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DNA Fingerprinting and Genotyping of Four Black Seed (*Nigella sativa* L.) Taxa

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Abstract. DNA fingerprints of four Taxa of black seeds (*Nigella sativa* L.) from Qassim (Saudi Arabia), Ethiopia, Egypt, and Syria were studied. The results showed that there are several genetic differences between these different black seeds Taxa, which could be considered as genotypic characteristics and lead to classifying them as varieties under the *sativa* species. To study the DNA fingerprinting of these Taxa, the Inter Simple Sequence Repeat (ISSR) method was employed in the PCR technique to determine the levels of polymorphism between their genetic makeups. The ISSR-PCR investigated the inter-microsatellites sequences in their three types (Di, Tri, and Tetra) of the Short Tandem Repeats. Seventeen proper primers representing these three primers' types were used. The obtained banding pattern indicated a high level of polymorphism. The scored bands of the DNA fingerprints in these Taxa were 108, 106, 100 and 81 in Qassim, Ethiopia, Syria, and Egypt, respectively. When the percentages of dissimilarity between them were computed, the range was between 21.5-36.3%. Such a relatively high level of polymorphism substantiated the objectives of the present study which supposed that black seeds grown in the different localities in the world over time have undergone genetic changes to the level that could make them different varieties. Twenty four genes representing 24 different enzymes and isozymes were selected and scanned *via* PCR technique using suitable SSR primers. The obtained results, showed some changes in the genetic structure of some of these genes. The differences in the DNA fingerprints and the number of comparable genes should be reflected on the gene expression manifested in the protein homology and hence on the metabolism.

Introduction

Nigella sativa L. *(Ranunculaceae* family), commonly known as black seed has been employed for thousands of years as spice and food preservative, as well as a protective and curative remedy for numerous disorders. In Islamic culture, it is regarded as one of the greatest forms of healing medicine available. Prophet Muhammad once stated that the black seed can heal every disease except death. *N. sativa* L. has been used traditionally for centuries in the Middle East, Northern Africa and India for the treatment of various diseases (Worthen, 1998, Burits and Bucar, 2000, Al-Ghamdi, 2001, and Gilani *et al*., 2004). Research from around the globe is giving increasing support for black seed's widespread healing powers. Extracts of the black seeds have many therapeutic effects such as antidiabetes, antibacteria, and antitumor (Khan *et al*., 2003, Kanter *et al*., 2004, and Hussein *et al*., 2005).

Despite the voluminous research published about the great medicinal benefits of the black seed consumed around the world, there is no published research, to our knowledge, on the application of molecular markers to study the genetic structure of *Nigella sativa* L. Moreover, the questions of whether the black seeds grown in different localities are different varieties of one species or not, have not been addressed. Hence, this imposes a second important question: Are all the black seed grown around the world having the same genetic/metabolic components in quality and quantity for medicinal effects?

The objective of the present study was to evaluate the genetic makeup by using the genetic markers in PCR technique to characterize and compare DNA Fingerprints in four Black Seed (*Nigella sativa* L.) Taxa that are widely consumed for food and medicine.

Materials and Methods

DNA Extraction

The black seeds *Nigella sativa* L. were collected from Qassim (Saudi Arabia), Ethiopia, Egypt, and Syria. After complete germination of seeds, seedlings were shock-frozen in liquid nitrogen and stored at -20°C until DNA isolation was performed. Plant genomic DNA was extracted using the DNA Extraction Kit from Roche Company (Roche, Germany). This

method has given a large amount of DNA with high degree of purity. DNA purity and concentration were checked by measuring absorbency ratio OD_{260}/OD_{280}, using a Gene-Quant spectrophotometer (Amersham Company, USA). Then samples' DNAs were diluted to the working concentration of 100 ng/ µL (Al- Huqail, 2006).

Primers

Anchored and nonanchored (di, tri and tetra) oligonucleotide primers were used for amplifications with specific and optimal annealing temperatures (Table 1). All Primers were supplied by Amersham Pharmacia Biotech, Sweden.

ISSR Amplifications

Each reaction contained 1.5 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 0.4 mM of each deoxyribonucleotide phosphate dNTPs; 0.5 µM primer; 100 ng DNA template/reaction and 1.25 units of Taq DNA Polymerase in a final reaction volume of 25 µl. Many factors of PCR amplification influence pattern quality. $MgCl_2$ used at final concentration of 1.5 mM was generally found to generate bands of high intensity. Template DNA concentration was found to influence band intensity. Thus, from different concentrations tested, 100 ng DNA per reaction gave the best amplification products, while the Primer concentration of 0.5 µM resulted in a higher number of bands. Reactions without DNA were used as negative controls (Al-Huqail, 2006).

PCR Program

Amplifications were carried out in a thermal cycler (AMPLITRON II Thermolyne. USA). ISSR-PCR amplifications and analyses were as described by Nagaraju *et al*. (2002), but with some modifications. The apparatus was programmed to execute the following conditions: Initial denaturation step of 4 min at 95°C, followed by 35 cycles each of which composed of 30 s. at 95°C, 45 s. at the primer's specific annealing temperature at 42-64°C and 2 min for extension at 72°C. A final extension step of 10 min at 72°C was run at the end of the last PCR cycle. Different annealing temperatures were tried for each primer and the temperatures ranged from 40 to 65°C (Table 1).

Table 1. Anchored and nonanchored oligonucleotide primers.

Primer	Type	Sequence	Ta
Di:			
$(CA)_6AC$	Anchored	5-CACACACACACAAC-3	45
$(CA)_6GT$	Anchored	CACACACACACAGT	45
$(CA)_6AG$	Anchored	CACACACACACAAG	45
$(CA)_6GG$	Anchored	CACACACACACAGG	45
$(CA)_6GA$	Anchored	CACACACACACAGA	45
$(CA)_6CT$	Anchored	CACACACACACACT	45
$(CT)_8TG$	Anchored	CTCTCTCTCTCTCTCTTG	45
$(AG)_8CC$	Anchored	AGAGAGAGAGAGAGAGCC	42
$(AG)_8CA$	Anchored	AGAGAGAGAGAGAGAGCA	42
$(AG)_8CT$	Anchored	AGAGAGAGAGAGAGAGCT	42
Tri:			
$(CAA)_5$	Non anchored	CAACAACAACAACAA	54
$(CAG)_5$	Non anchored	CAGCAGCAGCAGCAG	64
$(AGT)_6$	Non anchored	AGTAGTAGTAGTAGTAGT	42
$(GTG)_3GC$	Anchored	GTGGTGGTGGC	45
Tetra:			
$(GACA)_4$	Non anchored	GACAGACAGACAGACA	52
$(GATA)_4$	Non anchored	GATAGATAGATAGATA	42
$(GATA)_2(GACA)_2$	Non anchored	GATAGATAGACAGACA	42

Electrophoresis of PCR Amplicons

The PCR amplification products (amplicons) were analyzed by electrophoresis using a 2.25% agarose gel in 1x Tris Acetic cid EDTA (TAE). DNA was stained by soaking the gel in a0.5 mg/mL ethidium bromide solution and run at 60 v for 3 h. Stained gel was visualized and photographed on UV Transilluminator (Al-Huqail, 2006).

Index of Similarity

Similarity coefficient (index of similarity) was calculated to compare the results of the genetic fingerprints for the determination of the percentage of similarities between the Taxa. The following formula was used:

$$B_{ab} = 2\ N_{ab} / (N_a + N_b)$$

Where N_{ab} is the number of bands that appear common in all types a, b. While each of the N_a, and N_b are the total number of bands that appear in both a and b, respectively (Lynch, 1990).

Genes Scan via PCR Technique

Twenty four genes representing 24 different enzymes and isozymes were selected and scanned via PCR technique using suitable SSR primers. These genes were selected from the list of *Arabidopsis thaliana*, grains, and legumes cited in the gene bank (NCBI.org). The genes codes and numbers were checked in the web site http://www.Entrez PubMed to find their base pair sequence to select proper lengths of bases for the SSR primers design in both directions (Forward and Reverse). The Melting-Temperatures of the selected SSR primers were computed in the appropriate program on the website: http://www.promega.com/biomath/default.htm. (Al-Huqail, 2006).

Results and Discussion

DNA Fingerprinting

Results clearly indicate that the four Black Seed (*Nigella sativa*) Taxa (Qassim, Ethiopia, Egypt and Syria) differ in their DNA structures, as their DNA fingerprints show a high degree of polymorphism. Hence, the results mean that these four Taxa of *Nigella sativa* do have different relative frequencies of microsatellite motifs of their respective DNAs, as can be seen from the scored banding patterns. Furthermore, these banding patterns represent the ISSR-PCR markers obtained by the use of the selected 17 ISSR primers. The figures presented in this paper (Fig. 1 & 2) show the amplified fingerprints of 8 primers (the remainder ISSR primers results can be seen in Al-Huqail, 2006).

The total number of bands and their total base pairs yielded by the 17 ISSR primers are presented in Table 2 .The Qassim's taxon yielded a total of 108 bands with total bands lengths of 63795 base pair. While, the total bands of the Ethiopia's taxon was 106 with a total of 59240 base pair. Moreover, the total number of bands in the Egypt's taxon was 81 bands totalling 47995 base pair, whereas the total number of bands in the Syria's taxon was 100 bands with 60820 base pair of total lengths.

The index of similarity between bands of DNA fingerprinting of black seeds *Nigella sativa* Taxa for Qassim and Ethiopia (Table 3) was 0.78, between Qassim and Egypt 0.62, between Qassim and Syria 0.69, between Ethiopia and Egypt 0.71, between Ethiopia and Syria 0.68, and between Egypt and Syria 0.72.

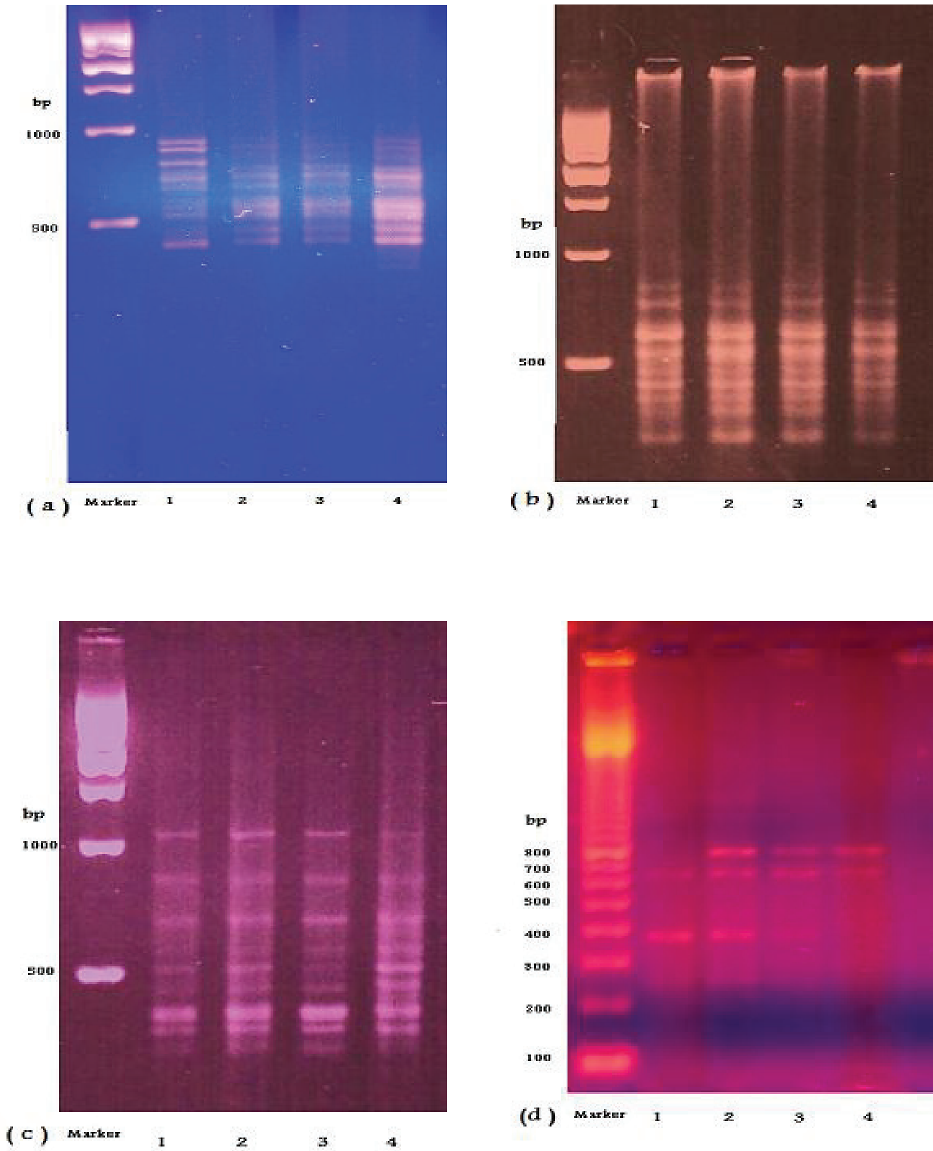

Fig. 1. Gel electrophoresis of ISSR bands obtained with primers: (a)- $(CA)_6GT$, (b) - $(CA)_6AC$, (c)- $(AG)_8$ CC, (d)- $(AGT)_6$. In the X-axis, numbers from (1-4) represent four black seeds (*Nigella sativa* L.) Taxa from Qassim, Ethiopia, Egypt, and Syria respectively.

On the other hand, when the percentages of dissimilarity between these Taxa were computed, they ranged between 21.5-36.3 %.

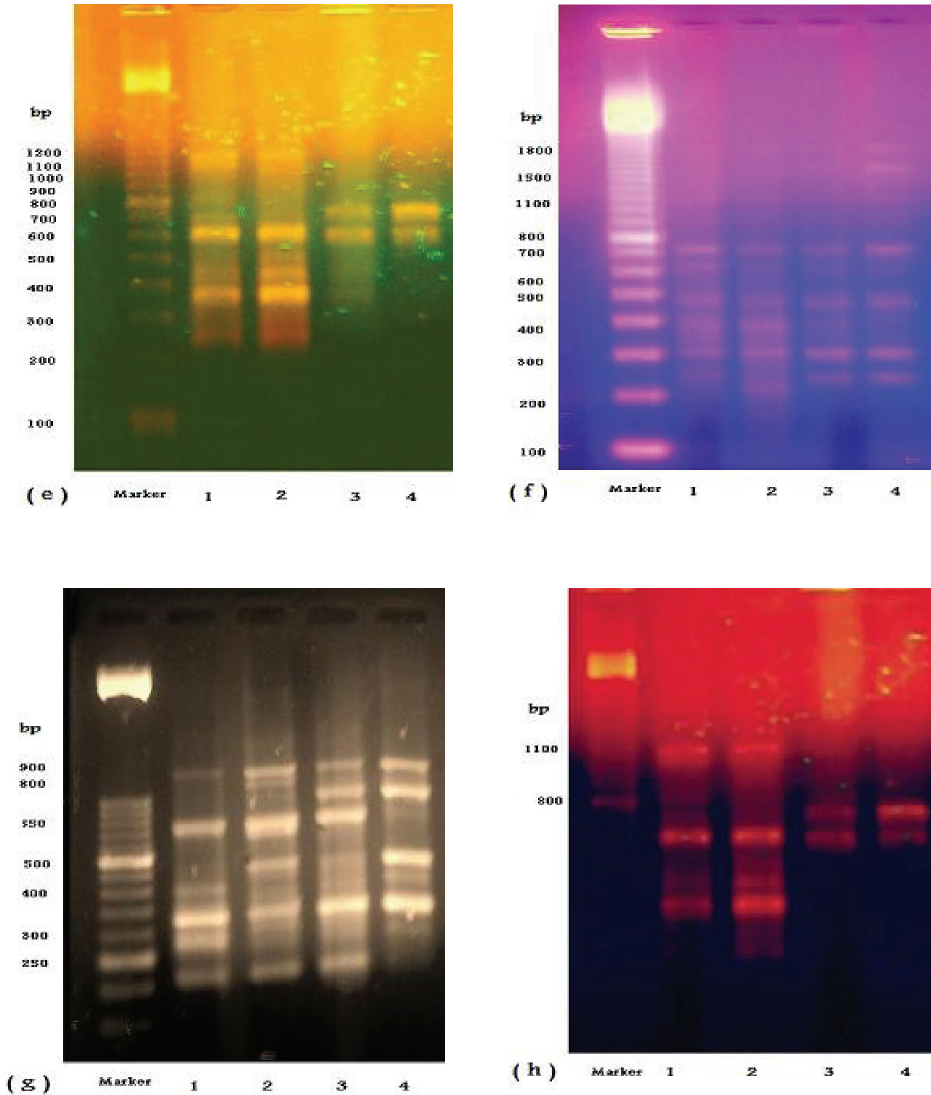

Fig. 2. Gel electrophoresis of ISSR bands obtained with primers : (e) - $(CAA)_5$, (f) -$(GTG)_3$ GC, (g)- $(CAG)_5$, (h)- $(GATA)_2(GACA)_2$.. In the X-axis, numbers from (1-4) represent four black seeds (*Nigella sativa* L.) Taxa from Qassim, Ethiopia, Egypt, and Syria, respectively.

Table 2. Total number of bands and total bands' base pair from DNA fingerprinting of black seeds (*Nigella sativa* L.) Taxa from Qassim, Ethiopia, Egypt, and Syria.

Primer	Qassim	Ethiopia	Egypt	Syria
$(CA)_6CT$	2	0	3	7
$(CA)_6AG$	11	6	2	6
$(CA)_6GT$	7	8	7	9
$(CA)_6GA$	8	6	4	8
$(CA)_6GG$	7	8	7	5
$(CA)_6AC$	8	9	9	7
$(CT)_8$ TG	6	7	7	6
$(AG)_8CA$	9	9	6	7
$(AG)_8CC$	9	9	9	9
$(AG)_8CT$	6	9	2	9
$(AGT)_6$	2	4	3	2
$(CAA)_5$	5	5	3	2
$(GTG)_3$ GC	8	6	4	7
$(CAG)_5$	6	5	5	5
$(GATA)_4$	4	4	4	5
$(GACA)_4$	4	4	4	4
$(GATA)_2(GACA)_2$	6	7	2	2
Total no. of bands	108	106	81	100
Total bands base pair	63795	59240	47995	60820

Such a relatively high level of polymorphism has substantiated the objectives of the present study which supposed that black seeds grown in different localities over time have undergone genetic changes to the level that could make them different varieties. The ISSR marker technique involves polymerase chain reaction (PCR) amplification of DNA using a single primer composed of a microsatellite sequence such as $(GACA)_4$, anchored at the 3' or 5' end by two to four arbitrary, often degenerate, nucleotides (Zietkiewicz *et al.* 1994).

Table 3. Index of similarity between bands of DNA fingerprinting of black seeds (*Nigella sativa* L.) Taxa from Qassim, Ethiopia, Egypt, and Syria.

Nigella sativa L. Taxa	Qassim	Ethiopia	Egypt	Syria
Qassim	-	0.78	0.62	0.69
Ethiopia	0.78	-	0.71	0.68
Egypt	0.62	0.71	-	0.72
Syria	0.69	0.68	0.72	-

The ISSRs have proven to be a reliable, rapid, simple, cost effective, easy to generate, and versatile set of markers that do not require previous knowledge of the genome sequence to generate DNA markers, unlike SSRs (Zietkiewicz *et al*., 1994, Gupta *et al*., 1994, Bornet and Branchard, 2001, & Bornet *et al*. 2002).

The flexibility to design primers containing a di-, tri-, or tetra-nucleotide repetitive motifs anchored by one or more nucleotides at the 3' or 5' end make them ideal to explore the genome of any species, including those without previous knowledge of DNA sequence (Zietkiewicz *et al*., 1994).

Furthermore, previous investigators have demonstrated that ISSR analysis usually detects a higher level of polymorphism than that detected with Restriction Fragment Length Polymorphism (RFLP) or Random Amplified Polymorphic DNA (RAPD) analyses (Kantety *et al.*, 1995 and Nagaoka and Ogihara, 1997). Thus, ISSR markers are useful in studies on genetic diversity, phylogeny, genetagging, genome mapping and evolutionary biology and are widely applied in plant genetic analyses (McClean *et al*., 2002, Reddy *et al*., 2002, Gonzalez *et al*., 2005, Hou *et al*., 2005, Manimekali *et al*., 2006, Martinez, 2006, Thomas *et al.*, 2006, and Li *et al*., 2006). ISSR has also been used in barley for detecting genetic diversity, genotype identification and genetic mapping (Matus and Hayes, 2002, and Hou *et al*., 2005). Furthermore, ISSRs have been used for cultivar identification in maize (Kantety *et al*., 1995), wheat (Nagaoka and Ogihara, 1997). In addition, ISSR-PCR markers are scored as dominant, highly reproducible and consistent because the anchors serve to fix the annealing of the primer to a single position of the target site, thus, resulting in a low level of slippage during amplification (Zietkiewicz *et al*., 1994). ISSR method has been considered an efficient molecular marker to reveal genetic relationships in traditional and evolved Basmati and semidwarf non-Basmati rice varieties (Nagaraju *et*

al., 2002). SSR and ISSR were compared and evaluated for the determination of the similarity degree between 41 commercial cultivars of apple (*Malus domestica* Borkh). The scored similarity coefficient between cultivars ranged from 0.20 to 0.87 for SSR analysis and from 0.71 to 0.92 for the ISSR methodology (Goulao and Oliveira, 2001).

Genotyping of Four Black Seed (Nigella sativa L.) Taxa

Results in Fig. 3 and 4 show some of the scanned twenty four genes representing 24 different enzymes and isozymes via PCR technique using the selected SSR primers (the results of the remainder scanned genes can be seen in Al-Huqail, 2006). The obtained results revealed some changes in the genetic structure of some of these genes. The Qassim taxon contained all 24 genes as indicated by the presence of their bands, while the Ethiopian and Egyptian Taxa gave 23 bands representing 23 genes. On the other hand, only 17 bands appeared in the Syrian taxon. These results indicate that there are several genotypic differences between these different Taxa of black seeds, which can be considered as genotypic characteristics. These genotyping results do add further evidence to the supposition put forth in this research that these four Taxa may be different varieties of the *Nigella sativa* species.

Although the traditional taxonomic classification of varieties has been based on analysis of the morphological traits, the development of molecular genetic markers has made it possible and more accurate to differentiate between Taxa even if they have the same morphological traits. The cited literature for genotyping is enormously voluminous and, thus, only some examples will be reported here. In previous studies, the genetic diversity of denitrifying bacteria was investigated by using two distinct PCR methods for the nitrite reductase genes to differentiate between the sampled Taxa (Braker *et al*., 1998). Moreover the polymorphic β -amylase gene loci in various barley varieties in Ukraine were studied by Stratula and Sivolap, (2007) and their results showed that the genotypes of the different barley varieties included different alleles of the β-amylase genes. Furthermore, in another study, alcohol dehydrogenase (*Adh*) genes were scanned in two distantly related legumes, and their sequences were used to examine the molecular evolutionary history of the basic nuclear gene (Fukuda *et al*., 2005). Hence, it can be concluded that genes can undergo evolutionary changes over time by generating different base pair sequences when compared

with the original base pair sequence present in the original gene. Such evolutionary changes will lead to different genetic makeup to the level that could classify the Taxa as different varieties of the pertaining species.

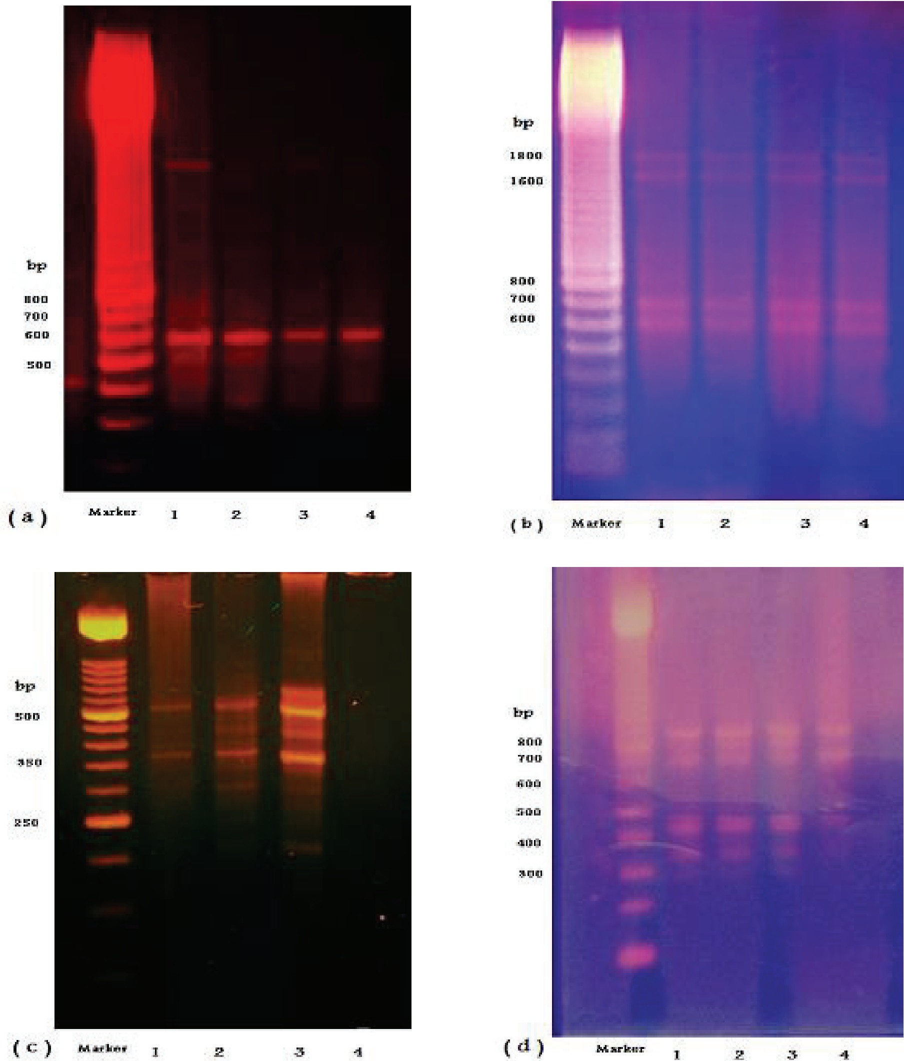

Fig. 3. Gel electrophoresis of genes scan using PCR technique with SSR primers (a) represent gene band of Alcohol dehydrogenase enzyme, (b)- Catalases, (c)- Glutamine synthetases, (d) Lipase. In the X-axis; numbers from (1-4) represent four black seeds (*Nigella sativa* L.) Taxa from Qassim, Ethiopia, Egypt, and Syria respectively.

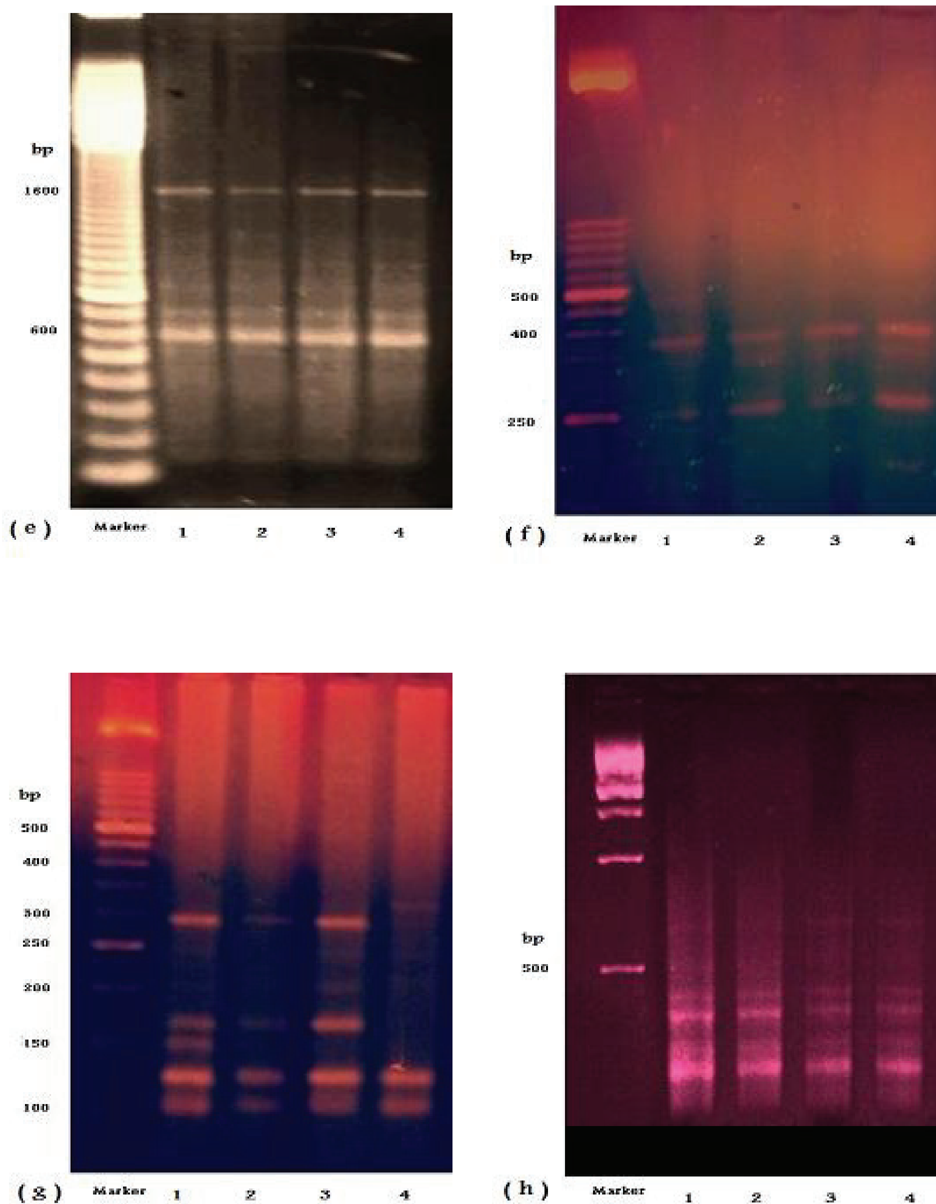

Fig. 4. Gel electrophoresis of genes scan using PCR technique with SSR primers (e) represent gene band of Amylases enzyme, (f) - Nitrate reductase and Nitrite reductase , (g)- GOT, (h) Cellulase and Kinases. In the X-axis; numbers from (1-4) represent four black seeds (*Nigella sativa* L.) Taxa from Qassim, Ethiopia, Egypt, and Syria respectively.

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البصمة الوراثية وفحص المورثات لأربع وحدات تصنيفية من الحبة السوداء *Nigella sativa* L.

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المستخلص. أجريت هذه الدراسة حيث تمت مقارنة بصمة الـدنا (DNA) في بذور وبادرات أربعة وحدات تصنيفية مختلفة من الحبة السوداء *Nigella sativa* L. من كل من القصيم (المملكة العربية السعودية)، وأثيوبيا، ومصر، وسوريا. ولقد بينت نتائج هذا البحث أن هناك فروقا وراثية بين هذه الوحدات التصنيفية من الحبة السوداء، ربما ترقى إلى تصنيفها بأصناف varieties. ولقد استخدم في هذا البحث طريقة الـ Inter Simple Sequence Repeat (ISSR) لتحديد البصمة الوراثية للوحدات التصنيفية من الحبة السوداء للكشف عن التغيرات في محيط الميكروستلايت Microsatellites الثنائية، والثلاثية، والرباعية الموجودة في الدنا الجينومي، باستخدام ١٧ من البادئات المناسبة. ولقد بينت نتائج بصمة الدنا وجود اختلافات في نمط الحزم في كل من العدد والأطوال لجميع الوحدات التصنيفية، فقد ظهر أكبر عدد لحزم بصمة الدنا في الوحدة التصنيفية من القصيم والتى تمثل ١٠٨حزمة، يليها الوحدة التصنيفية من أثيوبيا حيث بلغ عدد حزم بصمة الدنا ١٠٦ حزمة، بينما ظهرت في الوحدة التصنيفية من سوريا ١٠٠ حزمة، وقد ظهر أقل عدد لحزم بصمة الدنا في الوحدة التصنيفية من مصر

بـــ ٨١ حزمة فقط. وقد وجد أن نسبة الاختلاف بـــين الوحـــدات التصنيفية للحبة السوداء تتراوح بين ٢١,٥ – ٣٦,٣٪. وبمقارنـــة بعض المورثات في الوحدات التصنيفية الأربعة للحبة السوداء، تـــم اختيار ٢٤ مورثا ممثلة لـــ٢٤ إنزيماً متنوعاً، ومسحت باســـتخدام تقنية الـــPCR وبادئات الـــ SSR المناسبة. ودلت النتائج على وجود اختلافات وتغيرات في التراكيب الوراثية لبعض هـــذه المورثـــات، متمثلة في اختفاء بعض الحزم في نواتج الـــ PCR، حيث أن مواقع ارتباط البادئات في هذه المورثات قد تغيرت.