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Validation of DNA fingerprinting techniques in characterization of entomopathogenic nematodes

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Abstract
Three isolates of entomopathogenic nematodes (EPNs) *Steinernema feltiae, Heterorhabditis bacteriophora and Steinernema* sp. isolated from Egypt and Kingdom of Saudi Arabia have been, examined and evaluated for their molecular characterization. Genetic diversity evaluation among three EPNs genotypes were determined via RAPD and ISSR markers. The highest degree of polymorphism was selected from RAPD and ISSR primers. A total of 45 bands of polymorphic ISSR bands were revealed from 61 bands, with an average of 4.5 polymorphic fragments per primer. The number of amplified fragments with ISSR primers ranged from 2 to 11, with the size of amplicons ranging from 93 to 2043 bp. The polymorphism ranged from zero to 100.0, with an average of 73%. The three RAPD primers produced 12 bands across 3 genotypes, of which 10 were polymorphic, with an average of 3.3 polymorphic fragments per primer. The number of amplified bands varied from 3 to 5, the amplicons size ranging from 188 to 295 bp. The percentage of polymorphism using RAPD primers ranged from 33.3 to 100 with an average of 77.7%. The results of molecular characterization of isolated EPNs represent that *S. feltiae* and *S.* sp. are relatives more than *H. bacteriophora.*

Keywords: Entomopathogenic nematodes, fingerprinting, RAPD, ISSR

1. Introduction
Entomopathogenic nematodes (EPNs) considered being important parasites spread throughout the world [1]. EPNs could be effective biological control agents certainly which belongs to the family Steinernematidae and Heterorhabditidae [2]. The nematicidal effect of these nematodes is due to their bacterial symbionts which kill the host insects through septicaemia within 24-48 hours [3] and then exit from the cadaver en-masse within 4-9 days according to nematode species. Recently, great demand to the commercial usage of these (EPNs) as biocontrol tools. Various isolates have been found from everywhere except Antarctica [4]. The newly discovered isolates of (EPNs) have been increased over the last decade [5]. In Egypt, EPNs have been of great importance for use in biological control. Six Egyptian isolates of heterorhabditid nematodes were evaluated *in vitro* for control of the red palm weevil [6] as well as Egyptian and foreign entomopathogenic nematodes were used against the red palm weevil, *Rhynchophorus ferrugineus* in laboratory and field trials [7]. Electrophoretic analysis of DNA fragments can facilitate specific characterization of *Steinernema* and *Heterorhabditis* strains [8]. Genetic diversity among species of *Heterorhabditis* and *Steinernema* was explored via (RAPD) Random Amplified Polymorphic DNA [9]. ISSR markers have been used in many species for phylogenetic studies and fingerprinting, mapping and gene tagging [10]. In Egypt, although most researchers have investigated the nematicidal activities of entomopathogenic nematodes against various insect and nematode pests, no or little attention has been given to the molecular characterization of EPNs. Therefore, the present study was focused on the genetic diversity evaluation among three isolates of EPNs via RAPD and ISSR markers.

2. Materials and Methods
This study was carried out in the laboratory of Biology Department, Science Faculty, Taif University, Saudi Arabia during the period of 2016-2017.

2.1 Isolation and preparation of entomopathogenic nematodes
Entomopathogenic nematodes (EPNs) used in this study were isolated from rhizosphere of *Psidium guava* trees grown in Mansoura University farm in Egypt, and *Punica granatum* trees cultivated in Taif University gardens in Saudi Arabia using the *Galleria mellonella* baiting

method explained by [11]. The technique of white trap was used for culturing of EPNs [12]. Two isolates from Egypt and one from Saudi Arabia were morphologically identified as *Steinernema feltiae, Heterorhabditis bacteriophora* and *Steinernema* sp., respectively by examining morphometric for first-generation males and IJs [13].

2.2 Nematode molecular characterization

The isolates were molecularly characterized by ISSR and RAPD markers. DNA was extracted using lysis buffer [14]. The extraction buffer (1% SDS, 0.05 M EDTA, 400µl Proteinase K) were used to crush the IJs and, centrifuge for 10-15 minutes for 12,000 rpm. The supernatant was collected and equal volume of phenol: chloroform: isoamylalchol were added at the ratio 25:24:1. The tubes mixed carefully and centrifuge 12,000 rpm for 10 minutes. The upper layer was removed, then chloroform: isoamylalchol (24:1) was added, mixed and centrifuged 12,000 rpm for 10 minutes. The upper phase was separated and 3M ammonium was added for better DNA precipitation. The suspensions were frozen for 2 hours then, centrifuged for 30 minutes at 12,000 rpm. Aqueous phase was the precipitated DNA and, after dryness DNA was resuspended in TE buffer. The optical density of extracted DNA sample was measured at 260 nm according to the equation: Concentration (µg/ml) = OD260 x 50 x dilution factor.

2.3 Primer screening and ISSR- PCR optimization

A total of 10 primers based on repeats of dinucleotide were selected for generation of ISSR markers. [UBC -809: (AG) 8 G; UBC -810: (GA) 8 T; UBC -812: (GA) 8 A; UBC -826: (AC) 8 G; UBC -813: (CT) 8 T; UBC -821: (GT)8 A; UBC -828: (TG)8 C; UBC -846: (GA)8 A; UBC -864: (ATG) 6; UBC -880: (GGAGA)3]. The amplification reaction was performed in a 25-µl volume containing 1 X PCR buffer with 4mM $Mgcl_2$, 0.2 of mM dNTPs, 20 PM primer, 2 units of *Taq* DNA polymerase and template DNA 25 ng. As for the (PCR) polymerase chain reaction protocol were designed according to [15, 16] with few modifications. The preliminary step of the cyclic conditions of 4 min at 94 ºc for denaturation, and amplification was programmed to full 40 cycles of 4 min at 94 ºc, after an initial denaturation cycle. Annealing, 1 min at 40 ºc; extension at 72 ºc for 2 min and incubate 7 min at 72 ºc for final extension then stored at 4 ºc.

2.4 Primer screening and RAPD- PCR optimization

Five primers of RAPD were used in the investigation but three were successful only in generating amplicons, as follow: [(OPS-18): CTGGCGAACT; (OPO-08): CCTCCAGTGT; (OPC-15): GACGGATCAG]. PCR analysis was carried in 25-µl reaction as ISSR conditions and amplification was programmed also to complete 40 cycles after the first denaturation cycle for 4 min at 94 ºc. Each cycle considered as a denaturation step at 94 ºc for 1 min, the annealing step 1 min at 37 ºc and an extension step for 2 min at 72 ºc, followed by the final extension cycle at 27 ºc for 7 min.

2.5 Separation and detection of PCR products

Samples of PCR products of both ISSR and RAPD about 10-µl were separated and detected via agarose gel electrophoresis (1.4% in 1X TBE buffer) supported with ethidium bromide (0.3 µg/ml), running for 3.30 h at 80 V. Each agarose gel need DNA molecular size ladder (100 bp marker; RTU) and visually examined by UV transilluminator then photographed and documented using a CCD camera (UVP, UK). The controls and treated products of PCR were run in the same gel for more clarification in comparison between the results. All study reactions were carried twice to check the reproducibility of all obtained polymorphic bands laboratory.

2.6 Data analysis

The numerous bands of RAPD and ISSR were scored as absent (0) or present (1) each of which was treated as individualistic site despite of its intensity. Comparing the banding patterns of genotypes for a particular primer, genotype certain bands were identified. Pallid or unclear bands were not investigated. The binary data produced were used to evaluate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. The size of band was evaluated by comparing with 1 kb ladder (Invitrogen, USA) using Totallab, TL120 1D v2009 (nonlinear Dynamics Ltd, USA). The generated data by ISSR and RAPD primers were used to compile a binary matrix for cluster analysis (NTSYSpc Ver. 2.1).

3. Results

3.1 Polymorphisms detected by ISSR and RAPD markers

The studied genotypes of entomopathogenic nematodes appeared a high level of polymorphism between them (Table 1 and 2). The reproducible polymorphic products (Fig. 1 and 2) were successfully generated in presence of all primers. ISSR primers resulted in different numbers of DNA fragments, according to their simple sequence repeat motifs (Figure 1). The ISSR primers used for ISSR analysis detected a total of 61 fragments, with an average of 6.1 fragments per primer. The percentage of polymorphism ranged from 0% (UBC-880) to 100% (UBC-809), (UBC-810), (UBC-812), and (UBC-846) with an average of 73% of the 61 amplified bands, 45 were polymorphic with an average of 4.5 polymorphic bands per primer. Unique bands were identified out of the polymorphic ones and achieved a total of 27 bands. The number of bands resulted in each primer depended on the extent of variation in specific genotype and the primer sequence. The amplified fragments scored varied number from 2 (UBC -880) to 11 (UBC -813) and the applicant size varied from 93 bp (UBC -864) to 2043 bp (UBC -826). A total of 10 oligonucleotide primers were mainly used in RAPD-PCR fingerprinting of the three samples belonging to entomopathogenic nematodes isolates. The RAPD primers induced 12 bands across the three species; the number of polymorphic bands were 10 (Fig. 2). Out of the polymorphic bands a total of 3 unique bands were identified. The number of bands altered between three (OPO-08) to five (OPS-18) and the size of amplicon altered between 118 to 295 bP. Average number of bands and polymorphic bands concerning every primer were 4 and 3.3 respectively. The percentage of polymorphism ranged between 33.3% (OPO-08) and 100% (OPS-18, OPC-15).

Table 1: List of ISSR primers; the number of amplified products; the number of polymorphic and monomorphic bands and percentage of polymorphism obtained by analyzing entomopathogenic nematodes.

Primers	sequence (5´-3´) Primer	MW (bp) range	Total no. of bands	Monomorphic bands	Polymorphic bands	Unique bands	% Polymorphism
UBC -809	(AG) 8 G	165-1691	3	0	3	3	100%
UBC -810	(GA) 8 T	165-273	4	0	4	1	100%
UBC -812	(GA) 8 A	200-273	3	0	3	0	100%
UBC -826	(AC) 8 G	180-2043	8	3	5	2	62.5%
UBC -813	(CT) 8 T	213-1262	11	2	9	6	81.8%
UBC -821	(GT) 8 A	178-1130	10	2	8	5	80%
UBC -828	(TG) 8 C	143-1295	7	5	2	2	28.5%
UBC -846	(GA) 8 A	182-271	4	0	4	3	100%
UBC -864	(ATG) 6	93-682	9	2	7	5	77.7%
UBC -880	(GGAGA) 3	207-234	2	2	0	0	0%

Table 2: List of RAPD primers; the number of amplified products; the number of polymorphic and monomorphic bands and percentage of polymorphism obtained by analyzing entomopathogenic nematodes.

Primers	sequence (5´-3´) Primer	MW (bp) range	Total no. of bands	Monomorphic bands	Polymorphic bands	Unique bands	% Polymorphism
OPS-18	CTGGCGAACT	118-276	5	0	5	1	100%
OPO-08	CCTCCAGTGT	188- 227	3	2	1	1	33.3%
OPC-15	GACGGATCAG	188-295	4	0	4	1	100%

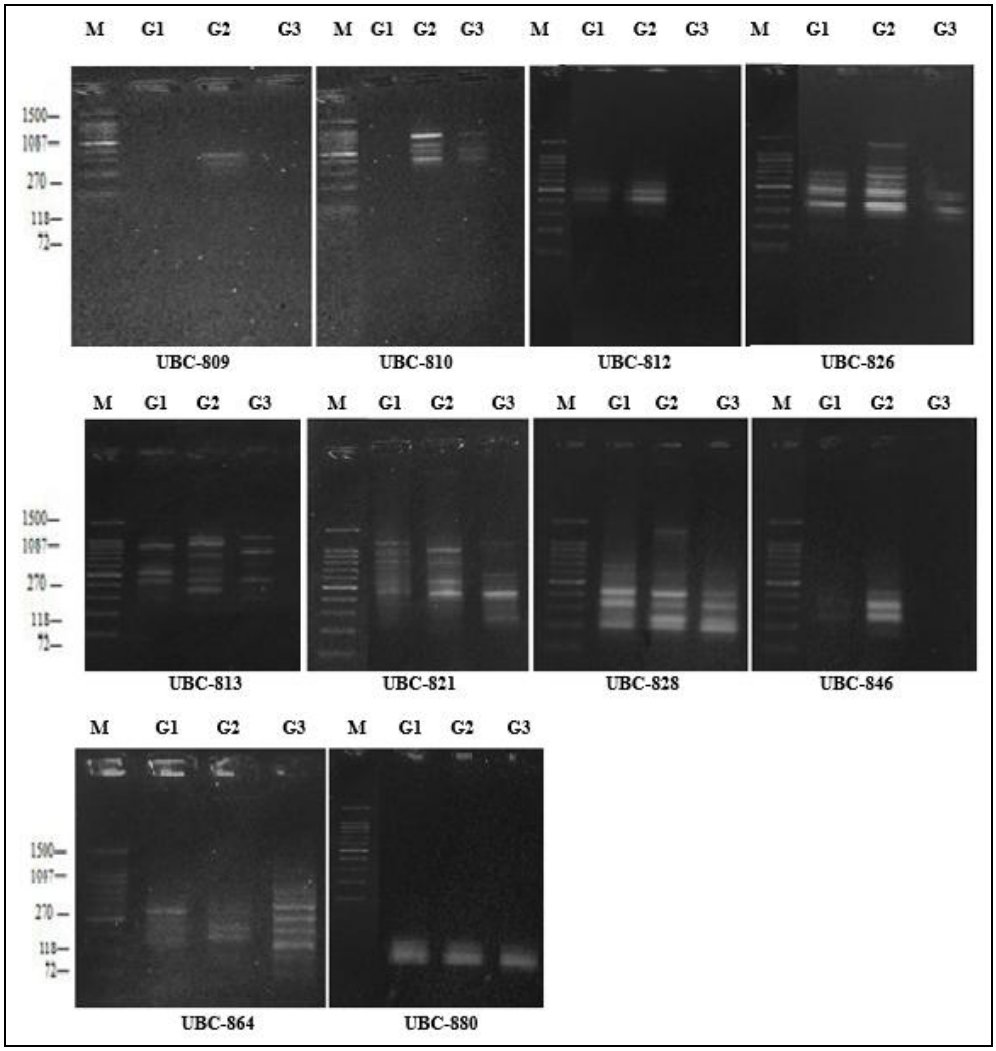

Fig 1: ISSR-PCR profile of DNA samples from different genotypes of entomopathogenic nematodes. M=DNA Marker; G1= *Steinernema feltiae*; G2= *Steinernema* sp.; G3= *Heterorhabditis bacteriophora*

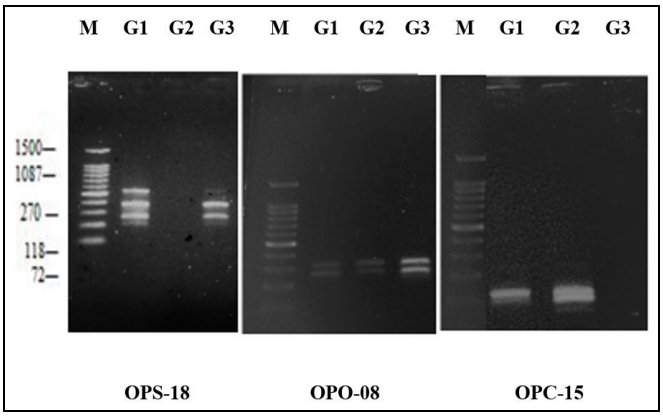

Fig 2: RAPD-PCR profile of DNA samples from different isolates of entomopathogenic nematodes. M=DNA Marker; G1= *Steinernema feltiae*; G2= *Steinernema* sp.; G3= *Heterorhabditis bacteriophora*

3.2 Comparison of RAPD and ISSR markers

It is a matter of interest to mention that different markers have various properties and will clear different sides of genetic diversity. The ISSR and RAPD surveys among the three isolates of entomopathogenic nematodes revealed 73 and 77.8% of polymorphic bands, respectively (Table 3). ISSR–PCR data presented that ISSR primers generated 9 common amplicons between *S. feltiae* and *S.* sp. Whereas 7 common bands were generated between *S.* sp. and *H. bacteriophora*. On the other hand, the less number of common bands (2) was exhibited between *S. feltiae* and *H. bacteriophora* (Table 4). Regarding RAPD- PCR analysis, it is obvious that no common bands were generated between *S.* sp. and *H. bacteriophora.* Meanwhile, 9 common bands were revealed between *S. feltiae* and *S.* sp. as well as 4 common bands was existed between *S. feltiae* and *H. bacteriophora* (Table 5).

Table 3: Comparison of DNA marker systems in entomopathogenic nematodes.

Marker system	Number of primer	Polymorphism (%)	Average number of bands /primer	Average number of polymorphic bands /primer
ISSR	10	73.05	6.1	4.5
RAPD	3	77.76	4	3.3
ISSR+ RAPD	13	74.13	5.6	4.2

Table 4: Number and molecular size of the common amplicons revealed by ISSR-PCR reaction among the studied nematodes.

Primers	Amplicons size (pb)	*S. feltiae*	*S.* sp.	*H. bacteriophora*
UBC-810	273		+	+
	214		+	+
	165		+	+
UBC-812	273		+	+
	219		+	+
	200		+	+
UBC-826	1306	+	+	
	674	+	+	
	337	+	+	
	281	+	+	+
	276	+	+	+
	268	+	+	+
UBC-813	1121	+	+	+
	936	+	+	
	284	+		+
	272	+	+	
	239	+	+	+
UBC-821	919	+	+	
	516	+	+	
	286	+	+	+
	279	+	+	+
	252		+	+
UBC-828	278	+	+	+
	258	+	+	+
	218	+	+	+
	167	+	+	+
	143	+	+	+
UBC-846	254	+	+	
UBC-864	280	+	+	+
	272	+		+
	238	+	+	+
	203	+	+	
UIBC-880	234	+	+	+
	207	+	+	+

Table 5: Number and molecular size of the common amplicons revealed by RAPD- PCR reaction among the studied nematodes.

Primers	Amplicons size (pb)	*S. feltiae*	*S.* sp.	*H. bacteriophora*
OPS-18	276	+		+
	229	+		+
	177	+		+
	118	+		+
OPO-08	227	+	+	+
	215	+	+	+
OPC-15	295	+	+	
	272	+	+	
	259	+	+	

4. Discussion

Accurate identification is important to understand geographical distribution and habitat specificity of any organism. Many EPN identifications are based on traditional morphological methods, but recently some researchers have used molecular techniques for the identification of EPNs. The use of molecular methods can greatly reduce the amount of time needed for identification of unknown nematode isolates [17]. There are many molecular analyses have been used to assess evolutionary relationships among *Steinernema* spp. and *Heterorhabditis* spp. [18-20]. ISSR and RAPD markers have advantages and disadvantages for evaluating genetic diversity [21]. Concerning the purpose of characterization the three isolates of entomopathogenic nematodes indicated high significant difference in their behavior, infectivity host range, mating and environmental tolerance. These variations have stimulated researchers to completely characterize their genetic diversity, perhaps new genotypes prove to be more profitable than the present species, which are recently used as biological control tool against important pests [22, 23]. True identification of these EPNs is importance for the success of integrated pest management (IPM) and biological control programmers. Recently, the description in the genus *Steinernema* has remarkable increase, because of the value of this group as biocontrol agents and due to the utilization of molecular techniques in species characterization carefully [24]. Genetic diversity among species of *Heterorhabditis* and *Steinernema*

was explored via (RAPD) Random Amplified Polymorphic DNA [9]. RAPD technique has been widely used by researchers in molecular taxonomy owing to the increment of generated molecular data, as well as necessity to previous genome information with the performed experiments. RAPD analyses were very sensitive to the quality of DNA template and parameters of PCR reaction. On the other hand, RAPD technique considered as a weak choice for use in systematics because of the failure in replication the results [25]. In the present study, RAPD analysis was successfully performed by using sets of random primers of variable lengths (93-2043bp) as recorded by [26] who studied genetic diversity in wild and laboratory populations of *H bacteriophora*. Furthermore, the feasibility of RAPD markers for the assessment of genetic variability among entomopathogenic nematode species and isolates has been demonstrated [27]. The molecular markers used here, i.e. ISSR and RAPD, evaluated the polymorphism among EPNs. These results agreed with those recorded by [10] who mentioned that ISSR markers have been used in many species for phylogenetic studies and fingerprinting, mapping, and gene tagging. *Heterorhabditis* have been found to be far more species than *Steinernema*. Due to the first generation in *Heterorhabditis* is hermaphrodite, except in *Steinernema*, with the remarkable exception of *S. hermaphroditum* it is amphimictic, thereby increasing the *Steinernema* potential for genetic diversity [28]. It is important to mention that, it was the first time to use ISSR DNA markers to estimate genetic variation of EPNs. Results confirm the performance of ISSR marker system to vent variation in the abundant microsatellite regions scattered, throughout the EPNs genome. This possibility of ISSR amplification to detect considerable numbers of polymorphic fragments was reported also by other authors [29, 30]. Actually, in spite of being ISSR is a qualitative method can only estimate the amount and nature of DNA effects; this technique was capable of detection of genetic variability in EPNs. Our genetic variation detected via ISSR markers was on bar and confirmed by [31] throughout their study about the morphometric measurements of *Heterorhabditis* sp. and *Steinernema* spp. They concluded that the length of IJs in *Heterorhabditis* sp. was less than 600µm, the IJs and male coincide well, tail length 101µm and head to excretory pore 98µm, whereas *Steinernema* spp. based on length of their IJs was 486µm, tail length 40µm, and head to excretory pore 35µm. The third stage of juveniles appears slender; esophagus is narrow, and long.

5. Conclusion

It can be concluded from this investigation that isolation and molecular identification of entomopathogenic nematodes are necessary demand for successful programs in biological control and classification. ISSR and RAPD analyses could be used to assess evolutionary relationships among *Steinernema* and *Heterorhabditis* species. This is the first documented record of *S. feltiae* and *H. bacteriophora* in Mansoura region, Egypt as well as *S.* sp. in Taif region, Saudi Arabia. The presence of an *S. feltiae* isolate in a temperate habitat as Egypt like that of Saudi Arabia as a desert habitat is a significant contribution to the biogeography of the species, as it confirms that it is able to adapt to a hotter and drier climate. This could lead to research that may enable this EPN to be used in the biological control of insect pests of temperate and desert regions.

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