

# Molecular characterization of some Brassica species

## Abstract

Nineteen ISSR were used to investigate polymorphism among five Brassica species. ISSR revealed 326 total bands the highest (89% polymorphism). 30SRAP primer pairs revealed 480 bands with polymorphism (93%). ISSR and SRAP, both were effective in studying genetic diversity in Brassica. Based on similarity matrix of overall analysis (ISSR and SRAP), the dendrogram was constructed and separated the five Brassica into two main clusters. Results showed that the length of the MYB28 gene for glucosinolates production at the fragment of 1257bp. This information can be used in the future for breeding and improvement programs and helps in national conservation programs.

**Keywords:** Brassica, ISSR, SRAP, glucosinolates and MYB28 gene

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## Introduction

Brassica genus (*Brassicaceae*) contains proteins, vitamins, minerals, glucosinolates and phenols. Glucosinolates contribute to reducing the risk of cancer.<sup>1</sup> That activity is mainly related to the presence of isothiocyanates and nitrates. Most species are of great economic importance since most of them are edible, a source of high-value edible oil, fodder, which helps to protect the food supply by expanding the range of genes available in the face of the challenges of the lack of agricultural production, and is of great medical importance such as: white mustard, black, brown, yellow, cabbage and broccoli help in the treatment of chronic, cough, asthma, chest pains, heart disease, headache, compresses the pain of rheumatism, joints, diuresis, stimulates the metabolism, clears the appetite, treats tumors, regulates blood sugar and prevents cerebral palsy. Molecular markers are effective tools for revealing genetic diversity, inter-sequence simple repeat (ISSR), is useful for detecting genetic polymorphisms among genotypes by generating a large number of markers that target multiple microsatellite loci distributed across the genome.<sup>2</sup> ISSR is a technique that overcomes most of other techniques limitations. This technology has been used to DNA fingerprint a wide range of species of medicinal plants, phylogeny and the geographic origin of some plant species as *Brassica napus*.<sup>3</sup> Sequence-related amplified polymorphism (SRAP) is a new and useful molecular marker system for tagging and mapping in *B. oleracea*.<sup>4</sup> The SRAP system is easy to perform, gives stable and moderate yields and a high proportion of co-dominance, and ultimately simplifies the cloning and sequencing of target segments. The SRAP technique is an effective tool for

constructing genetic and linkage maps,<sup>5</sup> carrying out comparative genomics and analyzing genetic diversity because sequences are amplified against open reading frames, and sequences near the centromeres and telomeres of genomes are only rarely amplified. The SRAP technique has been applied successfully in studies of sweet potato, rape, and Chinese cabbage and broccoli.<sup>6-8</sup> Although there are many genetic studies related to plant traits, there are few in terms of identifying genes and their characterization, especially specific of Brassica for the main active substance such as glucosinolates. There is a great challenge to be addressed in the inventory of genetic variation that helps us to improve crops. Glucosinolates, in turn, are resistant to pests and microbes, as well as the industrial significance of brassica in the work of bombs in wars, thus preserving genetic diversity in gene banks and characterizing them and defining the important genes that we can transfer to important crops to be used as food and source of important effective substances. (MYB28) gene was found to participate in regulating the aliphatic GSL biosynthesis glucosinolates and have been successfully identified in several plant species including *Arabidopsis thaliana*,<sup>9</sup> *Brassicajuncea*,<sup>10</sup> and *B. rapa*.<sup>11</sup> The objectives of this study were to investigate the level of genetic diversity and relationships in Brassica by molecular markers and provide valuable information for detecting glucosinolates gene.

## Materials and methods

Five Brassica species were collected from the Gene Bank of North Sinai Station, Desert Research, Egypt during February 2015-2017, as shown in (Figure 1) (Table 1).



**Figure 1** The five Brassica species collected from the Gene Bank of North Sinai Station, Desert Research, and Egypt

**Table 1** Taxa of the five studied Brassica species

No	Scientific name
1	<i>Brassica tournefortii</i>
2	<i>Brassica nigra</i> L.
3	<i>Brassica rapa</i> L.
4	<i>Brassica Juncea</i> L.
5	<i>Brassica carentia</i>

### DNA extraction

A total of 25 young fresh leaves from each of the 20 annual ryegrass plants were randomly collected and extracted using the DN easy Plant Mini kit (QiagenInc, Valencia, CA, USA). The quality and concentration of the DNA were confirmed by electrophoresis on 0.8% agarose gels with the standardized lambda DNA size markers.

### ISSR analysis

Finally, 15 primers (Life Technologies) (Table 2) were selected for ISSR analysis. The PCR amplification comprised a total volume of 20mL containing 3mL 20ng/mL DNA, 0.2mL Taq DNA polymerase, 1.4mL 2.5 mMdNTPs, 3mL 10mM primer, 1.4mL 25mM Mg, 2.5mL 10X buffer, and 8.3mL ddHO. PCR amplification was carried out on the Eppendorf PCR instrument and the reaction program comprised an initial 5 min at 94°C; 35 cycles of 45 s at 94°C, 45 s annealing at 50°C and a 90-s extension at 72°C; ending with a final extension of 5 min at 72°C and storage at 4°C. Amplified products were electrophoreses on 6% denaturing poly acrylamide gel, which was silver stained and photographed gels and visualized with the Gel Documentation System(Bio-Rad® Gel Doc-2001) (Germany).

**Table 3** The forward and reverse SRAP primer in this study

Primer name	Forward primers	Primer name	Reverse primers
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
Me6	TGAGTCCAAACCGGACA	Em6	GACTGCGTACGAATTGCA
Me7	TGAGTCCAAACCGGACG	Em7	GACTGCGTACGAATTCAA
Me8	TGAGTCCAAACCGGACT	Em8	GACTGCGTACGAATTCTG
Me9	TGAGTCCAAACCGGAGG	Em9	GACTGCGTACGAATTCAG
Me10	TGAGTCCAAACCGGAAA	Em10	GACTGCGTACGAATTCAT
Me11	TGAGTCCAAACCGGAAC	Em11	GACTGCGTACGAATTCTA
Me12	TGAGTCCAAACCGGAGA	Em12	GACTGCGTACGAATTCTC
DN06	TGAGTCCAAACCGGTAA	Em13	GACTGCGTACGAATTCTT
DN07	TGAGTCCAAACCGGTCC	Em14	GACTGCGTACGAATTGAT
DN08	TGAGTCCAAACCGGTGC	Em15	GACTGCGTACGAATTGTC
DN09	TGAGTCCAAACCGGTCA	Em16	GACTGCGTACGAATTCGA
DN10	TGAGTCCAAACCGGGCT	Em17	GACTGCGTACGAATTAGC
DN11	TGAGTCCAAACCGGTAG	Em18	GACTGCGTACGAATTGAG
DN12	TGAGTCCAAACCGGTGT	Em19	GACTGCGTACGAATTGCC

**Table 2** ISSR primer sequences

Primer name	Sequence	Primer name	Sequence
HB01	(CAA) 5	844B	(CT) 8 GC
HB02	(CAG) 5	HB9	(GT) 6 GG
HB04	(GACA) 4	HB10	(GA) 6 CC
17898A	(CA) 6 AC	HB11	(GT) 6 CC
17898B	(CA) 6 GG	HB12	(CAC) 3 GC
17899A	(CA) 6 AG	HB14	(CTC) 3 GC
17899B	(CA) 6 GG	HB15	(GTG) 3 GC
844A	(CT) 8 AC	814	(CT) 8TG
807	(AGA) 4GT	HB13	GAC)3 GC
HB8	(GA)6 GG		

### SRAP fingerprinting

Thirty selected pairs of primers (Life Technologies) (Table3) from 60 different primer combinations were used for detecting polymorphism in open reading frames (ORFs). Each 20-µLPCR mixture consisted of 1. 0U Taq DNA polymerase, 6X PCR buffer, 0.6mM MdNTP, 0.35µM primer, 1.5 mM Mg, and 25-200ng template DNA. Thermal cycling (Biometra T1 Thermo cycle) started with 5min at 94°C for initial denaturing, and 5 cycles of 30s at 94°C, 30s at 35°C, and 45 s at 72°C, followed by 40 cycles of 30s at 94°C, 30 s at 4°C and 45 s at 72°C. The last cycle was followed by a 7-min extension at 72°C. Amplified products were analyzed on 2% (w/v) gels and visualized with the Gel Documentation System (Bio-Rad® Gel Doc-2001) (Germany) (Table 3).

## Statistical analysis

Similarity matrix was developed by the statistical package for social science program SPSS based on combined analysis of overall molecular and biochemical markers.

## Detection of gene polymerase chain reaction (PCR) and sequencing

PCR was conducted using forward and reverse primers, which were designed based on these quence of MYb28 obtained from the Gen Bank (BLAST). These quinces of the primers were 5'-GGGACCATCACACAATTCATTCTC-3' (forward) and 5'-2' (reverse). A mixture of 20µL solution consisting of 10xEx Taq polymerase buffer, 2m MMgCl<sub>2</sub>, 200u Md NTPs, 25p mol primers, 1UTaqpolymerase and distilled water was used for each PCR reaction. The PCR program was seton 94°C for 2minutes pre heating continued with 35cycles consisting of 1minute denaturation at 94°C, 1minutes annealing at 56°C, and90 seconds extension at 72°C. The PCR product was visualizedon1,5% agarose gel and subjected to 100 volts for 1hr and then photographed using UV gel documentation system, UVP corporation- UK.

**Purification of PCR product and sequencing:** PCR products were

purified using High Pure PCR Product purification Kit (Roche) and sequenced (MWG, Germany).

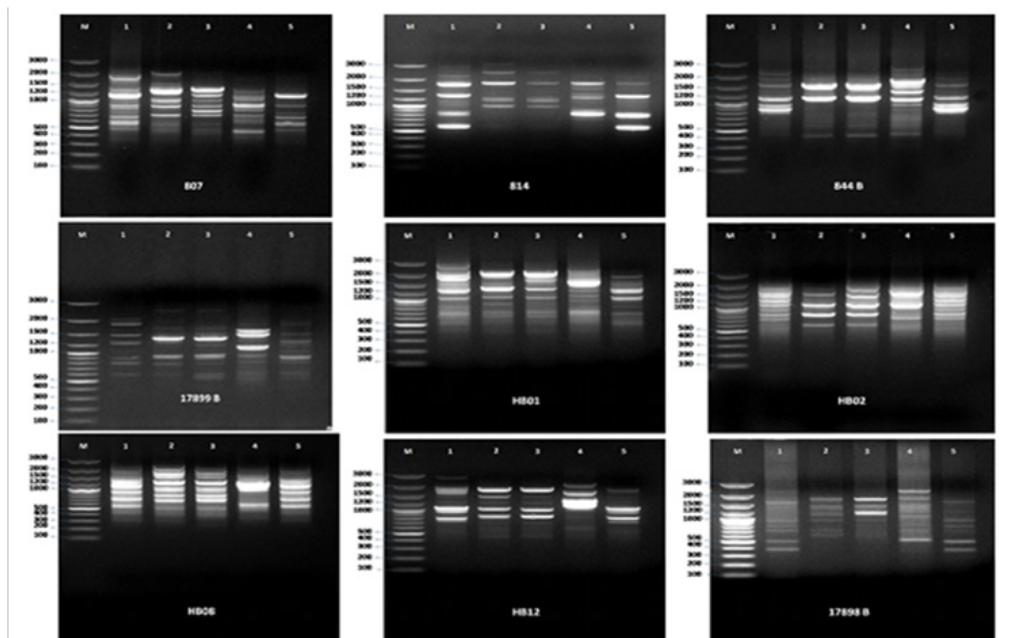
## Results and Discussion

### ISSR analysis

Nineteen preselected ISSR primers were used in the present investigation to study the genetic relationships among the five studied Brassica species as shown in ( Figure 2) and (Table 4) .326 total bands, 36 monomorphic and 290 polymorphic distinct bands (89% polymorphism) were generated by the nineteen ISSR primers. The results showed that 17898B, 17899A, HB1, HB4, HB9, HB11 and HB15 primers were highly polymorphic (100% polymorphism), while HB13 generated the lowest polymorphism (74%). These results agreed with the results obtained by Khalil)2010( who used fifteen preselected ISSR primers to identify eleven species of *Brassicaceae* and produced72 polymorphic distinct fragments (56.2% of polymorphism). Those results gave another dimension to detect the genetic variability in accurate differentiations and demonstrated significant levels of variation. Moreover, Santos et al., (2016) studied ISSR primers and were capable of detecting genetic polymorphism among *Mimosa caesalpiniaefolia*. A total of 7 ISSR primers generated 52.7% polymorphism.

**Table 4** Primer codes, length range (bp), monomorphic bands, polymorphic bands, total amplified bands and polymorphism percentages of nineteen ISSR primers amongfive *Brassicac pecies*

S. No	Primer code	Monom orphic bands	Polymorphic bands	Unique bands	Total amplified bands	Polymorphism percentages
1	807	2	18	7	20	90%
2	814	1	15	7	16	94%
3	844A	3	12	4	15	80%
4	844B	1	14	5	15	93%
5	17898A	1	14	5	15	93%
6	17898B	0	17	3	17	100%
7	17899A	0	13	3	13	100%
8	17899B	2	15	6	17	88%
9	HB1	0	13	3	13	100%
10	HB2	5	9	0	14	64%
11	HB4	0	20	7	20	100%
12	HB8	1	14	2	16	88%
13	HB9	0	8	1	8	100%
14	HB10	2	24	9	26	92%
15	HB11	0	14	1	14	100%
16	HB12	2	15	4	17	88%
17	HB13	6	17	3	23	74%
18	HB14	1	13	6	14	93%
19	HB15	0	21	7	21	100%
	Total	27	286	83	329	87%

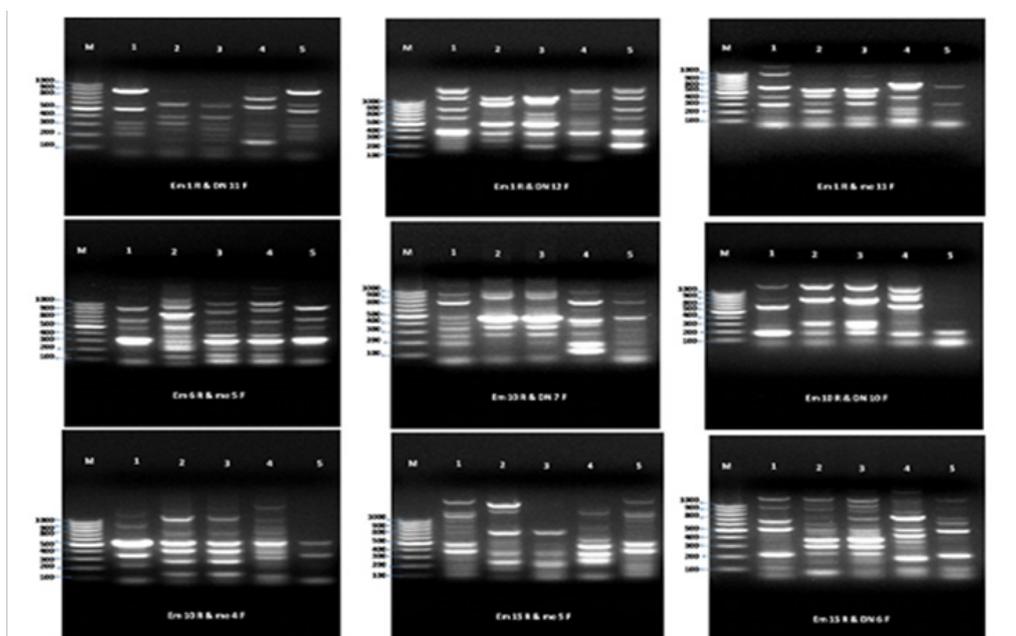


**Figure 2** ISSR primers of five Brassica species\*. \*\* (M) = Maker, \*(*Brassica tournefortii* (Gouam)) = 1, (*Brassica nigra* L.) = 2, (*Brassica rapa* L.) = 3, (*Brassica Juncea* L.) = 4, (*Brassica carentia*) = 5.

### SRAP analysis

Thirty pairs of preselected SRAP primers were used in the present investigation to study the genetic relationships among the five studied species of Brassica as shown in (Figure 3) and (Table 5) (Table 6). 480 total bands, 32 monomorphic bands and 448 polymorphic distinct bands (93% polymorphism) were revealed by the SRAP primers. The results showed that EM1RXme8F, EM6RXme5F, Em6RXme11F, EM10RXDN6F, EM10RXDN7F, Em10RXDN12F, Em13RXDN12F, Em17RXDN12F and Em18RXDN11F primer pairs were highly polymorphic (100% polymorphism). On the other hand,

EM6RXDm8F primer pair revealed the lowest polymorphism (75%). Polymorphic information content (PIC) and Marker indexes (MI) were equal between ISSR and SRAP markers (Table 6). These results agreed with the results obtained by Ahmad et al., (2014) who studied the genetic diversity and relationships among *B. napus* accessions using SRAP markers, which preferentially amplify open reading frames. Using 20 SRAP primers, a total of 60 spring-type *B. napus* accessions revealed 162 polymorphic fragments. Cluster analysis displayed five major groups. The clustering pattern mostly supported their respective pedigree and characteristic traits.



**Figure 3** SRAP primers among five Brassica species\*. \*\* (M) = Maker, \*(*Brassica tournefortii* (Gouam)) = 1, (*Brassica nigra* L.) = 2, (*Brassica rapa* L.) = 3, (*Brassica Juncea* L.) = 4, (*Brassica carentia*) = 5.

**Table 5** Primer codes, length range (bp), monomorphic bands, polymorphic bands, total

NO	Primer code	Monom orphic bands	Total polymorphic bands	Unique bands	Total amplified bands	Polymorphism percentages
1	Em1RXDN11F	1	12	3	16	94%
2	Em1RXDN12F	1	10	9	20	95%
3	EM1RXme8F	0	7	6	13	100%
4	EM1RXme11F	1	12	7	13	93%
5	EM2RXme12F	1	10	7	11	90%
6	EM6RXDm8F	3	9	3	12	75%
7	EM6RXme5F	2	17	7	19	100%
8	Em6RXme11F	0	18	4	18	100%
9	EM8RXme1F	1	8	1	9	88%
10	EM9RXme1F	1	16	5	17	94%
11	EM10RXDN6F	0	17	3	17	100%
12	EM10RXDN7F	0	20	6	20	100%
13	EM10RXDN8F	3	10	6	13	77%
14	Em10RXDN10F	2	10	4	12	83%
15	Em10RXDN12F	0	17	4	17	100%
16	Em10RXme4F	1	16	5	17	94%
17	Em12RXDN10F	1	15	8	16	94%
18	Em12RXDN12F	1	17	3	18	94%
19	Em13RXDN12F	0	14	4	14	100%
20	Em15RXDN6F	2	19	5	21	90%
21	Em15RXme10F	3	24	0	27	89%
22	Em15RXDN7F	1	18	3	19	95%
23	Em15RXDN9F	1	8	9	9	88%
24	Em15RXme11F	1	8	1	9	88%
25	Em17RXDN10F	0	18	6	18	100%
26	Em17RXDN12F	0	12	3	12	100%
27	Em18RXDN11F	0	10	5	10	100%
28	Em18RXDN12F	2	10	1	12	83%
29	Em20RXDN10F	1	16	1	17	94%
	Total	30	398	129	485	82%

**Table 6** Relative efficiency of molecular markers in determining polymorphism among five

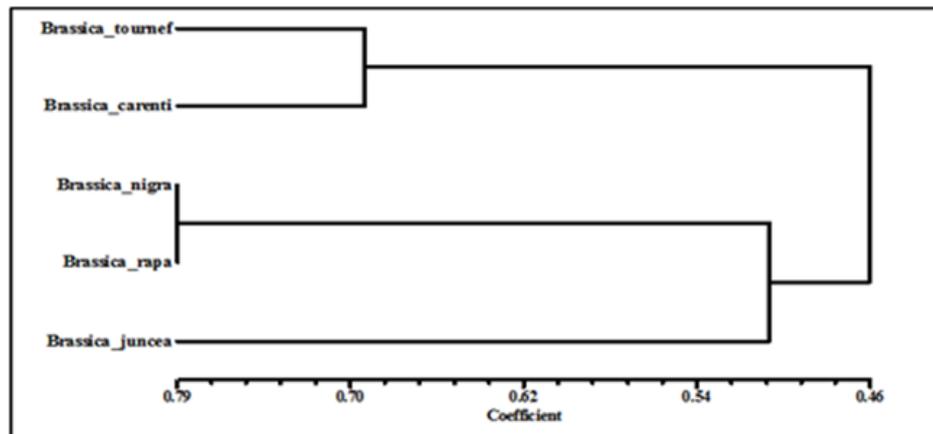
Parameters for marker efficiency	ISSR	SRAP
Number of individuals	19	30
Total number of bands (L)	326	480
Polymorphic bands (p)	290	448
Number of loci/assay unit (nu)	17.1	16
Total number of effective alleles (Ne)	1213	1975.8
Average number of polymorphic bands/assay unit(np/U)	15.26	14.93
Polymorphic information content (PIC)	0.97	0.97
Fraction of polymorphic loci ( $\beta$ )	0.88	0.93
Assay efficiency index (Ai)	63.8	65.8
Effective multiples ratio (E)	15.26	14.93
Marker index (MI) = $Hav \times MR$	14.82	14.62

Additionally, Yu<sup>12</sup> used SRAP and ISSR markers to assess the genetic diversity within and among 15 natural populations of *Stip abungeana* from the Loess Plateau of China. Using 15 SRAP primers, 504 (99.80%) polymorphic loci were detected, and 372 polymorphic loci (96.12%) were identified using 15 ISSR primers. The results showed that genetic variation within populations is lower than that among populations. The results demonstrated that both SRAP and ISSR markers are effective and reliable for assessing the genetic diversity of *S. bungeana*. In addition, these data inform conservation

and breeding strategies for *S. bungeana*. Besides, Ma et al.,<sup>13</sup> detected the genetic relationships of different *Cotoneaster schantungensis* natural populations and genetic diversity analysis based on SRAP markers. Twelve pairs primers were selected out, 93 bands were amplified, of which 91 bands (97.85%) were polymorphic. Guenni et al., (2016) used SRAP markers preferentially to amplify open reading frames and to study the genetic diversity of 43 Tunisian pistachio accessions. Using seven SRAP primer pairs, a total of 78 markers revealed (95.12%) that will help to design future conservation and breeding strategies concerning this species.

### Genetic and phylogenetic relationships

The generated ISSR and SRAP profiles were further used to assess similarities among the studied cultivars. The estimation of genetic distance was accomplished using a computer software (NTSYS-PC version 1.8) based on the formula originally proposed by Nei and Li (1979). The results are presented in (Table 7). Genetic distances ranged from (.385 to .762) in ISSR and SRAP Marker. The lowest genetic distance was found between *Brassica tournefortii* and *B. rapa*. The highest genetic distances were registered between *B. nigra* and *B. rapa*. The genetic distances were supported by the resulting dendrogram (Figure 4). Dendrogram revealed two main clusters, the first cluster splatted into two sub-clusters included *B. tournefortii* and *B. rapa*. The second cluster included *B. nigra* and *B. rapa* in one sub cluster and *B. juncea* another. The use of ISSR and SRAP marker is strongly supported by many studies dealing with the usefulness of such markers for investigating Brassica diversity. The current research is one of the attempts to use molecular markers in investigating the genetic relationships among various local genotypes geranium grown in desert Egypt.

**Figure 4** An un weighted pair-group method with arithmetic averages (UPGMA) dendrogram of genetic relationships among five genotypes of Brassica obtained using ISSR and SRAP combined data.**Table 7** Comparison by similarity of relationships Brassica spp. genotypes as revealed by ISSR, SRAP marker systems

Case	Matrix File Input			
	Brassica tournefortii	Brassica nigra	Brassica rapa	Brassica juncea
<i>Brassica tournefortii</i>				
<i>Brassica nigra</i>	0.386			
<i>Brassica rapa</i>	0.385	0.762		
<i>Brassica juncea</i>	0.462	0.457	0.48	
<i>Brassica carentia</i>	0.675	0.393	0.4	0.445

### MYB28 gene detection

The PCR product using specific primer of MYB28 gene indicated that the appearance of one band for each with fragment size 1257bp

as shown in (Figure 4) (Figure 5). These results agreed with the results obtain Ling et al.,<sup>14</sup> who isolated and characterized MYB28gene from *B. oleraceae*.

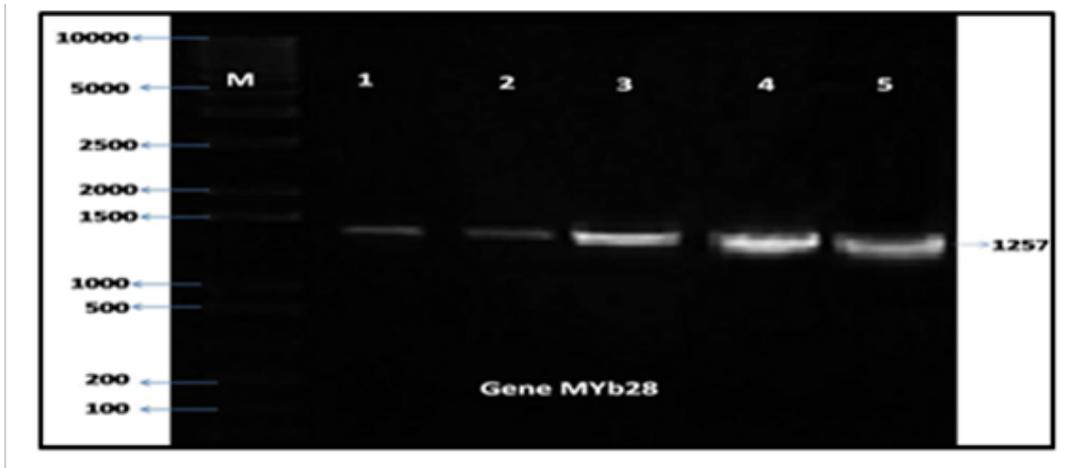


Figure 5 PCR product of MYB28gene from the five studied Brassica species

### Analysis of the MYB28 gene nucleotide sequence alignment

Sequencing and BLAST analysis showed that the length of TDC fragment shares high homology with the other known MYB28 as

shown in (Figure 6) (Figure 7). Homology search results in Gene Bank (NCBI) showed that MYB28 nucleotide had high identity to other plants such as *B. oleraceae* (94% identities, accession number (AB702694.1) and *Raphanus sativus* (89% identities, accession number XM018625413.1).The highest identities was 99% for *B. rapa*.

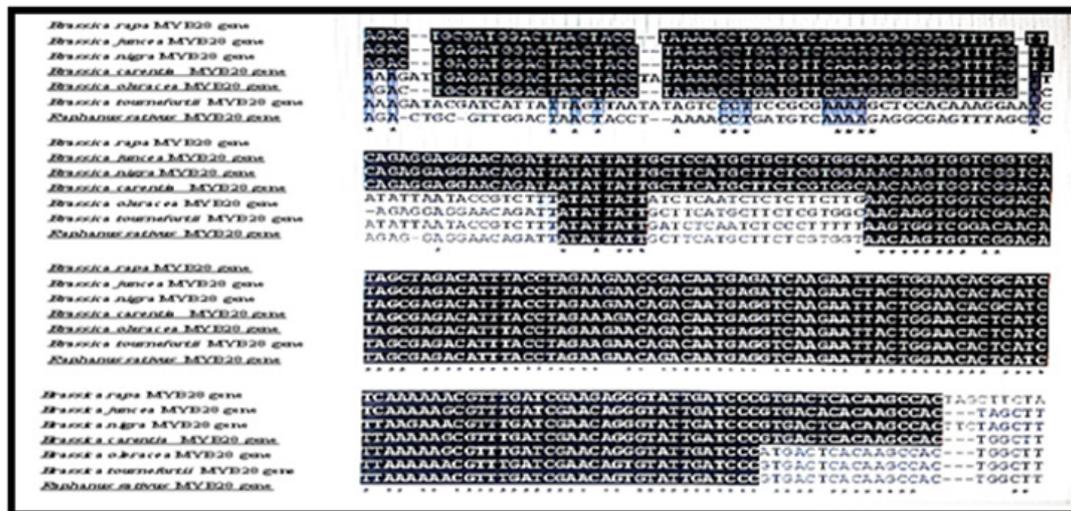
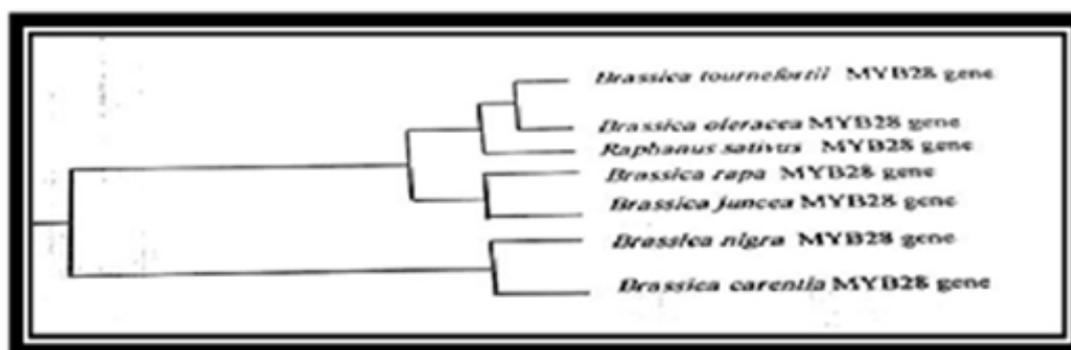


Figure 6 Sequence alignment of MYB28 gene among Brassica five species

Description	Max score	Total score	Query cover	E value	Ident	Accession
<input type="checkbox"/> <i>Brassica tournefortii</i> MYB28 gene	379	847	89%	0.0	90%	
<input type="checkbox"/> <i>Brassica nigra</i> MYB28 gene	442	1158	94%	0.0	96%	
<input type="checkbox"/> <i>Brassica rapa</i> MYB28 gene	1399	1886	99%	0.0	99%	
<input type="checkbox"/> <i>Brassica juncea</i> MYB28 gene	1284	1755	97%	0.0	98%	
<input type="checkbox"/> <i>Brassica carinata</i> MYB28 gene	1276	1723	94%	0.0	95%	
<input type="checkbox"/> <i>Brassica oleracea</i> MYB28 gene	399	838	92%	0.0	94%	AB702694.1
<input type="checkbox"/> <i>Raphanus sativus</i> MYB28 gene	240	245	86%	0.0	89%	XM 018625413.1

Figure 7 NCBI- BLAST analysis of MYB28 gene homology among Brassica five species



**Figure 8** Phylogenetic tree of the TDC gene sequences in zygophyllaceagenotypes

### Phylogenetic analysis of DNA sequence of TDC gene

Phylogenetic analysis was done by aligning DNA sequences using software Clustal X software to construct a phylogenetic tree (Figure 8). MYB28 gene among Brassica five species. *Brassica tournefortii*, *B. oleracea*, *B. juncea*, *B. rapa* and *Raphanus sativus* were grouped together and *B. nigra* and *B. crentia* were closer and group in another cluster.

### Conclusion

ISSR and SRAP used effectively to estimate genetic diversity among investigated five species of Brassica and may help further inbreeding programmers. Glucosinolates gene explorations are required to have better understanding of the presence of genetic variability in Brassica for improvement of other relative important crops.

### Acknowledgments

None.

### Conflicts of interest

The authors declared there is no conflict of interest.

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