JJBS

Volume 15, Number 3,September 2022
ISSN 1995-6673
Pages 489 – 500
https://doi.org/10.54319/jjbs/150319

Jordan Journal of Biological Sciences

Infraspecific identity of the wild *Brassica nigra* (L.) Koch. using morphological, cytogenetics and molecular (nuclear and chloroplast) approaches

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Received: December 10, 2020; Revised: May 20, 2021; Accepted: June 30, 2021

Abstract

Egyptian flora is still hosting the wild diploid *Brassica nigra* (L.) Koch. (Brassicaceae); as one of the most important wild crop relatives of the cultivated *Brassica* crops. Despite the field and herbarium observations indicating that the populations of this species showed considerable morphological diversity, the taxonomic identity of these variations is not yet resolved. This study covered the morphology, the cytogenetics and the genome diversity at the intraspecies level to resolve the taxonomic identity and their genetic relationships. Nine representative populations were morphologically investigated using 70 macro-and micro-morphological characters. The morphological results confirmed the presence of two varieties (var. *bracteolata* and var. *nigra*), and three forms, while the chromosome investigation revealed the presence of 2n=16), with notable karyotyping diversity among the studied varieties where satellite was observed on the chromosome pair no. "6" distinguished var. *bracteolata* from var. nigra. The retrieved accession numbers of the chloroplast (rps, ropC & trnK) and nuclear (ITS) markers have been deposited in the EMBL/GenBank/DDBJ nucleotide sequence data libraries; then compared with that of *B. nigra* from Gene-Bank database search via Basic Local Alignment Search Tool (BLAST). The constructed cladogram based on molecular markers supported the morphological and cytological results and confirmed the presence of the two varieties (*bracteolata* and *nigra*). This study recommends combining different taxonomic approaches in resolving the taxonomic identity of morphologically different wild *B. nigra* populations.

Keywords*: Brassica nigra* - Nuclear marker (ITS)- Chloroplast markers- Chromosome number- Karyotyping- infraspecific diversity- morphological diversity.

1. Introduction

Brassicaceae (Cruciferae) is a monophyletic family, distributed in temperate areas of all continents except Antarctica, species of this family retain high diversity in Irano-Turanian, Mediterranean, and West N. American regions (El Rabiai, 2015; Taiyan *et al.*, 2001). Brassicaceae is highly homoplasious in its morphological characters, which seems to play a limited role in resolving the phylogenetic affinities at the generic and family levels (Al-Shehbaz *et al*., 2006). It includes 3977 species (351 genera), in which the genus *Brassica* contains species with unique agricultural potentialities and wide-utilization range (Amer *et al.,* 2019; Rakow, 2004; Song *et al*., 1988). The generic boundary of this genus needs revision (Felger *et al.,* 2015).

In Egyptian flora, Brassicaceae is one of the four largest families, which is represented by 53 genera and 103 species (Boulos, 1995; Boulos, 1999). Genus *Brassica* in Egypt represented by five species from about 80 accepted *Brassica* species worldwide. These species are: *B. nigra* (L.) Koch, *B. rapa* L. and *B. tournefortii* Gouan , *B. deserti* Danin & Hedge and *B. juncea* (L.) Czernj. & Coss. (Boulos, 2009). This genus shows a high level of phenotypic variations with and within the wild Egyptian populations (Amer *et al*., 2019).

Genus *Brassica* L. received the attentions of taxonomists due to its great economic importance as oil seed and vegetable crops (Amer *et al.*, 2019; Warwick, 2011). *Brassica nigra* (L.) Koch. (Black Mustard) is widely cultivated as a chief source of edible oil, mustard and medicine (Tsunoda, 1980; Vaughan, 1977). It also possesses several agronomic potentialities as a pillar of the basic *Brassica* triangle for the diploid genome including *B. oleracea*, *B. nigra* and *B. rapa*, with 2n=18, 16 and 20; respectively and their shared hybrids (Schranz *et al*., 2002) and crossbreeding (Pires *et al*., 2004). Nevertheless, the study of chromosome numbers at the species level was carried out decades ago (Karpechenko, 1922). Later, the identity of the individual chromosome attains great attention. *Brassica nigra* harbors unique genomic ancestor genes in the genus Brassica (Yang *et al.,* 2002).

The taxonomic identity of the infraspecific taxa of *Brassica nigra* in Egypt is still unresolved. However, it

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was identified as three varieties namely var. *bracteolata* (Fisch. & Mey.) Spach, var. *torulosa* Alef. and var. *turgida* Alef. (Täckholm, 1974); while (Boulos, 1999, 2009) considered these varieties as synonyms of *B. nigra* (L.) Koch. Later, Amer *et al.,* (2019) studied the macro- and micro morphological characters of the infraspecific diversity of *B. nigra* (represented by 26 populations). The study revealed the presence of two distinctive varieties var. *bracteolata* and var. *nigra* and the later represented by two phenotypic forms. To date, the genetic variation, karyotyping, and molecular identity of this species at the infraspecific level are not yet investigated. Cytotaxonomy is the application of chromosome karyotyping in taxonomy (Greilhuber and Ehrendorfer, 1988), which can clarify the hereditary relationship along with the studied species or populations, and enable understanding their divergence (Guerra, 2008). The study of genetic variation of *B. nigra* accessions has significant implications in the crop improvement programs despite that the genome sequencing of *B. nigra* is still in progress (Negi *et al*., 2004; Jiang *et al*., 2015); and little is known about the magnitude and the genetic diversity among its wild population (Gomez-Campo, 1978; Roy, 1978).

Nowadays, the assessment of genetic diversity and DNA identity was performed by using molecular markers to overcome the morphological diversity within certain taxa (Ruangsuttapha *et al*., 2007; Li *et al.,* 2012). The nuclear ribosomal DNA (nrDNA) is a universal molecular marker which harbors both highly variable and conserved regions (Simon *et al.*, 2012). Bailey *et al.* (2006) reported that the plants and animals' phylogeny was estimated using the internal transcribed spacer (ITS); those ranges from 500-700 bp in angiosperms (Baldwin *et al*., 1995).

In plants, the chloroplast genome is of conserved genome compared to the mitochondria and nuclear genomes, so it is commonly used for genome identity (Haider, 2011). The highly conserved regions are interrupted by introns and non-coding cpDNA regions (Clegg, 1993); The variability in these regions can be utilized in the systematic studies even at lower taxonomic levels (Haider, 2011). Seol *et al*. (2015) reported that the *Brassica nigra* (accession no. KT878383) was a circular, measured 153633 bp with a pair of inverted repeat regions.

The present work aimed to: (1) Assess the morphological variation and its relationship to chromosome number variation within the infraspecific taxa (varieties and/or forms) of *B. nigra*. (2) Identify the molecular identity of the studied taxa using ITS and chloroplast markers. (3) Determine the genetic diversity within and between these taxa. (4) Check genotypic/karyotypic identity of these. (5) Identify the similarity between the status of Egyptian taxa and others available in the database.

2. Materials and Methods

2.1. Plant materials

Fresh *Brassica nigra* specimens were collected from different geographical regions during flowering and fruiting seasons, then grouped morphologically and represented in this study by 9 populations. Locations of the collected populations, for the *Brassica nigra* var. *nigra* Form 1, var. *nigra* Form 2 and var. *bracteolata* each represented by three populations collected from different localities (Table 1). The pollen grains of the collected populations were examined morphologically using Scanning Electron Microscope (by a Joel 1200 EX II SEM at 20 kv). The studied populations were identified based on the 70 macro-and micro-morphological characters as outlined in Table (2), according to (Amer *et al*., 2019).

Table (1): Geographic locations of the studied *Brassica nigra* populations.

St. No.	Localities	GPS Coordinates		
		N	E	Associated Field
1	Assuit –Dairout	27° 34' 3.348"	30° 48' 7.085"	Alfalfa
2	Sohag –Tema	26° 53' 41.863"	31° 26' 33.989"	Wheat
3	Minya – Samalot	28° 19 40.130"	30° 42 9.552"	Alfalfa
4	Minya – Matai	28° 23 58.125"	30° 46 14.230"	Lentil
5	Minya – Maghagha	28° 37' 38.189"	30° 49' 8.758"	Alfalfa
6	Minya – Abo Qorkas	27° 52 3.226"	30° 48 0.528"	Wheat
7	Beni Suef	29° 04' 22.45"	31° 05' 27.33	Wheat
8	El Giza	30°18' 33.14	31°10' 91.44	Wheat
9	Monufia - Ashmoon	30°12' 39"	30° 57' 23"	Wheat

Table 2 . The spectrum of *Brassica nigra* macro-& micro-morphological features in the studied 9 populations.

Characters	var. nigra						var. bracteolata		
	Form 1.1	Form 1.2	Form 1.3	Form 2.1	Form 2 .2	Form 2.3	Bract. 1	Bract.2	Bract. 3
Stem:									
Sculpture by SEM (Striate=0 & not so =0)	1	1	1	1	1	1	0	0	0
Presence of verrucate cuticular deposits by SEM (Dense =1 & spare =0)	0	0	0	0	0	0	1	1	1
Basal (Radical) leaf:									
Shape (Pinnatisect =1 & not so =0)	1	1	1	1	1	1	1	1	1
Margin Shape (Serrate=1 & not so =0)	1	1	1	0	0	0	0	0	0
Margin regularity (Regular=1 & Irregularly=0)	1	1	1	0	0	0	0	0	0
Petiole (Petiolate =1 & sessile =0)	1	1	1	1	1	1	1	1	1
Leaf Length (Up to 75 cm=1 & up to 50 cm=0)	1	1	1	0	0	0	0	0	0
Shape (Oblong ovate= 1 & not so 0)	1	1	1	1	1	1	1	1	1
Stomata shape (Kidney =1 & not so 0)	1	1	1	1	1	1	1	1	1
Apex (Acute=1 & not so =0)	1	1	1	1	1	1	0	0	0
Segmentation lobes (3-7 lobes=1 & not so =0)	1	1	1	0	0	0	0	0	0
Width of the upper lobe (up to 5 cm=1 & less than 5 cm=0)	1	1	1	1	1	1	0	0	0
Hairy allover =1 & not so =0	1	1	1	0	0	0	0	0	0
Hairy midrib=1 & not so =0	1	1	1	1	1	1	0	0	0
Stomata position by SEM (On raised ridges=1 & not so= 0)	1	1	1	1	1	1	0	0	0
Upper (Cauline) leaf:									
Shape (Ovate =1 & not so =0)	1	1	1	1	1	1	1	1	1
Apex (Acute =1 & not so =0)	1	1	1	1	1	1	1	1	1
Margin (Denticulate=1 & not so =0)	0	0	0	1	1	1	1	1	1
Petiole (Petiolate =1 & sessile =0)	1	1	1	1	1	1	1	1	1
Segmentation lobes (more than 2 =1 & not more than 2 =0)	1	1	1	0	0	0	1	1	1
Upper lobe L mm (Up to 2.5 =1 & not so =0)	1	1	1	0	0	0	0	0	0
Upper lobe W mm ((Up to 15 =1 & more than 15 =0)	0	0	0	1	1	1	0	0	0
Sepal:									
Sepal L mm (From 4-6 mm=1 & not so =0)	1	1	1	1	1	1	1	1	1
Sepal W mm (Up to 1.5 mm =1 & not so = 0	1	1	1	1	1	1	1	1	1
Shape (Linear=1 & not so =0)	1	1	1	1	1	1	1	1	1
Surface (Hairy =1 & Glabrous =0)	0	0	0	0	0	0	0	0	0
No. of main veins (3 veins=1 & others=0)	1	1	1	1	1	1	1	1	1
Apex (Obtuse=1 & others =0)	1	1	1	1	1	1	1	1	1
Margin (Entire=1 & others =0)	1	1	1	1	1	1	1	1	1
Petal:									
Petal L mm (Up to 14 mm=1 & less than 14 mm=0)	1	1	1	0	0	0	0	0	0
Petal W mm (Up to 6 mm=1 & less than 6 mm=0)	0	0	0	1	1	1	1	1	1
Petal blade L mm (Up to 9 mm=1 & less than 9 mm=0)	1	1	1	1	1	1	0	0	0
Shape of claw (Filiform=1 & not so =0)	1	1	1	1	1	1	1	1	1
Petal surface (Glabrous=1 & Hairy=0)	1	1	1	1	1	1	1	1	1
No. of main veins in petal (Up to 9=1 & less than 9=0)	0	0	0	1	1	1	1	1	1
Apex (Obtuse=1 & others =0)	1	1	1	1	1	1	1	1	1
Margin (Entire=1 & others =0)	1	1	1	1	1	1	1	1	1
Stamens: 6 (2+4)									

Filament L mm (Up to 7mm=1 & less than 7mm=0)	1	1	1	1	1	1	1	1	1
Filament W mm (Up to 0.5mm=1 & less than 0.5 mm=0)	1	1	1	1	1	1	1	1	1
Stamen shape (Linear =1 & not so =0)	1	1	1	1	1	1	1	1	1
Stamen surface (Glabrous=1 & Hairy=0)	1	1	1	1	1	1	1	1	1
Anther:									
Anther L mm (Up to 3mm=1 & less than 3mm=0)	1	1	1	1	1	1	0	0	0
Anther W mm (Up to 1.0 mm=1 & less than 1mm=0)	1	1	1	1	1	1	0	0	0
Anther shape (Oblong=1 & not so=0)	1	1	1	1	1	1	1	1	1
Anther apex (Obtuse=1 & not so=0)	1	1	1	1	1	1	1	1	1
Stigma shape (Capitate=1 & not so =0)	1	1	1	1	1	1	1	1	1
Pedicle									
Pedicle L mm (equal or more than 10=1 & not so=0)	1	1	1	0	0	0	1	1	1
Flower bract:									
Surface (Hairy=1 & Glabrous= 0)	1	1	1	0	0	0	0	0	0
Bract venation (Reticulate=1 & not so=0)	1	1	1	1	1	1	1	1	1
Bract margin (Dentate=1 & not so=0)	1	1	1	1	1	1	0	0	0
Apex (Acute=1 & not so=0)	1	1	1	1	1	1	1	1	1
Shape (Ovate=1 & not so=0)	1	1	1	1	1	1	0	0	0
Length cm (Up to 3.5cm=1 & less than 3.5cm=0)	1	1	1	0	0	0	0	1	0
Fruit:									
Fruit L mm (Up to 35mm=1 & more than 35mm=0)	1	1	1	1	1	1	0	0	0
Fruiting part L mm (Up to 20mm=1 & more than 20mm=0)	1	1	1	0	0	0	0	0	0
Fruit peak L mm (Up to 15mm=1 & less than 15mm=0)	1	1	1	1	1	1	0	0	0
Number of seeds/ fruit (Up to 12=1 & more than 12=0)	1	1	1	1	1	1	0	0	0
Surface (Hairy=1 & Glabrous= 0)	0	0	0	0	0	0	0	0	0
Receptacle shape (Flat=1 & not so= 0)	1	1	1	1	1	1	1	1	1
Pollen using SEM:									
Pollen shape (Oblate=1 & not so=0)	1	1	1	1	1	1	0	0	0
Number of colpi (3=1 & not so =0)	1	1	1	1	1	1	1	1	1
Pollen sculpture (Reticulate=1& not so=0)	1	1	1	1	1	1	1	1	1
Endexine sculpture (Warty=1 & not so=0)	1	1	1	0	0	0	1	1	1
Muri-wall (Dense warty=1 & not so=0)	1	1	1	1	1	1	0	0	0
Muri-pattern (Regular=1 & irregular =0)	1	1	1	0	0	0	1	1	1
Polar Axis (P) µm (Equal or less than 20=1 & more than 20=0)	1	1	1	1	1	1	0	0	0
Equatorial Axis L µm (More than 30=1 & less than 30=0)	1	1	1	0	0	0	1	1	1
P/ E µm (More than 0.5 =1 & equal or less than 0.5=0)	0	0	0	1	1	1	1	1	1
Colpus Length L µm (More than 22 =1 & equal or less than 22=0)	1	1	1	1	1	1	0	0	0

SEM: Scanning Electron Microscope.

2.2. Chromosome counting

Seeds from ten individual plants were randomly selected from each population of the studied three infraspecific taxa (varieties and Forms1 & 2), for chromosome counting. Ten seeds/taxa were soaked in distilled water for 2 hours, then germinated at 20ºC in the dark chamber. Then root tips of about 1.5 cm length were treated at room temperature for 4 hours with colchicine ($C_{22}H_{25}NO_6$, 0.05 %) then washed by distilled H_2O. Overnight fixation was carried out in a freshly prepared farmer's fixative (ethanol/glacial acetic acid, 3: 1). Then, root tips were hydrolyzed using 1 N HCl at 64 C for five minutes. Slides were prepared using 45% acetic acid for squashing the root tips and stained with Aceto-orcein solution. Chromosome counting was visualized in the clearly ten observable cells during the mitotic metaphase.

2.3. Data analysis and karyotyping

In order to prepare the karyotype and ideogram, the chromosome counting was carried out on metaphase cells using the light microscope (Nikon Eclipse EG00 microscope at initial magnification of 2.5X). Ten clearly observable/cells, of the well-spread chromosomes were photographed, using the high-resolution automated karyotyping software processing (Leica CW4000). The two arms of each chromosome were measured, the short arm length (p), the long arm length (q) and the total chromatid length (TL) was calculated (p+q) for each chromosome. The relative chromosome length (RL) was ($\frac{TL}{sumTL}\times 100$), the mean relative length (MRL)/chromosome pair to calculate the relative length of each chromosome pair. The centromeric index (CI) was estimated using the equation $\frac{P}{TL}\times 100$ and the mean centromeric index (MCI) was calculated/ each chromosome pair, which used for determining the centromere position (metacentric chromosome when the CI ranges between 45.0 and 50.0; submetacentric CI ranges from 35 to 44; subterminal for CI less than 35 and telocentric when CI was zero) as reported by (Hassan and Gawad, 2013). It was found more appropriate to use the relative length expressed in percentage of the total length of the complement (Rothfels and Siminovitch, 1958).

2.4. Plant genomic DNA extraction

To study the molecular identity using the DNA markers (nuclear ITS and chloroplast), the total genomic DNA was extracted from fresh young leaves that were grounded in liquid nitrogen, following the steps of CTAB protocol (Doyle, 1991). The extracted DNA pellet washed with 70% alcohol, dried at room temperature, and dissolved in TE buffer.

2.5. Polymerase Chain Reaction (PCR)

The complete ITS region of the ribosomal DNA was amplified with plant specific primer pair ITS-p5/ITS-u4 (Cheng *et al*., 2016), while the three chloroplast marker-intergenic spacers were amplified using three plastid primer pairs (rps7 & rpsl2.1), (rpoC1 intron.1 & 2) and (trnK intron.1 &2), according to (Haider and Wilkinson, 2011), the sequences of the used primers are given in Table (3).

Amplification was carried out using a thermal cycler (Thermo Hybaid, USA). The PCR mixture (25 µL) contained dNTPs (200 µM), KCL (50 mM), Tris-HCl (10 mm ; pH 9.0), $MgCl_2$ (1.5 mM), 0.1% Triton® X-100, 0.5µM /primer, one unit of *Taq* DNA Polymerase (Promega), and template DNA (50 ng). The PCR program was 94 °C (4 min), 34 cycles of 30 s at 94 °C, 40 s at 55 °C and 52°C for both of the rps7 & rpsl2.1 primer and 1 min (72 °C), the final step was 10 min at 72 °C. The developed PCR products were run in a 1.5% agarose gel with ethidium bromide and visualized under ultraviolet transilluminator.

2.6. Sequencing of amplified DNA

Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme; Applied Biosystems), according to the Sigma laboratory protocol supplied by the manufacturer. Single-pass sequencing was performed on each template using primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with BigDye XTerminator™ purification protocol. The PCR products were re-loaded in dis. H_2O and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems), then sequence was achieved by Clone Manager 9 (Sci-Ed, Cary, NC, USA). The obtained sequences were aligned by CLUSTAL W (Thompson *et al*., 1994). Similarities in nucleotide sequence were determined using BLAST, version 2.0 (National Center for Biotechnology Information databases); jModelTest 0.1.1 program was used to find the model of sequence evolution that fitting the data (Posada, 2008). Neighbor-Joining method NJ was used for phylogenetic analyses (Saitou and Nei, 1987) with the Maximum Composite Likelihood method (Felsenstein, 1992) in MEGA 6.0 (Tamura *et al*., 2013).

3. Results

3.1. Morphological features

The morphological investigations of the collected 9 populations using the 70 macro-and micro-morphological characters (Table 3), confirming the presence of two varieties namely var. *bracteolata* and var. *nigra.* The fruit peak and trichomes are the differential characters delimiting the studied taxa, the differential key is:

Leaves glabrous, fruit peak up to 1/4 fruit length
…..……. var. *bracteolata*
Leaves hairy, fruit peak up to 1/2 fruit length
……. var. *nigra*
2- Basal leaves to 30 cm, hairs on all over the surface
.....…… …… Form 1
Basal leaves to 75 cm, hairs found only on leaf mid-rips
……. Form 2

The retrieved heat map based on the detected morphological characters (Table 2 & Figure 1), showing two hierarchical clusters each/variety, one of them including the six populations of var. *nigra* (Form 1 & Form 2) indicating that the phenotypic features are distinctive to the infraspecific level.

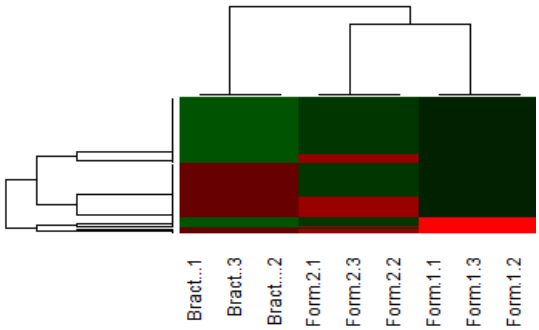

Figure 1. Heat map with hierarchical clustering of the 9 studied *Brassica nigra* populations (1-9; X-axis), Y-axis representing 70 micro-& macro-morphological characters. Red indicates a high level of expression; green represents a low level of expression; increasing color intensity is directly proportional with the value of the studied character. Using "R" software for windows version 3.5.1. (Bract. 1,2 & 3: var. *bracteolata.* Form 1.1,1.2 & 1.3: var. *nigra* Form 1 and Form 2.1,2.2 & 2.3: var. *nigra* Form 2). Cytogenetics (chromosome number and karyotyping):

Chromosome count of *Brassica nigra* (ten well-spread root cells/ population) in the mitotic metaphase revealed that all populations have the same diploid chromosome number (2n=16) as shown in Figure (2). Chromosomes were observed in metaphase and the karyotyping of the chromosome pairs were arranged in descending order (from 1 - 8; Figure 3), while the karyotyping measures as short arm (p), long arm (q) and total lengths (TL), the mean relative length (MRL), the mean centromeric index (MCI) were taken for each chromosome/taxa (Table 4).

The retrieved results showed that Chromosomes of *B. nigra* are small in size, the total genomic length was 27.05 µm in var. *nigra* Form 1, 25.13 µm in var. *nigra* Form 2, while the smallest value (24.6 µm) was in var. *bracteolata*. Moreover, the chromosomes are highly variable referring to their mean relative length (MRL; Fig. 4 and Table 4). The chromosome pair no. "1" is the longest among the studied taxa, its length ranges from 4.85 µm in var. *nigra* Form 1 to 4.53 µm in var. *bracteolate,* and its mean relative length (MRL) ranges from 17.93% in var. *nigra* Form 1 to 18.70% in var. *nigra* Form 2. The shortest chromosome pair is the chromosome pair number 8, its length ranges from 2.11 µm to 2.13 µm in var. *nigra* Form 1 and 2, respectively. The mean relative length (MRL) ranges from 7.80% to 8.62% for var. *nigra* Form 1 and var. *bracteolate*; respectively (Table 4).

The observed eight chromosome pairs are variable referring to their mean centromeric index (MCI), classifying the chromosomes based on the centromere position into two types: metacentric and acrocentric (Figure 5 and Table 4). The chromosome pairs 1-4 are metacentric while chromosomes 5-8 are acrocentric in all the studied taxa. Satellite was observed on the chromosome pair no. "6" distinguished var. *bracteolata* from *var. nigra* (Figure 6).

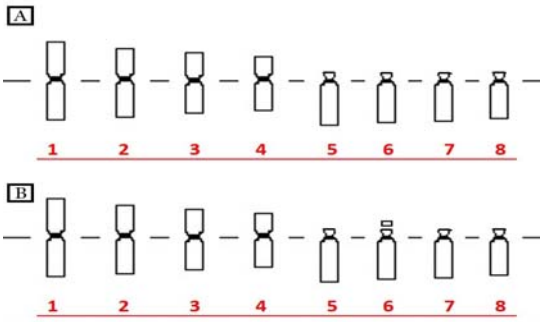

Figure (6): Idiogram of haploid set of chromosomes of *B. nigra* showing metacentric (1-4) and acrocentric (5-8) chromosomes. A: var. *nigra* Forms1 and 2 (without satellite); B: var. *bracteolata* (has satellite in chromosome no. 6).

3.2. Molecular identity

3.2.1. Amplification of internal transcribed spacer (ITS) nrDNA genes:

The amplified products of PCR using ITS-p5 and ITS-u4 plant specific primers showed one monomorphic band of c. 650 bp for the three representative populations/taxa (varieties and forms; Fig. 7). These amplified fragments were sequenced; var. *bracteolata* the length of the ITS region was 630 bp, and notable base divergence recorded in var. *nigra* Form 1 (652 bp) reached 656 bp in var. *nigra* Form 2, 161 bp for the 5.8S nrDNA gene and 197-216 bp for the ITS2 region.

3.2.2. Amplification of chloroplast markers:

3.2.2.1. Rps intergenic spacer:

Using rps7 & rps12.2 universal primers, single monomorphic band c. 400 bp was produced as PCR product of three representative populations in each studied taxa (Fig. 7). The amplified fragments were subjected to DNA sequencing. The length of rps region varied from 369 bp in var. *494racteolate*, 371 bp in var. *nigra* Form 1 to 372 bp in var. *nigra* Form 2.

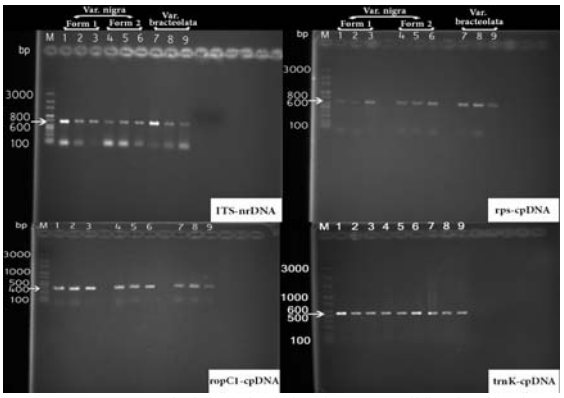

Figure 7.Agarose gel-electrophoresis of PCR amplification bands. M: DNA ladder (100 bp - 3000 bp). Lanes (1-9): amplicons of targeted locus in the studied 9 populations of var. *bracteolata* and var. *nigra*; denoting the close genetic affinity between the studied populations.

3.2.2.2. ropC1 intron:

The PCR products of ropC1 intron amplification show bands c. 620 bp for the populations/studied taxa (Fig. 7). The amplified fragments were subjected to DNA sequencing. The length of ropC1 intron varied form 612 bp in var. *494racteolate*, 618 bp in var. *nigra* Form 1 to 620 bp in var. *nigra* Form 2.

3.2.2.3. trnK intron:

The amplified products of PCR using trnK intron 1 and trnK intron 2 primers revealed one monomorphic band with about 550 bp in all the studied taxa (Fig. 7). The amplified fragments were subjected to DNA sequencing, the length of trnK intron varied form 568 bp in var. *bracteolata*, 587 bp in var. *nigra* Form 1 to 556 bp in var. *nigra* Form 2.

3.2.3. Molecular authentication:

Accession numbers of nucleotide sequences for the ITS region and the three studied barcodes (chloroplast non-coding regions) were obtained from complete sequence data that have been deposited in the EMBL/GenBank/DDBJ nucleotide sequence data libraries. The sequences of the studied *B. nigra* taxa were submitted to Gene-Bank database with accession numbers as outlined in Table (5), then compared with that of *B. nigra* retrieved from Gene-Bank database search via Basic Local Alignment Search Tool (BLAST) as outlined in Table (5). The constructed cladogram of ITS region and the three studied chloroplast non coding regions are the same, each exhibited two branches each representing variety, one for variety *bracteolata* and the other cluster including the two Forms (Form 1 & Form 2) of the variety *nigra* (Fig. 8).

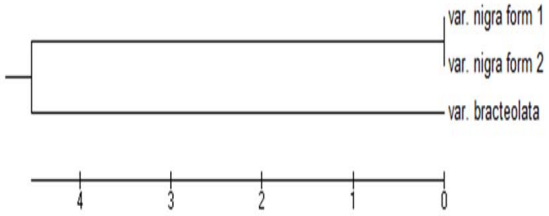

Figure 8. Cladogram showing varieties of *Brassica nigra* based on the nucleotide sequences of ITS region and three chloroplast regions (using Mega 6 program); denoting the genomic similarity of Forms 1 &2.

4. Discussion

The combined morphological data (70 characters) retrieved from the studied wild *Brassica nigra* populations (Table 3), and the developed consensus tree (Fig. 1), delimited all the morphological diversity under two distinct varieties var. *bracteolata* and var. *nigra.* This study distinguished the later variety into two Forms (Form 1 & Form 2), by their basal leaves up to 75 cm in Form 2 and 30 cm in Form 1); while var. *bracteolata* was identified earlier in the Egypt (Täckholm, 1974). The observed quantitative morphological (phenotypic) variations at the infraspecific level of *B. nigra* may be induced by the natural selection as claimed by (Österberg *et al*., 2002).

Table 3. The oligonucleotide sequences of forward and reverse primers used during this study.

Primers	Sequence
Forward primer ITS-p5	5'-CCTTATCAYTTAGAGGAAGGAG-3'
Reverse primer ITS-u4	5'-RGTTTCTTTTCCTCCGCTTA-3'
Forward primer rps7&rpsl2.1.1	5'-GGTGAAAATCTCATGTACG-3'
Reverse primer rps7&rpsl2.1.2	5'-TTTCTAGCGATTCACATGG-3'
Forward primer rpoC1 intron.1	5'-GAGTAACATGAAGCTCAG-3'
Reverse primer rpoC1 intron.2	5'-GTTTCCTTCATCCGGCT-3'
Forward primer trnK intron.1	5'-GTCTACATCATCGGTAGAG-3'
Reverse primer trnK intron.2	5'-CAACCCAATCGCTCTTTTG-3'

The morphological diversity of the studied *B. nigra* taxa, is not reflected to its chromosome number, where all these taxa are similar (2n=16; Fig. 2 & Table 4). This indicated that the studied wild populations were diploid *Brassica* (genome BB). Identification of this BB genome in Egypt will be of future applicability in breeding programs of *Brassica* crops (Westman *et al.,* 1999), as source of the drought tolerant genes (Groat, 2003; Pradhan *et al*., 2011), and its importance extended to the evolutionary lineages of both of the *nigra* and the *rapa/oleracea* lineages used in crop improvement (Yang *et al.*, 2002). The observed chromosome number in the *B. nigra* taxa was reported earlier (Nakayama and Fukui, 1997).

Table 4. The karyotype features of studied *Brassica nigra* taxa.

var. *nigra* Form 1						
Chromosome number	p µm	q µm	p+q µm	MRL%	MCI%	CP
1	2.4	2.45	4.85	17.93	49.48	metacintric
2	1.99	2.39	4.38	16.19	45.43	metacintric
3	1.97	2.4	4.37	16.16	45.08	metacintric
4	1.89	2.35	4.24	15.67	44.58	metacintric
5	0.15	2.3	2.45	9.06	6.12	acrocentric
6	0.15	2.25	2.4	8.87	6.25	acrocentric
7	0.15	2.1	2.25	8.32	6.67	acrocentric
8	0.11	2	2.11	7.80	5.21	acrocentric
Total genome length			27.05			
var. *nigra* Form 2						
Chromosome number	p µm	q µm	p+q µm	MRL%	MCI%	CP
1	2.3	2.4	4.7	18.70	48.94	metacintric
2	1.91	2	3.91	15.56	48.85	metacintric
3	1.87	1.95	3.82	15.20	48.95	metacintric
4	1.74	1.87	3.61	14.37	48.20	metacintric
5	0.13	2.25	2.38	9.47	5.46	acrocentric
6	0.14	2.2	2.34	9.31	5.98	acrocentric
7	0.14	2.1	2.24	8.91	6.25	acrocentric
8	0.13	2	2.13	8.48	6.10	acrocentric
Total genome length			25.13			
var. *bracteolata*						
Chromosome number	p µm	q µm	p+q µm	MRL%	MCI%	CP
1	2.21	2.32	4.53	18.41	48.79	metacintric
2	1.79	1.99	3.78	15.37	47.35	metacintric
3	1.77	1.9	3.67	14.92	48.23	metacintric
4	1.75	1.9	3.65	14.84	47.95	metacintric
5	0.1	2.26	2.36	9.59	4.24	acrocentric
6	0.15	2.2	2.35	9.55	6.38	acrocentric
7	0.14	2	2.14	8.70	6.54	acrocentric
8	0.12	2	2.12	8.62	5.66	acrocentric
Total genome length			24.6			

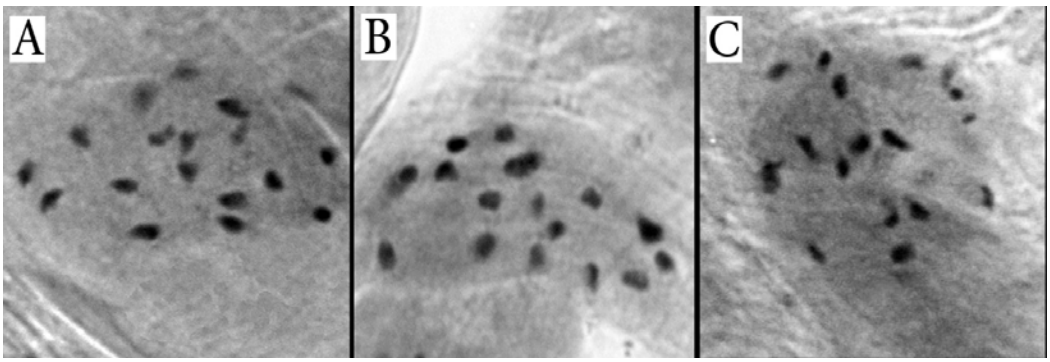

Figure (2): Metaphase photomicrographs of the studied *Brassica nigra* taxa indicating number of chromosomes (2n=16). A: var. *nigra* Form 1, B: var. *nigra* Form 2 and C: var. *bracteolata*

The observed karyotype analysis (Table 4; Figs 3,4 & 5) showed that the chromosomes are small in size; this result was supported by (Schmidt and Bancroft, 2011). The small-size chromosome as in *Brassica nigra,* makes its karyotyping study challenging (Kulak *et al*., 2002), however, some studies have provided information about

chromosome morphology of the B genome (Cheng and Heneen, 1995; Fukui *et al*., 1998; Hasterok and Mahszynska, 1997; Lan *et al*., 1991; Maćkowiak and Heneen, 1999; Robbelen, 1960; This *et al.*, 1990).

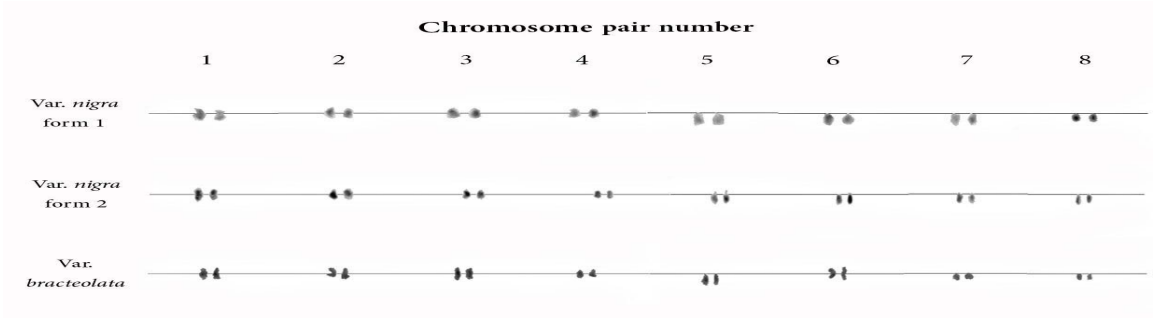

Figure (3): Karyotypes of the *Brassica nigra* taxa.

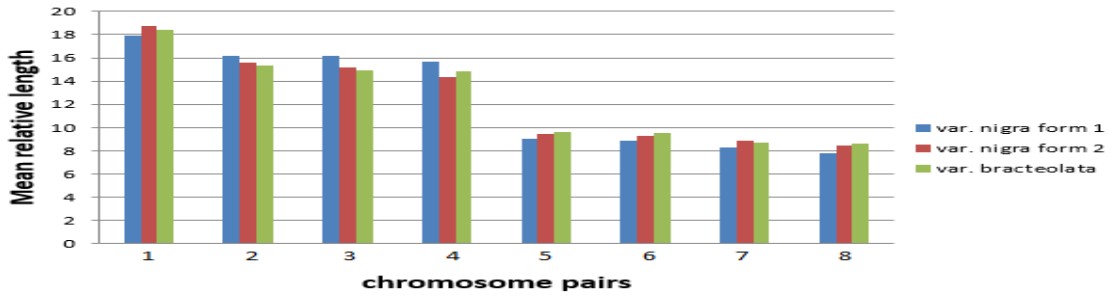

Figure (4): Mean relative length (MRL) of each chromosome pair in the studied *B. nigra* taxa**.**

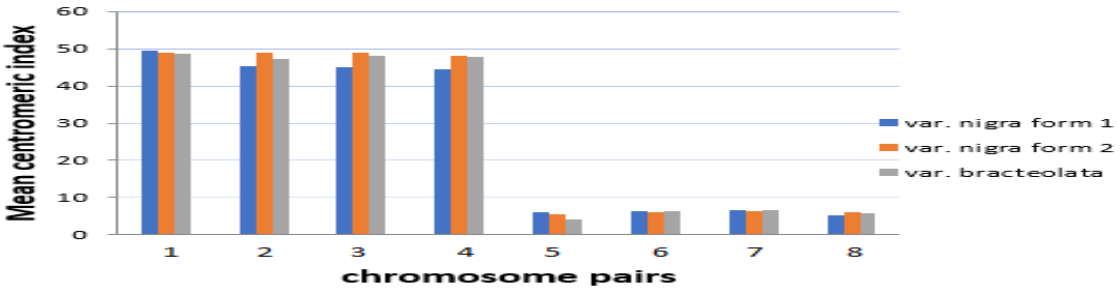

Figure 5. Mean centromeric index (MCI) of each chromosome pair in the studied *B. nigra* taxa.

The karyotyping of the studied *B. nigra* taxa showed chromosomes features distinguished between the studied varieties, referring to their mean relative length (MRL), the longest chromosome (chromosome no. 1; MRL ranges from 17.93% in var. *nigra* Form "1" to 18.70% in var. *nigra* Form "2"), where var. *bracteolata* is characterized by an intermediate value (18.41%); similar observation was noticed in the shortest chromosome (chromosome no. 8 ;Table 4 & Fig. 4). Consistent results were reported in *B. nigra* using the Fish technique (Wang *et al.,* 2017). This observed infraspecific chromosomal variability in the studied *B. nigra* taxa indicated that each taxon retains its genotypic identity.

However, the achieved results (Table 4 & Fig. 5), showed variable mean centromeric index (MCI), classifying the chromosomes into two types: metacentric and acrocentric in all the studied taxa. Wang *et al*., (2017), reported that all chromosomes of *B. nigra* are metacentric with an arm ratio of 1.12–1.49.

The karyotyping (Fig. 6) indicates the presence of satellite in the chromosome pair no. "6" which characterizes var. *bracteolata* from var. *nigra*. The presence of satellite was supported by Wang *et al.*, (2017), who found that the satellites were abundant in the whole genome of *B. nigra;* in the same study he reported the presence of 6 satellite repeats in metaphase cells of *B. nigra* root tips. Cheng and Heneen (1995) and Fukui *et al*. (1998) noticed the presence of three nucleolar chromosome pairs with satellites in mitotic divisions, while similar observation was reported during meiotic divisions of *B. nigra* by Maćkowiak and Heneen (1999). Only two pairs of chromosomes to be satellited in mitotic divisions were reported by other studies (Robbelen, 1960; This *et al.*, 1990) and in meiotic divisions (Lan *et al*., 1991; Sikka, 1940). Accordingly, it is expected that satellite associations among the nucleolar chromosomes were predominate during diakinesis in *B. nigra* (Maćkowiak and Heneen 1999).

The data retrieved from the nuclear DNA marker (ITS, Fig. 7) and the chloroplast DNA markers (rps, ropC & trnK; Table 5 & Fig. 7), showed that the genetic divergence of var. *bracteolata* is higher than that of var. *nigra* the divergence is more notable in ITS region. This is attributed to the rate of evolution (limited deletions-insertion and point mutation) of chloroplast genome being much lower than that in the nuclear genome (Zhu *et al.,* 2021). The obtained nuclear data (Table 5) elucidate the phylogenetic relationship between the studied wild taxa and that from other geographic regions; Zhu *et al.,* (2021) reported that several studies used the chloroplast genome

to study the phylogenetic relationships in family Brassicaceae. The congruent data of the nuclear and chloroplast markers enhance the author to support treating these identified morphological taxa as distinct varieties not as synonyms. This postulation was supported by Yang *et al*., (2002), who reported that the chloroplast noncoding region between trnD & trnT and that between trnT & trnF are useful for studying the interspecific relationships among angiosperms. The achieved results for the cpDNA markers reveal the intraspecific polymorphisms in Egyptian wild *B. nigra* taxa, is a useful tool for conservation and as marker assisted *Brassica* breeding programs, as reported by Sarin *et al.* (2015). The detected low divergence between the two forms of var. *nigra* (Table 5 & Fig. 8) indicate that these forms were recently diversified; relevant data were reported by Mummenhoff *et al*., 2001) in section *Lepia* (Brassicaceae), and other *Brassica* species (Zhu *et al*., 2021).

The studied 9 populations of wild *B. nigra* were collected from different localities along the Nile valley and Delta (Table 1), showing that the identified varieties are not correlated with geographical distribution, which could be explained by adaptive mechanism of natural selection (Österberg *et al.,* 2002).

The authentication of the molecular data in the GenBank confirmed that the chloroplast marker showed that 100% similarity to the accession number (KT878383; Table 5), with *var. nigra* Form "1" and Form "2". Moreover, the current results obtained using chloroplast DNA of *Brassica nigra* from Egypt as part from its native range, this range include North African and Mediterranean region (Mummenhoff *et al.,* 2001). These results may help to identify the species distribution range. The population origin and distribution range can be achieved by using chloroplast DNA and also for population genetics/ phylogeographic analyses (Oduor *et al*., 2015).

Table 5. Authentication of the studied *B. nigra* taxa compared to the GenBank data.

Studied Taxa		BLAST Result	Accession numbers retrieved from database	Locus	Similarity %
Name	Accession numbers				
var. bracteolata	MN480491	Brassica nigra clone 136	KX709349 (South Korea)	ITS	97.99%
	MN869001	Chloroplast complete genome	KT878383 (Korea)	rps	99.68%
	MN886246	Chloroplast complete genome	KT878383 (Korea)	ropC	93.50%
	MN886249	Chloroplast complete genome	KT878383 (Korea)	trnK	95.74%
var. nigra Form 1	MN480492	Clone 133	KX709348 (South Korea)	ITS	98.60%
	MN869002	Chloroplast complete genome	KT878383 (Korea)	rps	100%
	MN886247	Chloroplast complete genome	KT878383 (Korea)	ropC	98.84%
	MN886250	Chloroplast complete genome	KT878383 (Korea)	trnK	98.91%
var. nigra Form 2	MN480493	Clone 95	KX709346 (South Korea)	ITS	98.40%
	MN869003	Chloroplast complete genome	KT878383 (Korea)	rps	100%
	MN886248	Chloroplast complete genome	KT878383 (Korea)	ropC	99.42%
	MN886251	Chloroplast complete genome	KT878383 (Korea)	trnK	98.94%

Finally, this study recommends the application of multidiscipline approach in taxonomy to resolve the identity of the infraspecific level for the *Brassica nigra*, which possesses morpho-plasticity and wide geographic range crop similar to many wild plants' relatives.

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