International Journal of Entomology Research
ISSN: 2455-4758; Impact Factor: RJIF 5.24
Received: 01-02-2019; Accepted: 06-03-2019
www.entomologyjournals.com
Volume 4; Issue 3; May 2019; Page No. 10-15

Molecular diversity in *Earias insulana* populations from different Egyptian governorates

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Abstract
The present study was conducted to assess the molecular diversity among four populations of spiny bollworm from different insecticides treated Egyptian governorates (Gharbia, Kafr ELShaikh, Daqahlia and Behira) and a population from untreated field cotton. The protein profile displayed deviation in the resulted bands among the studied samples. The distribution of peroxidase and polyphenol oxidase isoenzymes and the intensity of peroxidase presented no difference between the tested samples when compared to control population and each other. While, there was a variation in the intensity of the detected polyphenol oxidase isoenzyme. Data of RAPD-PCR presented a variation in DNA bands of the studied populations. As well as, the similarity index revealed that the control population recorded low similarity index with the other studied populations. Furthermore, it the studied population could be clustered into 2 groups Daqahlia and Behira (100% similar) and Gharbia and Kafr El Shaikh (92%). Therefore, it could be concluded that *Earias insulana* larvae were adapted to the environment changes and the use of insecticides.

Keywords: *Earias insulana*, spiny bollworm, protein electrophoresis, isoenzymes, RAPD-PCR

1. Introduction
Cotton is the most important industrial crop worldwide. The cotton varieties differ significantly among themselves with regards to the infestation of insect pests [1]. *Earias insulana* (Boisd.), the spiny bollworm (SBW) is one of the most destructive pest of cotton, maize and okra pests in Egypt and worldwide [2]. In cotton cultivates, the larva feed on fruiting parts of the plant and may destroy 1 cotton boll in 1-2 plant [3]. The *Earias* spp. are a devastating pest that reduces about 50% of cotton qualitatively by staining lint and about 40 % of the yield [4]. Many insecticides belonging to different classes, containing pyrethroid, organophosphate and novel chemical are used to manage *Earias* spp. infestations [5, 6].
Many authors; Syed *et al.* [7]; Shan *et al.* [8] and El-Sayed [9] studied the distribution and development of *Earias* spp and they proved that it controlled by many abiotic factors, (temperature, place, humidity, cotton varieties as well as other natural factors). Furthermore, Gillott [10] announced that the adaptation to the different factors may occur, as genetically different populations capable of surviving within a different temperature range.
Numerous molecular markers e.g.: isoenzymes, Random Amplified Polymorphic DNA (RAPD-PCR), have been widely used to reveal intra and interspecific genetic differences, phylogenetic studies and species classification [11, 12]. Furthermore, expansion of molecular markers deliveries excellent information on the genetic diversity of the insect and helps to classify pesticide resistant populations in order to develop appropriate control strategies [13].
Therefore in the current study, molecular variations have been evaluated in 4 different SBW populations from different governorates against laboratory reared population through the alterations in protein profile, some isoenzyme (peroxidase (Px) and polyphenol Oxidase (PPO)) and RAPD-PCR markers.

2. Material and Methods
2.1 Insect collecting
At the end of the cotton season (first of September) 2017, four larval populations of *Earias insulana* (Boisd.) were collected from different insecticides treated governorates (Gharbia, Kafr ELShaikh, Daqahlia and Behira). The control sample was population from untreated field cotton.

2.2 Characterization of protein by polyacrylamide gel electrophoresis in total body tissue homogenate of *E. insulana*
Extraction of total body tissue proteins: Protein extraction was conducted by homogenizing 0.2 g of insect tissue with 1 M Tris-HCl buffer, pH 6.8 in clean eppendorf tube and left in refrigerator overnight, then centrifuged at 10.000 rpm for 10 min. The supernatant of each sample (contains protein extract) was kept in deep-freeze until use for electrophoretic analysis. Then boil for 5 minutes in water bath before loading in the gel.
Preparation of (SDS-PAGE) gel: Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate was performed as described by Smith [14] using an acrylamide gradient (12%) gel. A volume of 80 µl of the protein extract was loaded on the gels. Control wells were loaded with standard protein marker Medium range from 14.20 KDa to 66.00 KDa (Fermentas.Com).

2.3 Isozymes electrophoresis
For isozymes extraction; 0.5 g larvae were homogenized in 1 ml extraction buffer (10% glycrol). The extract was then transferred into clean eppendorf tubes and centrifuged at 10000 rpm for 5 minutes. The supernatant was transferred to new clean eppendorf tubes and kept at –20 ºC until use for electrophoretic analysis.
Native-polyacrylamide gel electrophoresis (Native-PAGE)

was conducted to identify isozyme variations among studied larvae using 2 isozyme systems according to Stegemann *et al.* [15]. The utilized isozymes were Peroxidase (Px) and Polyphenol Oxidase (PPO)**.**

Isozymes and protein gels were photographed, scanned and analyzed using Gel Doc VILBER LOURMAT system

2.4 DNA Extraction and RAPD-PCR

Larvae were ground with liquid nitrogen and the DNA was extracted using DNeasy mini spin columns as described by the manufacturer (Qiagen, Hilden, Germany) stored at -80ºC. RAPD-PCR reactions were conducted according to the method of Williams *et al.* [16] to determine the phylogenetic relationship between different samples. Six arbitrary primers (Table 1) DNA marker with high range molecular ruler (100 – 10000bp) were used for the PCR analysis.

The electrophoresis gel images were recorded using a gel Documentation system (UVP, UK) and analyzed using CLIQS 1D Pro (Totallab) to reveal the molecular sizes (bp) of the bands. The relationships among genotypes as revealed by dendrograms were done using SPSS windows (Version 10) program. DICE computer package was used to calculate the pairwise difference matrix and plot the phenogram among cultivars [17].

Table 1: The nucleotide sequences of the primers used for RAPD-PCR analysis:

Primer name	Sequence (5`-3`)
OP-A1	CAGGCCCTTC
OP-B7	GAAAGGGGTG
OP-B11	GTAGACCCGT
OP-C4	CCGCATCTAC
OP-C9	CTCACCGTCC
OP-D1	ACCGCGAAGG

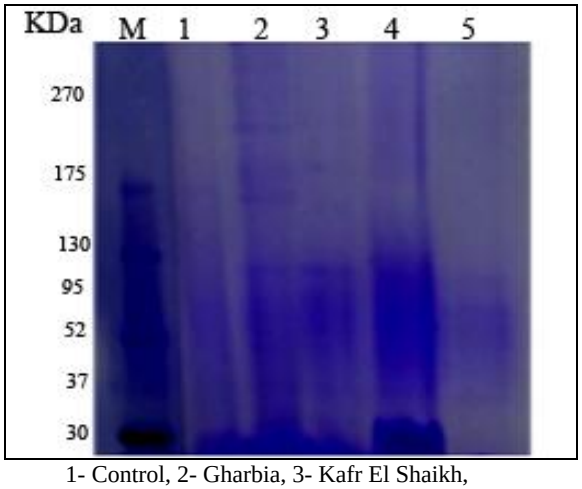
3. Results

Table (2) and Figure (1) presented the electrophoretic protein profile of total body tissue of *E. insulana* populations from the results indicated that 9 protein bands with molecular weights ranging from 34 - 225 KDa were electrophoretically separated. Protein bands of 68, 72, 85 and 225 KDa were detected as dominant bands in all populations. Data revealed increase in protein bands in populations from Gharbia, Kafr El Shaikh and Daqahlia when compared to the control population. Contrarily, Behira population presented less protein patterns than the other populations and the control.

Table 2: Molecular weights of protein fractions of total body tissue of *E. insulana* populations

Band No	M.W KDa	(1) Control	(2) Gharbia	(3) Kafr El Shaikh	(4) Daqahlia	(5) Behira
1	225	+	+	+	+	+
2	193	+	+	+	+	-
3	162	+	+	+	-	-
4	115	-	+	+	+	+
5	85	+	+	+	+	+
6	72	+	+	+	+	+
7	68	+	+	+	+	+
8	43	+	+	+	+	-
9	34	-	+	+	+	+
Total		7	9	9	8	6

+ present and – absent.



1- Control, 2- Gharbia, 3- Kafr El Shaikh,
4- Daqahlia, 5- Behira.

Fig 1: Electrophoretic pattern of proteins of total body tissue of *E. insulana* populations.

Fig. (2a) and table (3) showed the peroxidase (Px) zymograms of *E. insulana* in larval stage and patterns, while Table (3) represented an analysis for the distribution of the detected bands according to their mobilities and densities. The analysis reflected 3 distinguishable banding groups (Px1, Px2 and Px3). The 3 bands of peroxidases were detected in all studied populations, but with variable densities ranging from low to high according to its governorate. The higher staining intensity of the bands indicated that peroxidase activity was higher in those samples. All populations were found to be typically similar.

Table 3: Distribution of Peroxidase isozyme groups of *E. insulana* populations according to their relative mobilities and densities

Peroxidase groups	Relative Mobility	(1) Control	(2) Gharbia	(3) Kafr El Shaikh	(4) Daqahlia	(5) Behira
Px 1	0.4	1^{-}	1^{-}	1^{++}	1^{+}	1^{+}
Px2	0.5	1^{-}	1^{-}	1^{++}	1^{+}	1^{+}
Px3	0.6	1^{-}	1^{-}	1^{++}	1^{+}	1^{+}

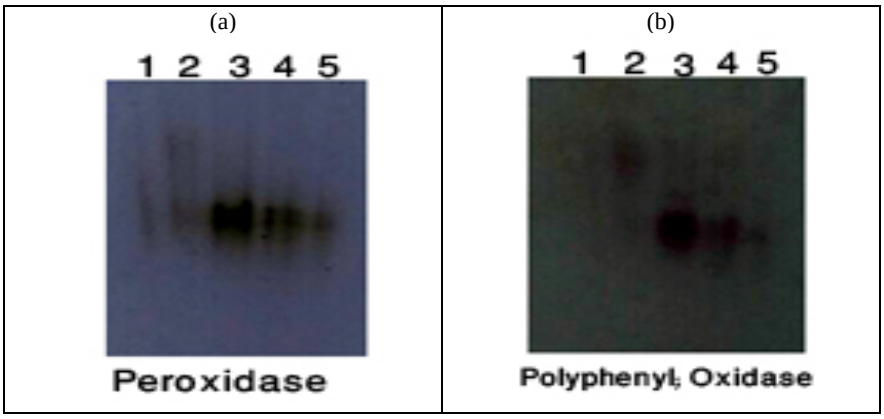
++ High density Band +Moderate density Band - Low density Band.

Polyphenol oxidase (PPO) isozyme patterns were presented in Fig. (2b). Table (4) presented that 4 PPO isozymes, PPO1, PPO2, PPO3 and PPO4 were detected and their relative mobility values were 0.2, 0.4, 0.5 and 0.6, respectively. The thickness and the staining degree of bands varied in all samples. Staining intensity of the bands in some samples indicated that PPO activity was higher in those samples. The 4 PPO isozymes were detected in the studied population but with variable densities according their region.

Table 4: Distribution of polyphenol oxidase isozyme groups of *E. insulana* populations according to their relative mobilities and densities

Polyphenol Oxidase Groups	Relative Mobility	Control	Gharbia	Kafr El Shaikh	Daqahlia	Behira
PPO 1	0.2	1^{-}	1^{+}	1^{+}	1^{-}	1^{-}
PPO2	0.4	1^{-}	1^{-}	1^{++}	1^{++}	1^{-}
PPO3	0.5	1^{-}	1^{-}	1^{++}	1^{++}	1^{+}
PPO4	0.6	1^{-}	1^{-}	1^{++}	1^{+}	1^{-}

++ High density Band, +Moderate density Band and - Low density Band.



Legend as Fig. (1).

Fig 2: Zymogram analysis of whole body tissue of *E. insulana* larvae

The RAPD analysis of the extracted DNA samples using OP-A1 primer showed occurrence of new band in *E. insulana* collected from Kafr El Shaikh with a M.W. of 1180 bp (Fig. 3a). In addition, OP-A1 showed 5 monomorphic bands among the studied samples with M.W. 1320, 860, 620, 500 and 370 bp (Table 5).

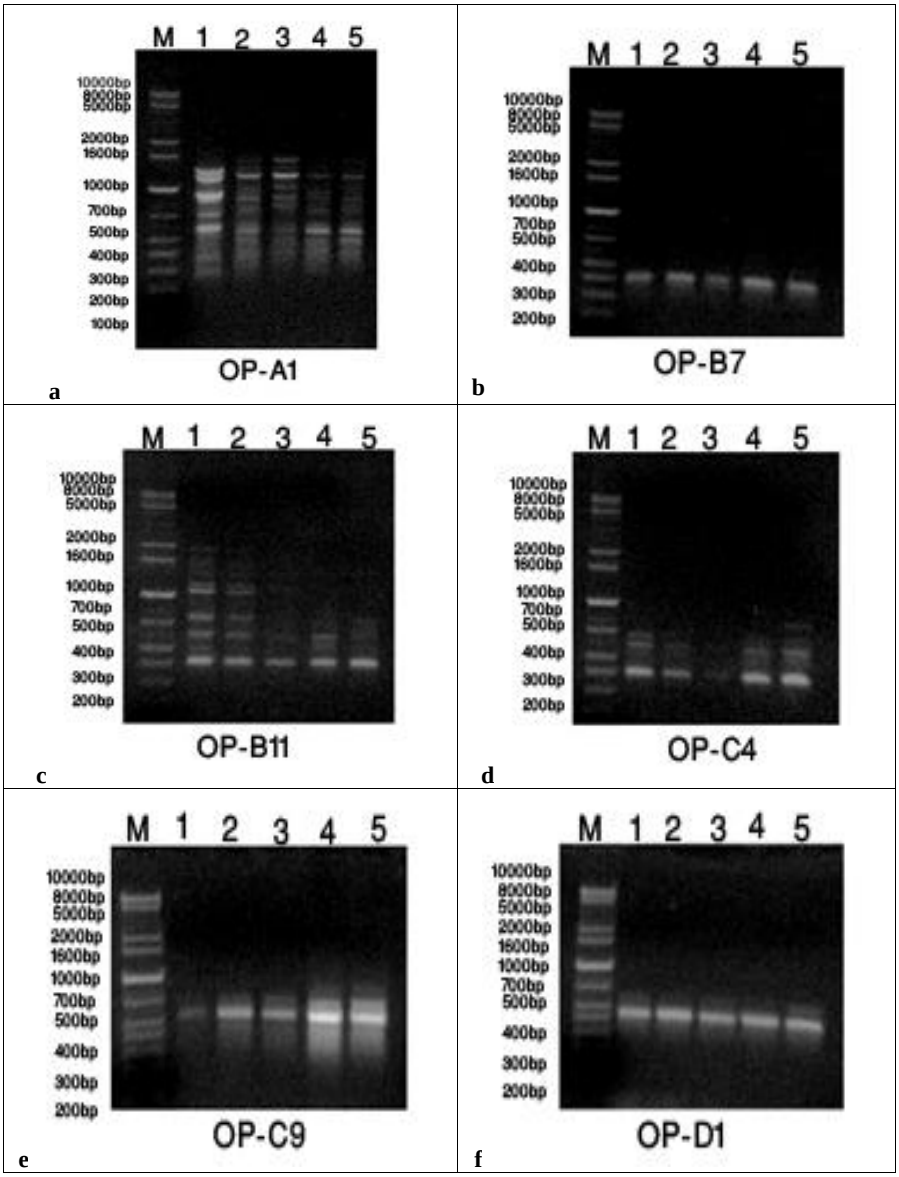
The created RAPD fragments in (Fig. 3b) revealed that the studied populations were identical when using OP-B7 primer; that showed 2 bands at M.W. 340 and 300bp.

Fig. (3c) manifested 2 common DNA bands when using OP-B11 with M.W. 470 and 340 bp. As well, disappearance of many bands when comparing with the laboratory population was obtained. Table (5) represented that OP- B11 primer had a polymorphism percentage of 77.77.

DNA fragments after using OP-C4 (Fig. 3d) revealed the existence of new band in Behira population with a M.W. 700bp. Also, they showed a common 2 bands. Data in table (5) proved that OP-C4 achieved 50% polymorphism.

Table 5: Polymorphism percentage of *E. insulana* populations using different random primers

Primer Name	Total Band	Polymorphic Band	Unique Band	% Polymorphism
A1	11	6	1	54.54%
B7	2	-	-	-
B11	9	7	-	77.77 %
C4	4	1	1	50 %
C9	3	2	0	66.6%
D1	2	1	-	50 %
Total	31	18	3	58 %



Legend as Fig. (1).

Fig 3: RAPD-PCR pattern resulting from amplification of genomic DNA of *E. insulana*

Using of OP-C9 primer presented a one band with a M.W. of 700bp in the populations collected from the studied governorates when comparing with the laboratory population (Fig. 3e). Furthermore, OP-C9 showed one common band with a M.W. of 600bp. Table (5) specified a 66.6% polymorphism when using OP-C9 primer.

The produced RAPD fragments in Fig. (3f) when using OP-D1 primer revealed the presence of one common band with M.W. of 460bp between the studied populations, with 50% polymorphism.

The data of similarity percentage in Table (6) and Fig. (4) demonstrated that populations from Daqahlia and Behira were similar to each other. Where, populations from Gharbia and Kafr El Shaikh were 92% similar. Moreover, the control population recorded low similarity index with the other studied populations. In addition, there was no similarity between Kafr El Shaikh and Behira.

Table 6: Similarity index among *E. insulana* populations (Dice method):

	Control	Gharbia	Kafr El Shaikh	Daqahlia	Behira
Laboratory	1.000				
Gharbia	.521	1.000			
Kafr El Shaikh	.230	.920	1.000		
Daqahlia	.416	.356	.013	1.000	
Behira	.614	.074	.000	1.000	1.000

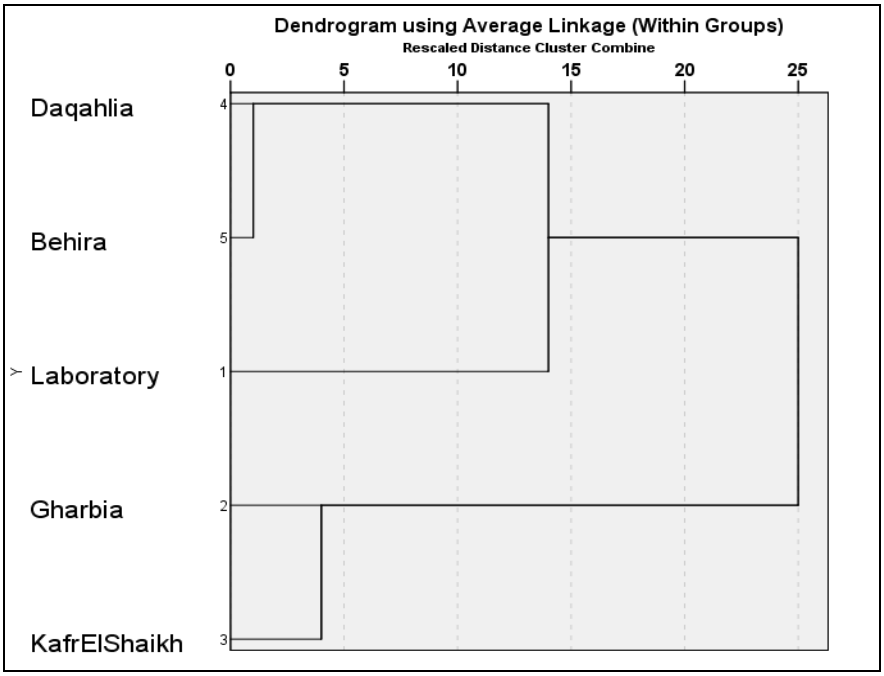


Fig 4: Dendrogram showing Phylogenetic relationship between *E. insulana* studied populations

4. Discussion

In the present study, 4 *E. insulana* populations collected from various regions in Egypt (Gharbia, Kafr ELShaikh, Daqahlia and Behira) and a laboratory reared population were evaluated for their molecular diversity. Nowadays, Molecular markers have become very useful for identifying variations between indistinguishable insect populations and to and to estimate the closely related populations based on the geographical region and different biotypes [18].

The obtained results of protein fraction reported that SBW from Gharbia, and Kafr ELShaikh had the same protein profile, while populations from Daqahlia and Behira were different than them and each other. However the variation in the protein profile of the studied population, they were similar in the distribution and intensity of the peroxidase and polyphenol oxidase isoenzyme.

DNA markers are specifically efficient tools in making deduction about movement between insect populations, because they existent selectively neutral individuality [19].

Moreover, the obtained data revealed that there was a variation between the studied populations. This was in harmony with Lopes *et al.* [20] on *Helicoverpa armigera* and *H. zea* and Kumar *et al*. [21] on *Plutella xylostella*. RAPD analysis provided evidence for grouping the studied populations as Daqahlia and Behira (100% similar) and Gharbia and Kafr El Shaikh (92% similar). This categorization was the same to that obtained from protein profile.

The molecular variability among the populations of *E. insulana* could be regarded to varied ecology and geography of the collection sites. This finding was previously proved by many authors. Where Syed *et al.* [7] declared that host plant and temperature had an effect of on the biology of *Earias vittella*. In addition, Shan *et al.* [8] and El-Sayed [9] discovered that, temperature played dynamic role in the development and growth rate of *E. insulana.* Also, Moustafa *et al.* [22] reported that the temperature and the cotton variety had highly significant effect on the biological aspects of *E. insulana.*

Otherwise, the molecular variation in the studied populations might result from the use of the insecticides in controlling them and the inherited effect. Whereas the sublethal effect of the insecticides contact could lead to physiological and molecular changes in the organism [23].

5. Conclusion

From aforementioned results, it could be concluded that all insect specimens collected from Egypt were geographically adapted. And the most resemblance populations in the study were Daqahlia to Behira (100% similar) and Gharpia to Kafr ElShaikh (92%).

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