varied in minute details. In meiosis, the two varieties were almost normal though they varied in their chiasma frequency. Kulak *et al.* (2002) also studied the karyotypes of three amphidiploid species (*B. napus*, *B. juncea* and *B. carinata*). They identified eight out of 19 pairs of chromosomes in *B. napus*, ten out of 18 pairs in *B. juncea* and six out of 16 pairs in *B. carinata*. *Brassica* species have small, morphologically similar chromosomes.

The chromosomes of *Brassica* species are relatively small, morphologically similar and numerous in allotetraploids. However, their analysis based on morphometric and karyological features only, is extremely difficult and requires additional markers (Olin-Fatih and Heneen, 1992; Kulak *et al.*, 2002). Therefore, isozyme and molecular markers have been established and used.

Biochemical markers

Biochemical markers including storage proteins and isozymes have been used to assess the genetic diversity and phylogenetic relationships of *Brassica* and several plant species.

Storage proteins

Proteins are the translational products of DNA molecules, and can form structural and enzymatic components of plant cells. The transcription and translation of the nucleotide sequences of genes result in the formation of amino acids (El-Esawi, 2008; Kephart, 1990). Therefore, the variation detected in proteins is as a mirror for genetic variations. Proteins have been extracted and assessed using different methodologies including, but not limited to,

chromatography, ultracentrifugation and electrophoresis. Electrophoresis proved to be the most suitable method for separation and comparison of proteins (Gordon *et al.*, 1988; El-Esawi, 2008), and could be used to characterize different plant genotypes (Sammour, 1990, 1999; Sammour *et al.*, 1994; DellaGatta *et al.*, 2002; Liang *et al.*, 2006; Toosi *et al.*, 2011). Electrophoretic analysis of seed storage proteins was used to assess the genetic variation, which provide a useful information for evaluating the taxonomic relationships at the plant species and subspecies levels (Vries, 1996; Rabbani *et al.*, 2001; Sihag *et al.*, 2004; Sammour *et al.*, 2007; Kakaei and Kahrizi, 2011; Toosi *et al.*, 2011; Khurshid and Rabbani, 2012; Khan *et al.*, 2014; Choudhary *et al.*, 2015; Mir *et al.*, 2015). Toosi *et al.* (2011) used sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to characterize the protein profiles of *B. juncea* var. Ensabi at different growth stages. Out of 11 protein bands detected in seed proteins, five bands matched the seed protein profiles of other *B. juncea* varieties. A comparison of the protein profiles at different growth stages revealed a steady expression of numerous genes encoding different proteins in *B. juncea*. Furthermore, SDS-PAGE was used to characterize the seed storage protein of 52 accessions of oilseed mustard germplasm from Pakistan (Rabbani *et al.*, 2001). The results indicated that the generated protein markers could not distinguish the closely related oilseed cultivars from each other. However, these polypeptides proved to be efficient to distinguish *B. juncea* and *B. campestris*. Khurshid and Rabbani (2012) also studied the genetic diversity of *Brassica* species based on protein markers using SDS-PAGE technique. The results revealed considerable degree of polymorphism and distinguished among the genotypes analysed.

Khan *et al.* (2014) used SDS-PAGE to analyse the seed storage proteins of a collection of 136 accessions of *B. napus* L. A total of 21 protein sub-units were detected and used to distinguish among the accessions. Out of these 21 bands, 16 (76.19%) were polymorphic and five (23.81%) were monomorphic. The similarity coefficient among these accessions varied between 0.83 and 0.98. The cluster analysis divided the accessions into five major clusters. The results also showed a low level of genetic variation. Further studies are recommended using two-dimensional (2D) gel electrophoresis along with molecular markers in order to reveal high levels of genetic variation among these accessions. Mir *et al.* (2015) evaluated the genetic variation and phylogenetic relationships among *B. juncea* accessions using SDS-PAGE. The cluster analysis divided the accessions into two main clusters and distinguished among the accessions analysed. SDS-PAGE of seed storage proteins proved to be an efficient method for distinguishing among plant populations.

Isozyme markers

Isozymes are molecular forms of an enzyme, having a different structure and a similar catalytic function (El-Esawi, 2008). Allozymes are allelic variants of enzymes encoded by structural genes of the same locus. Isozymes originate through the changes of amino acids, that change the net charge, the spatial structure of the enzyme molecules and their electrophoretic mobility. After specific staining, the isozyme profile of individuals can be detected (Dziechciarková *et al.*, 2004; Kumar *et al.*, 2009). Isozyme traits have been successfully utilized to study the genetic variation, phylogenetic relationships, population genetics, taxonomy and developmental biology as well as to direct utilization in plant genetic resources management and plant breeding (Dziechciarková *et al.*, 2004; Kumar *et al.*, 2009; El-Esawi, 2015a, b), for example, red clover (Mosjidis *et al.*, 2004), blue pine (Bakshi and Konnert, 2011), *Lactuca* (El-Esawi, 2008) and *Brassica* (Lázaro and Auginagalde, 1998a; Raybould *et al.*, 1999). Lázaro and Auginagalde (1998a) assessed the genetic diversity of *B. oleracea* based on five enzyme systems. The average values for the expected heterozygosity and percentage of polymorphic loci were 0.224 and 54%, respectively. The intra- and interpopulational variations were 67 and 33%, respectively. Raybould *et al.* (1999) also evaluated the genetic diversity in natural populations of *B. oleracea* using four isozymes and seven microsatellite loci. All loci were polymorphic, and the diversity index of microsatellite loci was similar to that of isozymes. Genetic differentiation among accessions (F_{ST}) was significant for all loci. The above studies have definitely proven that isozyme traits are efficient for characterizing genetic variability, taxonomic relationships and species identity.

Molecular markers

Molecular markers are regions in the genome that are heritable as simple Mendelian traits (Schulman *et al.*, 2004) and can be used to assess the genetic variation and phylogenetic relationships in plants. Several criteria should be considered in choosing molecular techniques for genetic diversity studies including the following: whether the techniques are highly reproducible between laboratories and whether the data that are generated can be reliably transferred; whether markers are dominant or codominant, allowing heterozygotes and homozygotes to be distinguished; the amount of genomic sequence information required; and whether the markers detect highly polymorphic loci (Osman *et al.*, 2003; El-Esawi, 2008, 2012). At present, various dominant and codominant molecular markers are available for assessing genetic diversity in plants, such as random amplified polymorphic DNA

(RAPD), amplified fragment length polymorphism (AFLP), restricted fragment length polymorphism (RFLP), microsatellites (simple sequence repeats (SSRs)) and single nucleotide polymorphisms (SNPs).

Random amplified polymorphic DNA

RAPD is a PCR-based technology based on enzymatic amplification of target or random DNA fragments with arbitrary primers (El-Esawi, 2008; Kumar *et al.*, 2009). Each product is derived from a genome region, which contains two short segments in inverted orientation, on opposite strands which are complementary to the primer. Amplified products are generally separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet light (El-Esawi, 2008; Kumar *et al.*, 2009). The RAPD system has been used in characterization of different resistance genes, hybrid origin identification (Friesen *et al.*, 1997) and breeding utilization (Baril *et al.*, 1997). The RAPD technique has also been used for assessing the genetic variation in plant species and to establish differences among lines of apparently closely related populations in germplasm collections, for example, rice (Bibi *et al.*, 2009), *Lactuca* (El-Esawi, 2008) and *Brassica* (Lázaro and Auginagalde, 1998b; Crockett *et al.*, 2000; Ali *et al.*, 2007; Saha *et al.*, 2008).

Lázaro and Auginagalde (1998b) used RAPD markers to evaluate the genetic diversity in *B. oleracea* group. The average value of Nei genetic distance among taxa was 1.825. Analysis of molecular variance (AMOVA) detected the highest amount of genetic variation in *B. oleracea* subsp. *cretica* (variance = 0.519), followed by *B. oleracea* subsp. *rupestris* (variance = 0.420). Saha *et al.* (2008) also studied the genetic diversity and relationship among *Brassica* species based on RAPD markers. The highest percentage of polymorphic loci (37.29%) was found in the accession of BARI sarisha-12 (*B. rapa*). In conclusion, genetic analysis with dominant RAPD markers is often quick, less technical and less expensive than other molecular markers (Kumar *et al.*, 2009).

Restricted fragment length polymorphism

RFLP is a technique in which plants and organisms may be differentiated by analysis of patterns derived from cleavages of their DNA (Kumar *et al.*, 2009). RFLP markers have been used in studying genetic variability and relationships in plant species, for example, *Lathyrus* (Chtourou-Ghorbel *et al.*, 2001) and *Pleurotus eryngii* (Urbanelli *et al.*, 2007). RFLP markers have also been used in assessing genetic variation and developing detailed genetic maps of *Brassica* (Diers and Osborn, 1994; Diers *et al.*, 1996; Pradhan *et al.*, 2003) and lettuce (Landry *et al.*, 1987;

Kesseli *et al.*, 1994), assessing polymorphisms in *Brassica* lines resistant and susceptible to Xcc (Malvas *et al.*, 2003; El-Esawi, 2012), and identification of origin of cultivated lettuce (Kesseli *et al.*, 1991). Malvas *et al.* (2003) identified two DNA fragments of *RPS2* gene homologues in two *Brassica* lines resistant and susceptible to Xcc. The digestion of these fragments with restriction enzymes showed polymorphisms at the *XbaI* restriction sites. In addition to their high genomic abundance and their moderate polymorphism, RFLP markers are codominant and having high reproducibility (Kumar *et al.*, 2009).

Amplified fragment length polymorphism

AFLP is a DNA fingerprinting method that detects restricted DNA fragments by PCR (Kumar *et al.*, 2009; El-Esawi, 2012). The method is based on selectively amplifying a subset of restriction fragments from a mixture of DNA fragments obtained after genomic DNA digestion with restriction endonucleases (Kumar *et al.*, 2009). Polymorphisms can be detected from the length differences of the amplified fragments by PAGE or by capillary electrophoresis. The technique involves four steps: (1) restriction of DNA and ligation of oligonucleotide adapters, (2) preselective amplification, (3) selective amplification and (4) gel analysis of amplified fragments. The major advantages of AFLPs scored as dominant markers, include the short time required to assay large numbers of DNA loci, the effectively unlimited number of loci, and the greatly enhanced performance in terms of reproducibility, sensitivity, resolution and time efficiency (Dziechciarková *et al.*, 2004; Kumar *et al.*, 2009).

AFLP is a widely valued technology for accelerating plant improvement and gene mapping studies (Vos *et al.*, 1995). AFLP markers have been successfully used for evaluating the genetic diversity and relationships in plant species, for example, sesame (Laurentin and Karlovsky, 2006), common bean (Kumar *et al.*, 2008), potato (Wang *et al.*, 2011) and *Brassica* (Lombard *et al.*, 2000; Warwick *et al.*, 2000, 2008; Genet *et al.*, 2005; Ren-Hu and Jin-Ling, 2006; Watson-Jones *et al.*, 2006; van Hintum *et al.*, 2007; Christensen *et al.*, 2011; Faltusová *et al.*, 2011; El-Esawi, 2012). The studies on *Brassica* populations showed considerable levels of genetic variation.

Genet *et al.* (2005) demonstrated that AFLP is a reliable tool for assessing the genetic diversity of *B. carinata*. Polymorphic rates varied from 50 to 80%. Cluster analysis divided these genotypes into seven distinct clusters. Watson-Jones *et al.* (2006) used AFLP markers to evaluate the genetic variation within three species of *Brassica* (*B. nigra*, *B. oleracea* and *B. rapa*). The results revealed higher diversity within *B. oleracea* populations than the other two species and it had the highest range of diversity

among populations. van Hintum *et al.* (2007) also studied the genetic diversity in *B. oleracea* using AFLP markers. The average genetic diversity within single accessions was 0.13 and the total diversity (H_T) was 0.24.

Warwick *et al.* (2008) assessed the genetic variation and relationships among taxa of *B. rapa* using seven AFLP primer pairs which displayed similar amounts of polymorphisms (84–97%) among accessions. Christensen *et al.* (2011) also studied the diversity and genetic structure among 17 *Brassica* accessions using AFLP markers. Several landraces showed higher levels of diversity than the wild populations. An AMOVA showed that 62% of the total variation was found within accessions. Furthermore, Faltusová *et al.* (2011) evaluated the genetic diversity of *B. oleracea* using AFLP markers. A total of 806 polymorphic fragments were found across the accessions. The accessions were clustered into two main groups. Special subgroups, reflecting origin, were observed within these groups.

Microsatellites (SSRs)

Microsatellites, alternatively known as SSRs or short tandem repeats, are sections of DNA consisting of tandemly repeating mono-, di-, tri-, tetra- or penta-nucleotide units that are arranged throughout genomes of most eukaryotic species (Powell *et al.*, 1996; Kumar *et al.*, 2009; El-Esawi, 2012). The advantages of microsatellites include the codominance of alleles, their high abundance in eukaryotes and their random distribution throughout the genome, with an association in low-copy regions (Morgante *et al.*, 2002). Due to using long PCR primers, the microsatellites reproducibility is high, and their analyses do not require a high quality or quantity of DNA. Although microsatellites are codominant markers, mutations in the primer annealing sites may result in the appearance of null alleles (no amplification of the intended PCR product) (Kumar *et al.*, 2009). Microsatellites are ideal markers in gene mapping studies (Jarne and Lagoda, 1996), and assessing genetic variation and relationships in germplasm collections, for example, *Oryza sativa* (Chakravarthi and Naravaneni, 2006), wheat (Iqbal *et al.*, 2009), *Prunus avium* (Ercisli *et al.*, 2011) and *Brassica* (Flannery *et al.*, 2006; Hasan *et al.*, 2006; Louarn *et al.*, 2007; Ofori *et al.*, 2008; Moghaddam *et al.*, 2009; Wang *et al.*, 2009a, b; El-Esawi, 2012; El-Esawi *et al.*, 2012b; Wu *et al.*, 2014). The above studies on *Brassica* populations helped in understanding their genetic variability and taxonomic relationships.

Flannery *et al.* (2006) used ten plastid SSR primer sets to detect polymorphism in *Brassica*, *Arabidopsis*, *Camelina*, *Raphanus* and *Sinapis*. Eight loci were polymorphic. SSR data separated the individuals of Brassicaceae into taxon-specific groups (*Arabidopsis*, *Camelina*, *Sinapis* and *Brassica* genera). Within *Brassica*, *B. oleracea* is separated

from *B. napus* and *B. rapa*. Louarn *et al.* (2007) also evaluated 59 *B. oleracea* cultivars for microsatellites polymorphisms. All SSR markers, except one, reported a polymorphic information content (PIC value) of 0.5 or above. Ofori *et al.* (2008) used 16 microsatellite markers to evaluate the genetic diversity in European winter *B. rapa*. The results showed that the majority of genetic variation (83%) resided within cultivars. Furthermore, Moghaddam *et al.* (2009) used microsatellite and RAPD markers to assess the genetic variability among 32 rapeseed cultivars. The PIC of microsatellite markers varied from 0.60 to 0.91. Na12-C01, a microsatellite marker amplifying two different genomic regions in the *Brassica* genome was observed in the spring cultivars in one of the regions.

Genome sequencing and SNPs

Genomics technologies apply recombinant DNA, DNA sequencing methods, and bioinformatics to sequence, assemble, and analyse the structure and function of genomes (NHGRI, 2014). Sequencing aims to determine the exact order of the bases in a DNA strand. Next-generation sequencing (NGS), known as high-throughput sequencing, is a term used to describe a number of different novel sequencing technologies including Illumina (Solexa), Roche 454, Ion torrent Proton/PGM and SOLiD (NHGRI, 2014). These recent technologies conduct sequencing DNA and RNA quicker and cheaper than the previously used Sanger sequencing, and as such have revolutionized the studies of genomics and molecular biology. DNA sequencing can be used to assess mutations that may play a role in developing diseases (NHGRI, 2014). The mutations may be substitution (SNPs) or insertion and deletion (INDELs) of a single base pair or a deletion of thousands of bases. Besides their abundance in genomes, SNP markers have the advantages of being codominant and amenable to high-throughput automation (Tsuchihashi and Dracopoli, 2002). Whole-genome sequencing and SNP markers have been recently used in the genetic analyses of *Brassica* and other plants, such as phylogenetic analysis, taxonomy, estimation of genetic variation and population structure, genome-wide association studies and construction of genetic linkage maps (Trick *et al.*, 2009; Bancroft *et al.*, 2011; Lai *et al.*, 2012; Huang *et al.*, 2013).

Lai *et al.* (2012) sequenced leaf transcriptomes across a mapping population of *B. napus*. Analysis of sequence variation and transcript abundance helped constructing single nucleotide polymorphism linkage maps of *B. napus*, comprising 23,037 markers. They also used these analyses to align the *B. napus* genome with that of *Arabidopsis thaliana*, and to genome sequence assemblies of *B. rapa* and *B. oleracea*. Huang *et al.* (2013) identified a total of 892,536 bi-allelic SNPs throughout the *B. napus*

genome. Using the GoldenGate genotyping platform, 94 of 96 SNPs sampled could effectively differentiate genotypes of 130 lines from two mapping populations, with an average call rate of 92%. SNPs identified in this could also be used to directly identify causal genes in association studies. In conclusion, it is expected that current advances in *Brassica* genomics will stimulate researchers to use these commercially available genechips based on SNPs markers for fast and cost-efficient breeding and population genetic studies in the near future. Sequencing platforms will also continue to improve the output length and quality, and that the complementary algorithms and bioinformatic software required to handle large genomes, will be enhanced.

Conclusions

In this paper, the issues and recent knowledge of the genetic diversity and phylogenetic studies of *Brassica* genetic resources were elucidated at different levels extending from morphological traits to advanced molecular markers passing with cytological and biochemical traits which have proven to be efficient for assessing the genetic variability, relationships and species identity. This information could be potentially used for enhancing future *Brassica* breeding programmes of highly agronomic *Brassica* species as well as improving their phylogeny, propagation and conservation strategies. Furthermore, it may be utilized for marker-aided selection and quantitative trait loci analyses.

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