Brazilian Journal of Biology
ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

https://doi.org/10.1590/1519-6984.226889
Original Article

Population structure and phylogenetic relationships in *Brassica rapa* L. subspecies by using isozyme markers

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Received: August 1, 2019 – Accepted: February 1, 2020 – Distributed: August 31, 2021
(With 1 figure)

Abstract

The present study aimed to assess population structure and phylogenetic relationships of nine subspecies of Brassica *rapa* L. represented with thirty-five accessions cover a wide range of species distribution area using isozyme analysis in order to select more diverse accessions as supplementary resources that can be utilized for improvement of *B. napus*. Enzyme analysis resulted in detecting 14 putative polymorphic loci with 27 alleles. Mean allele frequency 0.04 (rare alleles) was observed in *Cat4A* and *Cat4B in* sub species *Oleifera* accession CR 2204/79 and in subspecies *trilocularis* accessions CR 2215/88 and CR 2244/88. The highest genetic diversity measures were observed in subspecies *dichotoma,* accession CR 1585/96 (the highest average of observed (H_0) and expected heterozygosity (*He*), and number of alleles per locus (*Ae*)). These observations make this accession valuable genetic resource to be included in breeding programs for the improvement of oilseed *B. napus*. The average fixation index (F) is significantly higher than zero for the analysis accessions indicating a significant deficiency of heteozygosity. The divergence among subspecies indicated very great genetic differentiation (F_{ST} = 0.8972) which means that about 90% of genetic diversity is distributed among subspecies, while 10% of the diversity is distributed within subspecies. This coincides with low value of gene flow (Nm = 0.0287). *B. rapa* ssp. *oleifera* (turnip rape) and *B. rapa* ssp. *trilocularis* (sarson) were grouped under one cluster which coincides with the morphological classification.

Keywords: Brassica rapa L., isozyme markers, F-statistics, genetic relationships.

Estrutura populacional e relações filogenéticas em subespécies de Brassica rapa L. utilizando marcadores isoenzimáticos

Resumo

O presente estudo teve como objetivo avaliar a estrutura populacional e as relações filogenéticas de nove subespécies de Brassica rapa L. representadas com 35 acessos, cobrindo uma ampla gama de áreas de distribuição de espécies usando análise isoenzimática, a fim de selecionar acessos mais diversos como recursos suplementares que podem ser utilizados para melhoria de B. napus. A análise enzimática resultou na detecção de 14 loci polimórficos putativos com 27 alelos. A frequência média de 0,04 alelo (alelos raros) foi observada em Cat4A e Cat4B, nas subespécies Oleifera CR 2204/79 e nas subespécies trilocularis CR 2215/88 e CR 2244/88. As maiores medidas de diversidade genética foram observadas na subespécie dicotômica CR 1585/96 (a média mais alta observada (H0) e heterozigosidade esperada (He) e número de alelos por locus (Ae). Essas observações tornam esse acesso um valioso recurso genético a ser incluído em programas de melhoramento de oleaginosas B. napus. O índice médio de fixação (F) é significativamente maior que 0 para os acessos à análise, indicando uma deficiência significativa de heterozigose. A divergência entre as subespécies indicou uma grande diferenciação genética (FST = 0,8972), o que significa que cerca de 90% da diversidade genética é distribuída entre as subespécies, enquanto 10% da diversidade é distribuída nas subespécies. Isso coincide com o baixo valor do fluxo gênico (Nm = 0,0287). B. rapa ssp. oleifera (nabo) e B. rapa ssp. trilocularis (sarson) foram agrupados conforme a classificação morfológica.

Palavras-chave: Brassica rapa L., marcadores de isoenzima, estatística F, relações genéticas.

1. Introduction

Brassica rapa L. em. Metzg. (syn. *Brassica. campestris* L.) belongs to family *Brassicaceae* (Takuno et al., 2007). It is consisted of various cultivated subspecies including *B. rapa* ssp. *rapa* L., *B. rapa* ssp. *oleifera* (DC.) Metzg., *B. rapa* ssp. *pekinensis* (Lour.) Hanelt, *B. rapa* ssp. *chinensis* (L.) Hanelt, subsp. *dichotoma* (Roxb.) Hanelt, subsp. *trilocularis* (Roxb.) Hanelt and ssp. *Brassica rapa subsp. campestris* (L.) A.R.Clapham, *Brassica rapa* f.

biennis (Metzg.) Thell. and *Brassica rapa* f. *annua* (Metzg.) Thell. (Stace, 1997).

It is thought that *B. rapa* L. is originated in the mountainous areas near the Mediterranean Sea (Tsunoda, 1980). However, the time of domestication is unknown. There are two main centres of origin for *B. rapa* L.: one is the Mediterranean and the other is the Afghanistan-Pakistan region (Sinskaia, 1928).

B. rapa, with the exception of the Indian yellow sarson form (subspecies *trilocularis* (Roxb.) Hanelt), is an obligate outcrosser due to the presence of self-incompatible genes (Kimber and McGregor, 1995).

Subspecies of *B. rapa* L. species are widely cultivated as leaf and root vegetables, fodder and oilseed crops. In addition, they can be a weed of cultivated land and disturbed sites (Shahzadi et al., 2015). Some of the plants of *B. rapa* L. subspecies includes both annual and biennials forms, e.g. subsp. *Chinensis*, subsp. *oleifera* and *B. rapa* subsp. *rapa* L.; others are biennials e.g. subsp. *pekinensis.*

Genetic diversity is essential for long-term survival in time and in place as it supplies the species by the plasticity to cope with the new conditions brought about by the environment. It has a leading role in competition, symbiosis, parasitism, and the impact of climate and the effect of deficiencies (Tanhuanpää et al., 2016). The knowledge of the genetic diversity of plant genetic resources is essential for saving the present genetic diversity and for subsequent utilization in crop improvement. In *Brassica* genetic diversity study is major requirement for success in plant breeding and crop improvement (Shahzadi et al., 2015). Knowledge of genetic diversity in *Brassica* gene pool may be a valuable genetic source to overcome obstacles in genetic improvement (Bird et al., 2017). *Brassica rapa* L is one of the valuable crop species in Brassicaceae family. It is known by its common names field mustard, turnip, and/or Chinese cabbage. It is characterized by its broader global distribution than most of other *Brassica* species (OECD, 2016). The assessment of genetic distances within *B. rapa* L. may help to broaden the genetic diversity in *B. napus* since it suffered a series of bottlenecks during its development and has reduced genetic diversity (Becker et al., 1995; Cowling, 2007). Moreover, landraces of *B. rapa* L. are adapted to a broad range of environments, e.g. cold or high temperatures, across a very wide geographic area (Dixon, 2007) which means that they contain valuable genetic traits which can be used for *B. napus* improvement (Annisa et al., 2011).

The aim of the present study was to assess of genetic diversity and population structure of *Brassica rapa* L. subspecies in order to select more diverse accessions as Supplementary Material resources that can be utilized for improvement of *B. napus*.

2. Material and Methods

2.1. Biological Material

Thirty- five accessions of *B. rapa* L. were obtained as donation from IPK gene bank Gatersleben Germany. The accessions belong to nine subspecies of *B. rapa* L. The represented subspecies are: *chinensis*, *pekinensis*, *rapa*, *oleifera* f. *annua* (annual turnip rape, summer turnip rape), *Brassica rapa subsp. campestris* (L.), *oleifera* f. *biennis* ((biennial turnip rape, winter turnip rape), *dichotoma*, *oleifera*, *trilocularis*. The origin and the accession number of these accessions are recorded as shown in Table 1.

Table 1. Subspecies names, accession code and origin of 35 accessions of *Brassica rapa* subspecies.

Subspecies. Names		accessions code	Origin	Subspecies. Names		accession code	origin
Chinensis	1.	BRA 1602/98	Guam	*oleifera annua*	19	CR1027/01	Canada
	2.	BRA 1321/91	UNG		20	CR 1538/00	DEU
	3.	BRA 1308/ 88	Taiwan	*campestris (L.)*	21	CR 2238/99	Italy
	4.	BRA 116/ 80	China		22	CR 2272/93	UNG
	5.	K 9420/95	U.S.A.		23	CR 1578/93	SUN
	6.	RA 1637/94”K89	Kupa	*oleifera biennis*	24	CR 289/84	DDR
	7.	BRA 1638/95	Japan		25	CR 1586/87	Sweden
pekinensis	8.	BRA 1620/97	UNG		26	CR 1600/96	Nederland
	9.	BRA 1125/77	Japan		27	CR 1447/90	Poland
	10.	BRA 122/81	China	*dichotoma*	28	CR 1585/96	Canada
	11.	BRA 201/76	Kor DVR		29	CR 2332/92	China
	12.	BRA 1601/93	Indonesia		30	CR 2218/88	India
	13.	BRA 214/93	DDR		31	CR 2596/87	UNG
Rapa	14.	BRA 337/79	UNG	*Oleifera*	32	CR 2203/79	AME
	15.	BRA 1014/85	Nederland		33	CR 2204/79	Italy
	16.	BRA 1116/99	U.S.A.	*trilocularis*	34	CR 2215/88	India
	17.	BRA 1224/87	Georgian		35	CR 2244/88	UNG
	18.	BRA 1705/98”K 9188	Tunesien				

UNG: Unknown geographic origin. Kor DVR: South Korean. U.S.A.: United State of America. DEU: Germany. SUN: USSR (Union of Soviet Socialist Republics). DDR: Deutsche Demokratische Republik. AME: Africa and Middle east.

2.2. Isozymes extraction, electrophoresis and staining

The seeds of the studied accessions were surface sterilized in 70% v/v ethanol for 1 min before germination at 25ºC in sterilized Petri dishes with three moist filter papers. Three-day-old seedlings of each accession 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 isozymes.

15 µL clear supernatants were mixed with equal volumes of loading buffer (50% glycerol containing 1% bromophenol blue), then applied directly on 7% vertical PAGE at 4°C in a Mini Protean III unit (BioRad, USA) according to the method of Manchenko (1994). Electrophoresis was carried out at 15 mA/gel for 60 min.

The electrophoresed gels were stained for acid phosphatase, catalase, α-esterase and peroxidase (Pasteur et al., 1988). The gels of acid phosphatase were incubated in 100 ml solution of 0.1 M sodium phosphate buffer, pH 5.1, at 37 ^{0}C for 3 to 5 h, then stained in solution formed of 10 mM I_2 mixed with 14 mM KI developing white bands on a dark blue background. The chromatic or light brown bands appeared at the bottom of the gels were amylase bands. The gels of catalase were stained by immersing in 1:1 mixture of solutions 2% potassium ferricyanide and 2% ferric chloride after incubation in a solution of 3% H_2O_2 for about 15 min. The gels were then washed and gently agitated for a few minutes in water. Yellow bands of catalase activity appeared on a blue-green background. The gels of α-esterases were incubated at 37°C for 15 min in 100 ml staining solution consisted of 0.05 M phosphate buffer, pH 7.2, containing 1% α-naphthyl acetate and 50 mg Fast Blue RR until brown colored bands appeared. The stained gels were photographed as quickly as possible and stored in 3% acetic acid. The gels of peroxidase were incubated in 100 ml 0.05 M acetate buffer (pH 5.0 containing 65 mg benzidine dissolved in 1ml of ethanol. 2 ml of 0.1 M $CaCl_2$ were added as co-enzyme. Finally, 2 ml of H_2O_2 were added as a substrate and incubated in refrigerator until dark brown bands appeared. Stained gels were washed with distilled water and fix in 50% glycerol (Soltis et al., 1983). At least 5 and generally 10 plants per accession were examined for isozyme patterns.

2.3. Data analysis

The data of isozymes were analyzed using POPGENE version 1.31 Microsoft Window-based Freeware for Population Genetic Analysis. The construction of a dendrogram was made based on Nei's genetic distance using UPGMA (Nei, 1978). The banding patterns were first encoded using Microsoft Excel for easier editing before being transformed into a POPGENE data file.

3. Results

3.1. Loci and alleles scored

Enzyme electrophoresis resulted in detecting 14 putative polymorphic loci with 27 alleles. The mean allele frequency ranged 0.04 in loci *Cat4A* and *Cat4B* to 0.62 in *Per1B*. The alleles having frequency 0.04 were termed rare alleles (as shown in Supplementary Table 1). These alleles were observed for *Cat4A* and *Cat4B in* sub species *Oleifera* accession CR 2204/79 and in subspecies *trilocularis* accessions CR 2215/88 and CR 2244/88. It also showed that the total number of alleles in each accession for the 14 loci ranged from 4 in subspecies *chinensis* accession RA 1637/94"K89 and subspecies *rapa* accessions BRA 337/79, BRA 1116/99 and BRA 1224/87 to 17 in subspecies *chinensis* accession BRA 116/ 80 with a mean of 6.8 (total = 27). The percent of polymorphic loci varied from 0.29 in accession RA 1637/94"K89 of subspecies *chinensis* and accessions BRA 337/79, BRA 1116/99 and BRA 1224/87 of subspecies *rapa* L. to 1.21 in accession BRA 116/ 80 of subspecies *chinensis* (as shown in Supplementary Table 1).

3.2. Genetic diversity and accession-level homozygosity

The mean number of alleles per locus (*A*) and the effective number of alleles per locus (*Ae*) varied respectively from 1.13 in subspecies *chinensis*, accession BRA 1014/85 to 1.63 in subspecies *rapa*, accession BRA 1321/91 with a mean of 1.35 and from 1.13 in subspecies *chinensis*, accession BRA 1014/85 to 1.75 in subspecies *dichotoma,* accession CR 1585/96 with a mean of 1.281 (as shown in Table 2).

The average of observed heterozygosity (*Ho*) was 0.31, ranging from 0.09 (sub species *chinensis* accession K 9420/95, subspecies *rapa* accessions BRA 1116/99, subspecies *Brassica rapa subsp. campestris* (L.) accession CR 1578/93) to 1.75 (subspecies *dichotoma,* accession CR 1585/96) and the average of expected heterozygosity (*He*) was 0.24 ranging from 0.09 in sub species *chinensis* accession K 9420/95, subspecies *rapa* accessions BRA 1116/99 and subspecies *campestris* (L.) accession CR 1578/93 to 1.75 in subspecies *dichotoma,* accession CR 1585/96 (as shown in Table 2).

The average fixation index (*F*) is significantly higher than zero for the analysis accessions (as shown in Table 2) indicating a significant deficiency of heteozygosity. In general, it was found that the number of loci with significant deficiency of heterozygosity for all accessions was extremely higher than the number of loci with excessive heterozygosity. The non significant inbreeding coefficients (NS) were ranged between 1.00 in subspecies *dichotoma* accession CR 1585/96 to 7.00 in sub species *oleifera biennis* accessions CR 289/84 & CR 1586/87and subspecies *Oleifera* accession CR 2203/79 (as shown in Table 2).

3.3. Genetic structure and gene flow

The mean breeding index was significantly higher than zero (F_{IT} = 0.8228), which means that there is excess of average heterozygotes in the studied accessions. A high and significant value was obtained for F_{ST} (The coefficient of genetic differentiation) with mean = (0.8972) suggesting the occurrence of random mating system for the studied accessions (as shown in Table 3). The mean value of F_{IS} all over the analyzed loci was -0.7235 confirming the self-incompatibility of *B. rapa*. The estimate of gene flow based on Wright (1951) equation was very low:

Table 2. Accessions of *Brassica rapa* subspecies, mean allele number per locus (*A*), mean of effective allele number per locus (*Ae*), observed heterozygosity (*Ho*), expected heterozygosity (*He*), average fixation index (*F*), number of loci with a significant excess heterozygosity (HE), number of loci with a significant deficiency of heteozygosity (HD), non significant inbreeding coefficients (NS).

No.	Origin	*A*	*Ae*	*Ho*	*He*	*F*	HE	HD	NS	No.	Origin	*A*	*Ae*	*Ho*	*He*	*F*	HE	HD	NS
1.	BRA 1602/98	1.29	1.29	0.29	0.29	0.40	2	8	5	19	CR1027/01	1.50	1.50	0.50	0.5	0.60	2	11	2
2.	BRA 1321/91	**1.63**	1.37	0.23	0.26	0.46	4	8	3	20	CR 1538/00	1.33	1.33	0.33	0.22	0.46	2	9	4
3.	BRA 1308/ 88	1.30	1.26	0.25	0.22	0.24	3	5	7	21	CR 2238/99	1.33	1.33	0.33	0.33	0.20	3	6	6
4.	BRA 116/ 80	1.64	1.41	0.21	0.26	0.23	5	7	3	22	CR 2272/93	1.57	1.37	0.31	0.27	0.49	4	9	2
5.	K 9420/95	1.00	1.00	**0.09**	**0.09**	0.53	0	8	7	23	CR 1578/93	1.00	1.00	**0.09**	**0.09**	0.40	0	6	9
6.	RA 1637/94”K89	1.33	1.33	0.33	0.33	0.73	1	12	2	24	CR 289/84	1.17	1.17	0.17	0.17	0.26	2	6	7
7.	BRA 1638/95	1.29	1.29	0.29	0.19	0.40	2	8	5	25	CR 1586/87	1.22	1.22	0.22	0.22	0.26	2	6	7
8.	BRA 1620/97	1.38	1.29	0.28	0.23	0.32	3	7	5	26	CR 1600/96	1.33	1.33	0.33	0.33	0.46	2	9	4
9.	BRA 1125/77	1.45	1.33	0.22	0.26	0.20	5	4	6	27	CR 1447/90	1.25	1.25	0.25	0.25	0.33	2	7	6
10.	BRA 122/81	1.40	1.38	0.37	0.25	0.10	4	5	6	28	CR 1585/96	1.75	1.75	**0.75**	**0.75**	0.53	3	11	1
11.	BRA 201/76	1.50	1.48	0.40	0.37	0.06	4	5	6	29	CR 2332/92	1.25	1.20	0.19	0.15	0.37	2	7	6
12.	BRA 1601/93	1.17	1.17	0.17	0.167	0.53	1	9	5	30	CR 2218/88	1.17	1.17	0.17	0.17	0.53	1	9	5
13.	BRA 214/93	1.38	1.38	0.38	0.38	0.26	3	7	5	31	CR 2596/87	1.25	1.25	0.25	0.25	0.60	1	11	3
14.	BRA 337/79	1.33	1.33	0.33	0.33	0.73	1	12	2	32	CR 2203/79	1.30	1.19	0.13	0.14	0.32	2	6	7
15.	BRA 1014/85	**1.13**	1.13	0.13	0.13	0.40	1	7	7	33	CR 2204/79	1.33	1.33	0.33	0.33	0.46	2	9	4
16.	BRA 1116/99	1.00	1.00	**0.09**	**0.09**	0.73	0	11	4	34	CR 2215/88	1.40	1.39	0.38	0.27	0.09	4	5	6
17.	BRA 1224/87	1.33	1.33	0.33	0.33	0.73	1	12	2	35	CR 2244/88	1.50	1.50	0.50	0.29	0.20	4	7	4
18.	BRA 1705/98”K 9188	1.00	1.00	0.10	0.10	0.40	0	6	9										
	Mean											1.35	1.28	0.31	0.24	0.39			

NmW = 0.0287 which explained the low average of observed heterozygosity (0.31) and expected heterozygosity (0.24).

3.4. Genetic distance and dendrogram

Dendrogram constructed based on isozyme showed four main clusters at Nei's genetic distance 0.60 (see Figure 1). Accessions of subspecies *oleifera* and *trilocularis* were collected with each other in cluster (C2). The accessions of subspecies *oleifera biennis* and *oleifera annua* were separated in clusters (C1) and (C2) respectively. The accessions of other subspecies (*chinensis*, *pekinensis*, *rapa, dichotomy*, *campestris* L.) were clustered in two clusters each.

The matrix of eigenvectors and values of the principle components (PCs) resulting from the interaction of the isozyme data influenced 54.93% of the variability accumulated up to the first four components of the PCA (as shown Table 4). The accessions of subspecies *rapa*, *dichotoma*, *oleifera* and *trilocularis* were separated on PC1 and the accessions of *oleifera biennis* on PC2. The other accessions were separated on more than one access.

Table 3. Genetic divergence measures and gene flow of the studied accessions of *Brassica rapa subspecies*.

No.	Locus	Sample size	F_{is}	F_{it}	F_{st}	*Nm*
1.	ACP1	70	-0.8708	0.8281	0.9081	0.0253
2.	ACP2	118	-0.5411	0.7367	0.8291	0.0515
3.	ACP3	134	-0.8063	0.3219	0.6246	0.1503
4.	ACP4	38	-1.0000	0.8357	0.9178	0.0224
5.	Cata1	88	-0.3773	0.9453	0.9603	0.0103
6.	Cata2	52	0.000	1.0000	1.0000	0.000
7.	Cata3	122	-0.7363	0.5600	0.7466	0.0849
8.	Cata4	30	-1.000	0.9226	0.9613	0.0101
9.	Est1	64	-0.7087	0.957	0.9748	0.0065
10.	Est2	38	0.8231	0.9932	0.9616	0.0100
11.	Est3	52	-0.6571	0.9136	0.9479	0.0137
12.	Est4	104	-0.9091	0.8443	0.9184	0.0222
13.	Per1	94	-0.9481	0.6336	0.8119	0.0579
14.	Per2	56	-0.6514	0.9357	0.9611	0.0101
	Mean	71	-0.7235	0.8228	0.8972	0.0287

F_{IS}: Within-accessions inbreeding coefficient. F_{IT}: Total accession inbreeding coefficient. F_{ST}: Among-accessions genetic differentiation coefficient. ***Nm:*** Gene flow.

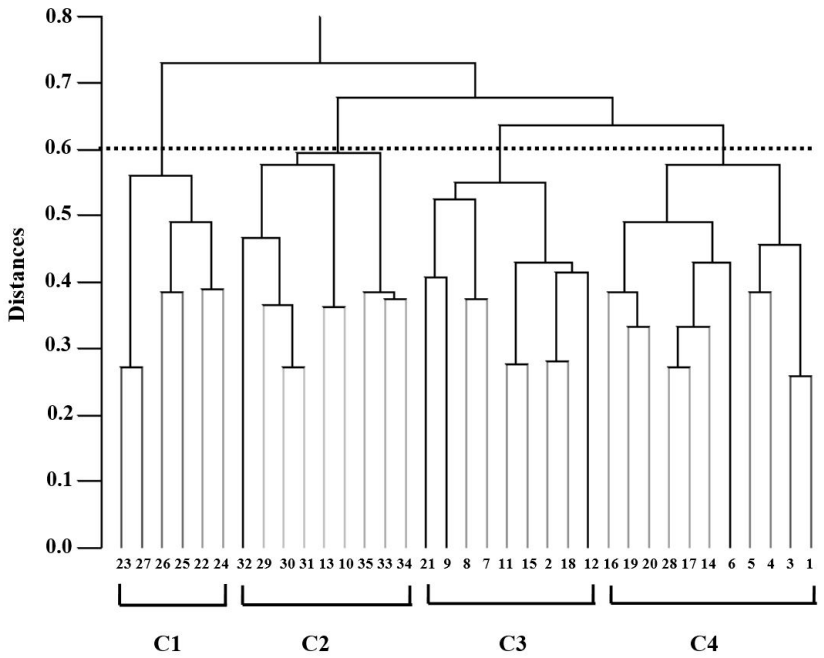

Figure 1. UPGMA dendrogram showing the relationships among the 35 accessions of the studied accessions of *Brassica rapa* subspecies.

Table 4. Sub species of *Brassica rapa, a*ccessions numbers and values of the principal components for isoyme data.

No.	PC1	PC2	PC3	PC4	No.	PC1	PC2	PC3	PC4
1.	-0.044	-0.335	0.046	-0.678	19	0.234	0.152	0.104	-0.558
2.	0.184	-0.490	-0.151	-0.627	20	0.688	-0.370	-0.051	-0.314
3.	0.692	0.177	0.180	0.180	21	0.335	0.543	0.282	-0.499
4.	0.513	-0.236	0.337	-0.421	22	0.522	0.225	-0.340	0.106
5.	0.321	-0.230	0.117	-0.398	23	0.201	0.781	0.003	-0.307
6.	-0.114	0.262	0.065	0.133	24	0.176	0.458	0.024	0.049
7.	0.616	0.044	0.419	0.395	25	0.606	0.500	-0.255	-0.019
8.	0.383	0.279	0.533	0.147	26	0.136	0.555	-0.251	-0.254
9.	0.396	0.203	0.720	-0.131	27	0.248	0.687	0.013	-0.420
10.	0.494	-0.299	0.250	0.258	28	0.453	-0.240	-0.570	0.041
11.	0.657	-0.042	0.477	-0.008	29	0.525	-0.137	-0.554	0.092
12.	0.708	-0.023	-0.012	0.225	30	0.550	-0.106	-0.606	-0.115
13.	0.369	-0.545	0.222	-0.190	31	0.507	-0.254	-0.358	-0.115
14.	0.466	-0.240	0.022	0.299	32	0.515	-0.525	0.002	0.049
15.	0.677	-0.117	0.533	0.237	33	0.464	0.286	-0.277	0.110
16.	0.398	-0.335	0.067	0.051	34	0.538	0.411	-0.323	0.259
17.	0.520	0.012	-0.465	-0.122	35	0.313	0.065	-0.528	0.097
18.	0.865	0.081	0.108	0.084					
Variance Explained by Components						8.085	4.298	3.947	2.893
Percent of Total Variance Explained						23.100	12.281	11.277	8.265

4. Discussion

The surprising phenotypic diversity of *B.rapa* has made it a precious multi-use crop for food, fodder, and oil. Although the complex domestication history and intense selective breeding of *B. rapa* had an effective role in generating and shaping this diversity, they highly complicated the elucidation of population structure and evolutionary relationships in the species. With using isozyme data, we provide a more detailed analysis of population structure and the relationships of *B. rapa* subspecies.

4.1. Loci and alleles scored

The alleles with low mean allele frequency (0.04) or what were known as rare alleles were observed in *Cat4A* and *Cat4B in* sub species *oleifera* accession CR 2204/79 and in subspecies *trilocularis* accessions CR 2215/88 and CR 2244/88. The presence of this allele could be due to deleterious mutations or may be due to evolutionary relics (Sammour et al., 2019). The detection of rare allele in combination with low allelic frequency of other loci leads to the conclusion that the studied accessions had narrow genetic differentiation.

Although the studied accessions except the accessions of subspecies *trilocularis* are an obligate outcrosser due to the presence of self-incompatible genes, the mean allele frequency in general was low. This was attributed to breakdown and mismatch of the differently adapted gene complexes or what is known outbreeding depression. The great variation in allele frequency in studied subspecies could be attributed to these subspecies include many varieties or forms or cultivars. The selected forms of some subspecies e.g. *chinensis* exhibited significantly different morphological traits which made early botanists classified them as separate species (OECD, 2016).

4.2. Genetic diversity and accession-level homozygosity

There was wide variation in mean number of allele per locus (*A*) and effective number of allele per locus (*Ae*). Such wide variation was reported by Karam et al. (2014). However, the subspecies which showed low and high number of allele per locus (*A*) and effective number of allele per locus in Karam et al. (2014) were different from that observed in the present study. This might be attributed to that Karam et al. (2014) carried out their study at population level and we carried out study at accessions level.

Low average of observed heterozygosity (*Ho*) and expected heterozygosity (*He*) which observed in sub species *chinensis* (accession K 9420/95), subspecies *rapa* (accessions BRA 1116/99) and subspecies *campestris* (L.) (accession CR 1578/93) might be attributed to these accessions at the edge of the subspecies distribution centre; the position at which population sizes gradually decrease as does genetic diversity or the origin of these accession could be associated with some sort of bottleneck event followed by the absence of inter-population gene flow (Meeus et al., 2012; Surina et al., 2014; Radosavljevic et al., 2015).

The highest average of observed and expected heterozygosity, and the highest effective number of alleles per locus (*Ae*) were observed in subspecies *dichotoma,* accession CR 1585/96 from Canada. The variation in genetic structure between subspecies *dichotoma,* accession CR 1585/96 from Canada and other accessions of subspecies *dichotoma* from Asia could be due to Asian

accessions were subjected to long term selection which narrow their genetic variation or/and the variation of geographical position of the accessions within subspecies distribution range or severe effects of small population sizes (Radosavljevic et al., 2015).

The highest genetic diversity measures were observed in subspecies *dichotoma,* accession CR 1585/96 (the highest average of observed and expected heterozygosity, and number of alleles per locus (*Ae*)), These observations make subspecies *dichotoma,* accession CR 1585/96 valuable genetic resources to be included in breeding programs for the improvement of oilseed *B. napus*.

4.3. The population structure and gene flow

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. The inbreeding coefficient of the individuals within each subspecies was relatively low (F_{IS} = -0.7235) which agreed with the self- incompatibility of *B. rapa*.

The coefficient of genetic differentiation (F_{ST}) was 0.8972 which was considered very great based on the guidelines suggested by Wright (1978). This guideline considered F_{ST} 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. The divergence among subspecies indicated very great genetic differentiation (F_{ST} = 0.8972) which means that about 90% of genetic diversity is distributed among subspecies, while 10% of the diversity is distributed within subspecies. This was coincided with low value of gene flow (Nm = 0.0287).

Although *Brassica rapa* was classified as obligate self-incompatible (Koch and Al-Shehbaz, 2009) the inbreeding coefficient of the individuals in the entire studied populations (within all subspecies) was relatively high (F_{IT} = 0.8228). 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). Variation in genetic structure was observed among accessions of the same subspecies and was attributed to severe selection, domestication and low gene flow (Snowdon et al., 2007).

4.4. The relationships among B. rapa subspecies

The subspecies were differentiated at Nei s genetic distance 0.60 into four main clusters, each cluster contained more than a subspecies confirming that *Brassica rapa* had a polyphyletic origin (Tanhuanpää et al., 2016). The accessions of each of *oleifera*, *oleifera annua*, *oleifera biennis*, *trilocularis* were collected with each other in a specific cluster. This could be due to the accessions of each of these subspecies collected from same sub-geographic region, which did not have a high fluctuation in environmental and ecological factors: the factors that may cause change/modifications in isozymes markers (Horáček et al., 2009). Conversely, the accessions of other subspecies were distributed between two clusters exhibiting a wide variation in genetic structure, e.g. subspecies *chinensis*, *pekinensis*, *rapa, dichotomy*, *campestris* L. The variation in the genetic structure of these subspecies based on isozyme analysis coincides the distinct variation of the morphotypes of these subspecies (OECD, 2016).

It was reported *B. rapa* L. classified into two main groups based on morphological and restriction fragment length polymorphism (RFLP) markers: one group consists of ssp. *rapa* and ssp. *oleifera* in Europe and another is the group of leafy vegetables, such as ssp. *pekinensis*, ssp. *chinensis* and ssp. *nipposinica* in East Asia (Takuno et al., 2007). However, our results based on isozyme analysis did not support this classification. The discrepancy between our results and Takuno et al. (2007) conclusion was attributed to Takuno et al. (2007) did not include ssp. *rapa* and ssp. *oleifera* from East Asia in their studies (Tsunoda, 1980).

B. rapa ssp. *oleifera* (turnip rape) and *B. rapa* ssp. *trilocularis* (sarson) were grouped in one cluster. Similar relationship was observed by Song et al. (1991) based on RFLP and Karam et al. (2014) based on isozyme. 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.

The separation of *dichotoma* and *trilocularis* with each other on PC1 confirmed the suggestion of Bird et al. (2017) for collecting of these two subspecies in a separate subpopulation by using a high-throughput GBS method that leverages next-generation sequencing and multiplexing of RRLs. However, the suggestion of Bird et al. (2017) to assign each of subspecies *chinensis* and *pekinensis* to a subpopulation, since these two species had many morphotypes, great variation in genetic structure based on isozyme (Karam et al., 2014) and RFLP analysis (Takuno et al., 2007).

These two subspecies and other subspecies of *B. rapa* need more study on a big collection of accessions covering their distribution area and using more than one molecular markers covering majority of their genome to assess genetic diversity and relationships within and among them.

5. Conclusion

In conclusion, enzyme electrophoresis resulted in detecting 14 putative polymorphic loci with 27 alleles. The rare alleles, alleles with mean frequency 0.04 were observed in *Cat4A* and *Cat4B in* subspecies *Oleifera* accession CR 2204/79 and in subspecies *trilocularis* accessions CR 2215/88 and CR 2244/88. The average fixation index (F) is significantly higher than zero for the analysis accessions indicating a significant deficiency of heteozygosity. The highest genetic diversity measures were observed in subspecies *dichotoma,* accession CR 1585/96 (the highest average of observed (H_0) and expected heterozygosity (*He*), and number of alleles per locus (*Ae*)) which made this accessions valuable genetic

resources to be included in breeding programs for the improvement of oilseed *B. napus*. Low heterozygosity in some accessions might be due to these accessions at the edge of the subspecies distribution centre; the position at which population sizes gradually decrease as does genetic diversity or origin of these as these accession could be associated with some sort of bottleneck event followed by the absence of inter-population gene flow. *B. rapa* ssp. *oleifera* (turnip rape) and *B. rapa* ssp. *trilocularis* (sarson) were grouped in one cluster which coincides with their morphological classification. These two subspecies and other subspecies of *B. rapa* need more study on a big collection of accessions covering their distribution area and using more than one molecular markers covering majority of their genome to assess genetic diversity and relationships within and among them.

Acknowledgements

This project was supported by King Saud University, Deanship of Scientific Research, College of Science, Research Center, Saudi Arabia.

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Supplementary Material

Supplementary material accompanies this paper.

Supplementary Table 1. Allele frequencies at the 14 analyzed enzyme loci for 35 accessions of Brassica rapa subspecies.

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