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Original Research Article

Assessment of genetic relationships within *Brassica rapa* subspecies based on polymorphism

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K e y w o r d s

Brassica rapa; genetic diversity; isozymes; oilseed rape.

A B S T R A C T

Genetic relationships of 10 subspecies of *B. rapa* was estimated using four isozyme systems. The interpreted genotypes were used to calculate genetic diversity measures (Mean number of alleles per locus (*A*), Proportion of polymorphic loci (*P*), observed and expected average heterozygosity, and F-statistics). Genetic distance matrix was used for clustering the collected subspecies. The UPGMA clustering based on Nei's genetic distance matrix separated *B. rapa* ssp. *dichotoma* and *B. rapa* ssp. *oleifera ruvo-gruppe* in one main cluster. In the remaining subspecies, *B. rapa* ssp. *oleifera* and *B. rapa trilocularis* were grouped together in a subcluster supporting the suggestion based on RFLPs that *B. rapa* ssp. *oleifera* had been derived from *B. rapa* ssp. *trilocularis*. The groupimg of *B. rapa* ssp. *rapa, B. rapa* ssp. *chinensis* and *B. rapa* ssp. *pekinensis* also supports the hypothesis that Chinese cabbage "*B. rapa* ssp. *pekinensis*" originated from inter-specific hybridization between *B. rapa* ssp. *rapa* or *B. rapa* ssp. *oleifera* with *B. rapa* ssp. *chinensis*.

Introduction

Among the *Brassicaceae*, the genus *Brassica* is the most widely cultivated in the world. *Brassica* crops contribute both to the economies and health of populations (e.g. *via* antioxidants, vitamins, anti-carcinogenic compounds). The major crop types are derived from three species, *B. rapa* (turnips, swede and Chinese cabbage A genome, n = 10), *B. nigra* (mustards B genome, n = 8) and *B. oleracea* (cabbages, brussel sprouts, kale C genome, n = 9). The amphiploid combination among these genomes results in three major crop species *B. juncea* L. (AB), *B. napus* L. (AC) and *B. carinata* Braun (BC) (U, 1935).

There are well defined groups of *Brassica rapa* based on their center of origin and morphological characteristics. Oleiferous or oil type rape, often referred to summer turnip rape, of which canola is a specific form having low erucic acid in its oil and few glucosinolates in its meal protein, has

been originated mainly in Europe (Gomez Campo, 1999; Chen et al., 2000; Guo et al., 2002, Zou et al., 2010). The second group is the Leafy type *Brassica rapa*, including c*hinensis* (Pack choi), *pekinensis* (Chinese cabbage), originated mainly in East Asia (He et al., 2003). The separate breeding tradition in India led to the development of the Sarson types, (yellow sarson *Brassica rapa* ssp. *trilocularis* and brown sarson *Brassica rapa* ssp. *dichotoma*) which are self-compatible (Gomez Campo, 1999).

As compared to its progenitor species *B. rapa* and *B. oleracea*, *B. napus* has limited variability (Prakash and Hinata, 1980). Intensive breeding has also exhausted the progenitor's variability to a considerable extent. High salt tolerance of amphidiploids with respect to their diploid relatives, suggests that salt tolerance has been obtained from A and C genomes (Ashraf and McNeilly, 2004). Thus, resynthesis of *B. napus* by utilizing the wider gene pool of the extant diploids provides new avenues for extending the range of genetic variation available to breeders (Roy and Tester 2013). So that this study was focused on the assessment of genetic relationships and genetic diversity of *Brassica rapa* subspecies in order to select more diverse accessions as supplementary resources that can be utilized for improvement of *B. napus*.

Materials and Methods

Plant material

A total of eighty-seven accessions of *B. rapa* were obtained that represent a wide geographical distribution of the genus (Table 1). Of these accessions, 80 were supplied by IPK gene bank Gatersleben Germany and the other 7 were purchased from seed markets in Egypt. The distribution of accessions was (23) accessions of *B. rapa* ssp. *chinensis*, (17) of *B. rapa*. ssp. *pekinensis*, (12) of *B. rapa* ssp. *rapa*, (5) of *B. rapa* ssp. *oleifera annua*, (5) of *B. rapa* ssp. *silvestris*, 5 *B. rapa* ssp. *oleifera biennis*, (5) of *B. rapa* ssp. *dichotoma*, (5) of *B. rapa* ssp. *oleifera ruvo-gruppe*, (5) of *B. rapa* ssp. *oleifera* and (5) of *B. rapa* ssp. *trilocularis*.

Seed germination

Seeds were surface sterilized by soaking in 70% (v/v) ethanol for 1 min, then rinsed several times with sterile distilled water. After sterilization, the seeds were germinated for 7 days at 25ºC in sterilized Petri dishes with three moist filter papers.

Isozyme extraction

Seedlings (3-day-old) were macerated in 5 ml saline solution containing 0.8% NaCl and 0.2% $NaNO_3$, then centrifuged at 12000 rpm for 15 minutes. Supernatants were collected in pre-chilled tubes and stored at -20ºC until use for electrophoretic separation of isozyme.

Isozyme electrophoresis

Mini vertical slabs of 7.5% acrylamide concentration were prepared according to Laemmli (1970). Aliquots (15 µl) of extracts were mixed with equal volumes of loading buffer (50% glycerol containing 1% bromophenol blue) and loaded onto the gels. Electrophoresis was carried out at 15 mA/gel for 60 min. The gels were stained for four isozymes according to Eduardo Vallejos (1983). The isozymes are acid phosphatase (Acp), catalase (Cat), esterase (Est), and peroxidase (Per).

Isozyme phenotypes were interpreted genetically according to standard principles (Wendel and Weeden, 1989)

and were scored collectively for all studied accessions. The genotypes were used in assessing genetic variability by the computation of allele frequency (A_f), mean alleles per locus (*A*), effective allele number (*Ae*), percentage of polymorphic loci (*P*), average observed and expected heterozygosity per locus (*Ho and He* respectively) as well as number of polymorphic alleles per locus (*A*p) according to Hedrick (1984). Genetic divergence among *B. rapa* subspecies were quantified by computing *F*-statistic (Wright, 1978), gene flow and pairwise values for Nei's Genetic distance (*D*) (Nei, 1978). The Nei's genetic distance was used to create the dendrogram using UPGMA (Sneath and Sokal, 1973).

Results and Discussion

A total of 15 polymorphic locus were observed allover the studied *B. rapa* subspecies (Table 2). These loci exhibited 30 alleles and their frequencies were computed for each subspecies. Alleles within a locus (*a* or *b*) having frequency $A_f \leq 0.20$ were termed rare alleles. These alleles were Acp1a in *B. rapa* ssp. *pekinensis*; Acp3a in *B. rapa* ssp. *dicotoma* and *B. rapa* ssp. *oleifera biennis*; Cat2a in *B. rapa* ssp. *chinensis*, *B. rapa* ssp. *rapa* and *B. rapa* ssp. *oleifera ruvo-gruppe*; Per1a and Per2a in *B. rapa* ssp. *pekinensis*; as well as Per3a in *B. rapa* ssp. *chinensis* and *B. rapa* ssp. *silvestris*. On the other hand, The loci showing allele "b" as the rare one were: Acp1b in *B. rapa* ssp. *silvestris;* Acp2b *B. rapa* ssp. *oleifera biennis*; Est1b in *B. rapa* ssp. *chinensis*; Est2b in *B. rapa* ssp. *pekinensis;* Est4b in *B. rapa* ssp. *silvestris* and *B. rapa* ssp. *chinensis;* Per1b in *B. rapa* ssp. *rapa*.

The alleles within a locus (a or b) with equal frequency (A_f = 0.50) were considered balanced alleles. This was observed in Acp1 in *B. rapa rapa*, *B. rapa* ssp. *oleifera* and *B. rapa* ssp. *trilocularis*; Acp2 in *B. rapa* ssp. *silvestris*; Acp3 in *B. rapa* ssp. *oleifera*, *B. rapa* ssp. *oleifera ruvo-gruppe*; Acp4 and Cat 4 in *B. rapa* ssp. *trilocularis*; Cat2 in *B. rapa* ssp. *trilocularis*; Cat3 in *B. rapa* ssp. *oleifera* and *B. rapa* ssp. *trilocularis*; Est2 in *B. rapa* ssp. *chinensis*; Est3 in *B. rapa* ssp. *rapa* and Per1 in *B. rapa* ssp. *oleifera biennis*. The remaining *B. rapa* subspecies were observed to be monomorphic (the frequency of allele a or b A_f =1). For example *B. rapa* ssp *oleifera annua* and *B. rapa* ssp *oleifera biennis* were monomorphic for Acp1a. On the other hand, *B. rapa* ssp. *chinensis* and *B. rapa* ssp. *oleifera* were monomorphic for Acp1b.

Genetic diversity among the collected sub-species (Table 3)

Mean alleles per locus (*A*) varied from 1.3 in *B. rapa oleifera ruvo-gruppe* to 1.75 in *B. rapa* ssp. *chinensis* with a mean of 1.33. The mean number of effective alleles per locus *Ae* ranged from 1.18 in *B. rapa* ssp. *oleifera* to 1.5 in *B. rapa* ssp. *trilocularis* with a mean of 1.33. The number of polymorphic alleles per subspecies (*Ap*) varied from 3 in *B. rapa* ssp. *oleifera* and *B. rapa* ssp. *oleifera annua* to 9 in *B. rapa* ssp. *chinensis* with a mean of 5.2. The lowest percentage of polymorphic loci (*Pp* = 20%) was observed in *B. rapa* ssp. *oleifera* and *B. rapa* ssp. *oleifera annua* while the highest value (60%) was observed in *B. rapa* ssp. *chinensis* with a mean of 34%. The observed heterozygosity (*Ho*) ranged from 0.125 in *B. rapa* ssp. *oleifera ruvo- gruppe* to 0.5 in *B. rapa* ssp. *trilocularis* with a mean of 0.247. The expected heterozygosity (*He*) varied from 0.134 in *B. rapa* ssp. *oleifera*

ruvo-gruppe to 0.297 in *B. rapa* ssp. *dicotoma* with a mean of 0.218.

The population structure and gene flow per sub-species

F_{IS}, F_{IT} and F_{ST} were calculated for polymorphic loci. "F_{IS}" is the inbreeding coefficient of individuals in each subspecies, "F_{IT}" is the inbreeding coefficient of individuals over all the studied subspecies and "F_{ST}" is the coefficient of genetic differentiation among the subspecies. The mean value of F_{IS} all over the analyzed loci was -0.0611 while that of F_{IT} over all loci was 0.6409 and mean F_{ST} over all loci was 0.7152. The value of Nm as indicator for gene flow was 0.0955.

The relationships among *B. rapa* subspecies

The subspecies were differentiated at Nei's genetic distance 0.68 into two main clusters (Figure). The first includes *B. rapa* ssp. *dicotoma* and *B. rapa* ssp. *oleifera ruvo-gruppe*. The remaining subspecies were sub-divided at Nei's genetic distance 0.54 in such a way that *B. rapa* ssp. *oleifera* and *B. rapa* ssp. *trilocularis* ocuupied one subgroup, whereas the second subgroup included the remaining subspecies. At Nei's genetic distance 0.39, two secondary subgroups were observed with *B. rapa* ssp. *oleifera biennis, B. rapa* ssp. *silvestris* and *B. rapa* ssp. *oleifera annua* in one cluster. The final group included *B. rapa* ssp. *rapa* separated from *B. rapa* ssp. *chinensis* and *B. rapa* ssp. *pekinensis.*

Genetic diversity measures of the studied subspecies

The highest genetic diversity measures were observed among accessions of *B. rapa* ssp. *chinensis* (the maximum mean number of alleles per locus, the highest polymorphic alleles per locus and the highest percentage of polymorphic loci), *B. rapa* ssp. *trilocularis* (the maximum mean number of effective alleles per locus and the maximum observed heterozygosity) and *B. rapa.* ssp. *dichotoma* (the maximum expected heterozygosity). These observations make these subspecies valuable genetic resources to be included in breeding programs for the improvement of oilseed rape *B. napus* (Girke et al., 2012; Roy and Tester 2013).

Genetic divergence (F- statistics)

The population structure and gene flow were analyzed in the term of *F* statistic. Genetic divergence was quantified by computing *F* statistic as an indicator for genetic diversity and gene flow among subspecies. Wright (1978) suggested guidelines for the interpretation of F_{ST} (based on allozyme loci). He considered ranges 0.0 to 0.05, 0.05 to 0.15, 0.15 to 0.25 and above 0.25 as indicator for little, moderate, great and very great genetic differentiation respectively. Although *Brassica rapa* was classified as obligate self-incompatible (Jorgenson and Andersen, 1994; Koch and Al-Shehbaz, 2009) the inbreeding coefficient of the individuals in the entire studied populations (within all subspecies) was relatively high (F_{IT} = 0.640). This can be attributed to the geographic isolation of the individuals of the studied subspecies. As a result of ongoing breeding depending on local preferences in different parts of the world, *B. rapa* has been undergone selection that increased genetic variation within the species (Gomez Campo, 1999; Koch and Al-Shehbaz, 2009).

On the other hand, the inbreeding

coefficient of the individuals within each subspecies was relatively low (F_{IS} = -0.261) which agreed with the self-incompatibility of *B. rapa*. The divergence among subspecies indicated very great genetic differentiation (F_{ST} = 0.7152) which means that about 72% of genetic diversity is distributed among subspecies, while 18% of the diversity is distributed within subspecies. This was coincides with low value of gene flow (*Nm* = 0.0955). Variation in genetic structure was observed among populations of the same species and was attributed to severe selection, domestication and low gene flow (Snowdon et al 2007).

Genetic relationships

Crop improvement through conventional breeding relies mainly on genetic relationships among utilized species (Kimber and McGregor, 1995). The genetic relationships among the studied subspecies of *B. raba* was assessed using agglomerative clustering based on Nei's genetic distance (Fig 1). The dendrogram based on Nei's genetic distance resulted in the grouping of *B. rapa* ssp. *dichotoma* and *B. rapa* ssp. *oleifera ruvo-gruppe*, that share the expression of 46% of the observed loci. The collected accessions of *B. rapa* ssp. *dichotoma* is mainly of Asian origin (Table 1) while *B. rapa* ssp. *oleifera ruvo-gruppe* have unknown place of collection. China has been suggested by Inaba and Nishio (2002); Wawick and Hall (2009) and Koch and Al-Shehbaz (2009) as the center of origin of ssp. *oleifera*. The grouping of these two subspecies under one cluster can indicate close correlation suggesting Asian origin of the collected acessions *B. rapa* ssp. *oleifera ruvo-gruppe*.

B. rapa ssp. *oleifera* (turnip rape) and *B. rapa* ssp. *trilocularis* (sarson) were grouped under one cluster at D=0.54. Similar relationship was observed by Song et al. (1991) based on RFLP. These subspecies shared the occurrence of 48% of the examined loci and were discriminated by the expression of alleles Acp3b, Cat3a and Cat3b. The grouping of these two subspecies coincides with the morphological classification suggested by Inaba and Nishio (2002) that sarson had been derived from turnip rape and was selected and developed in India.

At Nei's genetic distance 0.45, two subgroups were observed. The first involved *B. rapa* ssp. *oleifera annua* the other includes *B. rapa* ssp. *silvestris* and *B. rapa* ssp. *oleifera biennis* the subspecies in this subcluster also sharing alleles Acp2a, Cat2b, Est1a, Est3b Per2b, and Per3b, while *B. rapa silvestris* and *B. rapa oleifera biennis* can be distinguished from *B. rapa oleifera annua* by the presence of Est4a and Est4b. The accessions of the three subspecies represent oleiferous forms that represent the dominating forms in the European center of origin (Wawick and Hall 2009; Zhao et al., 2009).

The grouping of *B. rapa* ssp. *rapa, B. rapa* ssp. *chinensis* and *B. rapa* ssp. *pekinensis* (leafy vegetables) supports the hypothesis of Inaba and Nishio (2002); Wawick and Hall (2009) Zhao et al. (2009) that Chinese cabbage (*B. rapa* ssp. *pekinensis*) has been originated in China from inter-specific hybridization between turnip (*B. rapa* ssp. *Rapa*) or turnip rape (*B. rapa* ssp. *oleifera*) with pak choi (*B. rapa* ssp. *chinensis*).

Table.1 The number and origin of the collected *B. rapa* subspecies.

Subspecies	**Number and origin of collected accessions**
B. rapa ssp. *chinensis*	12 China, 5 unknown, 2 Taiwan, 2 Japan, 1 Cupa, 1 USA
B. rapa. ssp. *pekinensis*	5 Japan, 4 unknown, 3 China, 3 Korea, 1 Indonesia, 1 Germany
B. rapa ssp. *rapa*	7 Egypt, 1 unknown, 1 Goregia, 1 Tunisia, 1 USA, 1 Holland
B. rapa ssp. *oleifera annua*	1 Canada, 1 unknown, 2 Germany, 1 Sweden
B. rapa ssp. *silvestris*	1 Italy, 3 unknown, 1 Russia
B. rapa ssp. *oleifera biennis*	1 Unknown, 1 Germany, 1 Sweden, 1 Holland, 1 Poland
B. rapa ssp. *dichotoma*	2 China, 1 Canada, 1 India, 1 unknown
B. rapa ssp. *oleifera ruvo-gruppe*	5 unknown
B. rapa ssp. *oleifera*	5 Italy
B. rapa ssp. *trilocularis*	4 unknown, 1 India

Table.2 Allele frequency allover the observed loci in the collected *B. rapa* subspecies

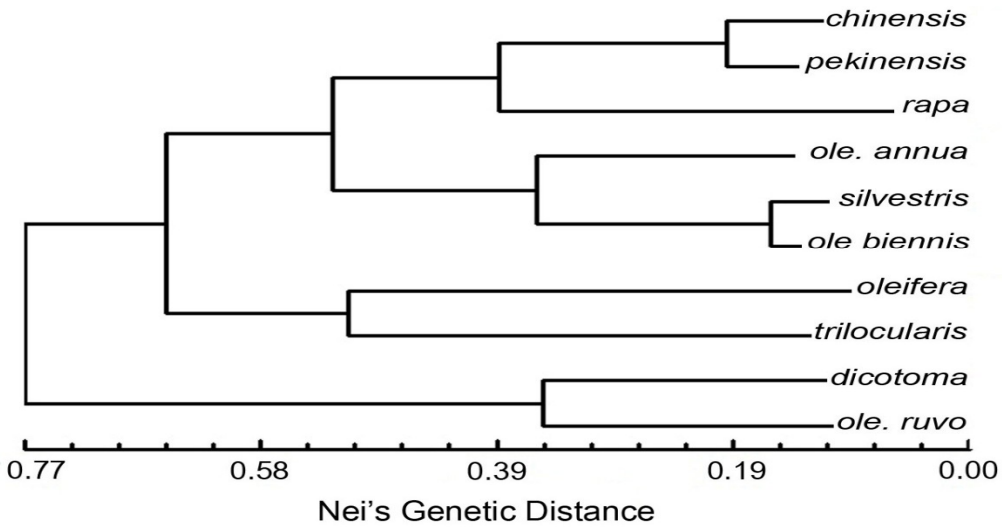
Sub species		***chinensis***	***pkinensis***	***rapa***	***o.annua***	***silvestris***	***o.biennis***	***dichotoma***	***o.ruvo gruppe***	***oleifera***	***trilocularis***
Loci	**alleles**										
Acp1	a	0.00	0.20	0.50	1.00	0.90	1.00	0.00	0.00	0.50	0.50
	b	1.00	0.80	0.50	0.00	0.10	0.00	0.00	1.00	0.50	0.50
Acp2	a	0.29	0.53	0.00	0.25	0.50	0.80	0.00	0.00	0.00	0.00
	b	0.71	0.47	1.00	0.75	0.50	0.20	1.00	1.00	1.00	1.00
Acp3	a	0.33	0.58	0.57	0.50	0.25	0.20	0.13	0.75	0.00	0.00
	b	0.67	0.42	0.43	0.50	0.75	0.80	0.88	0.25	0.00	1.00
Acp4	a	0.00	0.00	0.00	0.00	1.00	1.00	0.33	0.50	0.50	0.50
	b	0.00	0.00	0.00	0.00	0.00	0.00	0.67	0.50	0.50	0.50
Cat1	a	1.00	1.00	0.35	0.00	0.00	0.00	0.00	1.00	0.00	0.00
	b	0.00	0.00	0.65	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cat2	a	0.17	0.35	0.20	0.00	0.00	0.00	0.40	0.13	0.30	0.50
	b	0.83	0.65	0.80	1.00	1.00	1.00	0.60	0.88	0.70	0.50
Cat3	a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.50
	b	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.50	0.50
Cat4	a	0.00	0.00	0.00	0.00	1.00	1.00	0.38	0.50	0.50	0.50
	b	0.00	0.00	1.00	0.00	0.00	0.00	0.63	0.50	0.50	0.50
Est1	a	0.81	1.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00	1.00
	b	0.19	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00
Est2	a	0.50	0.80	1.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00
	b	0.50	0.20	0.00	0.00	1.00	1.00	0.00	0.00	1.00	0.00
Est3	a	0.75	0.30	0.50	1.00	1.00	1.00	0.00	0.00	0.00	0.00
	b	0.25	0.70	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Est4	a	0.18	1.00	1.00	0.00	0.80	0.60	0.00	0.00	1.00	0.00
	b	0.82	0.00	0.00	0.00	0.20	0.40	1.00	1.00	0.00	0.00
Per1	a	0.00	0.18	0.83	0.40	0.10	0.20	0.00	0.00	0.00	0.00
	b	1.00	0.82	0.17	0.60	0.90	0.80	1.00	1.00	1.00	0.00
Per2	a	0.77	0.17	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00
	b	0.23	0.83	1.00	1.00	0.00	1.00	1.00	0.00	0.00	0.00
Per3	a	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00
	b	0.95	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00

Table.3 Estimates of genetic diversity, average fixation index (F), and summary of results of tests for deviations of genotypic from Hardy - Weinberg equilibrium in 10 sub species of *Brassica rapa*.

Subspecies	N	A	Ae	Ap	P	Ho	He
chinensis	25	1.75	1.4181	9	60	0.1987	0.2657
pekinensis	18	1.6667	1.4377	8	53.33	0.2749	0.2762
rapa	9	1.4286	1.3321	6	40	0.1276	0.1955
o.annua	5	1.3333	1.2803	3	20	0.2556	0.1735
silvestris	6	1.4167	1.2091	5	33.33	0.1583	0.145
o.biennis	7	1.3636	1.2123	4	26.67	0.1455	0.1455
dichotoma	5	1.4545	1.3547	5	33.33	0.3288	0.2972
o.ruvo gruppe	6	1.3	1.188	3	20	0.125	0.1345
oleifera	7	1.4	1.3724	4	26.67	0.36	0.261
trilocularis	6	1.5	1.5	4	26.67	0.5	0.2937
Mean	9.2	1.31	1.33	5.2	34	0.247	0.218

N number of genotypes posses this locus, *A* mean allele number per locus, *A*e mean of effective allele number per locus, *A*p number of polymorphic alleles per locus , *P* % of polymorphic loci, *F* mean of wright's index per population, *H*o observed heterozygosity, *H*e expected heterozygosity, Tests number of loci for which tests could be performed, HE number of loci with a significant excess heterozygosity, HD number of loci with a significant deficiency of heteozygosity, NS non significant inbreeding coefficients

Figure.1 The UPGMA clustering based on genetic distance among the studied *Brassica rapa* subspecies



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