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Genetic profile characterization of *Earias insulana* (Lepidoptera:Noctuidae) in Egypt

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Abstract:

Genetic profile of *Earias insulana* (Boisd.) (Lepidoptera:Noctuidae) larvae was identified by two genetic techniques (SCoT and ISSR) using PCR. Seven SCoT primers (SCoT1, SCoT2, SCoT4, SCoT7, SCoT11, SCoT12 and SCoT15) resulted in twelve different bands with molecular weight between 975 and 165bp, while, five ISSR primers (49A, 49B, HB-10, HB-11 and HB-12) resulted in fifteen different bands with molecular weight between 1630 and 175 bp. The genome profile characterization using SCoT technique for *E. insulana* was studies for the first time in the present work. *E. insulana* field strains were collected from five okra fields in different Governorates ; Behera, Alexandria, Menofia, Qaluobya and Sharkeya during season 2019 compared to the reared laboratory strain to investigate genetic study. Results of isozymes showed differences among larvae in five Governorates , polyphenyl oxidase was different in all tested larvae. Whereas, two bands were appeared in larvae from Alexandria governorate of peroxidase, while, larvae from Qaluobya and Sharkeya Governorates have only light different band as control (Laboratory).

Introduction

The spiny bollworm (SBW) *Earias insulana* (Boisd.) (Lepidoptera: Noctuidae) is one of the most dangerous pests attacking many plants of Malvaceae family, especially okra *Abelmos esculentaus*. It's one of the most important vegetable crops (Kandil, 2013), larvae of *E. insulana* feeds on terminal shoots, flower buds and fruits. It has been recorded for several countries of Asia and Africa and is also known as a cotton pest (Kumar *et al*., 2014).

In recent years, many new marker techniques have been developed for genomic research (Gupta and Rustgi, 2004). A novel marker system called Start Codon Targeted (SCoT) Polymorphism (Collard and Mackill, 2002) was developed based on the short conserved region. SCoT markers are generally reproducible, and it is suggested that primer length and annealing temperature are not the sole factors determining reproducibility, SCoT markers have been used to evaluate genetic polymorphism, identify genotypes, and DNA fingerprinting in various species.

Inter simple sequence repeat (ISSR) markers are considered very useful in in distinguishing, fingerprint and assessment genetic diversity studies of genetic diversity, phylogeny, genomics and evolutionary biology

(Reddy *et al*., 2002 and Havlíčková *et al*., 2014). ISSR-PCR was already known as a very effective method to understand intra-specific and genetic structure of populations (Fang and Roose, 1997; Ge and Sun, 1999; Zhou *et al*., 1999 and Nagaraju *et al*., 2001) to generate species-specific genomic fingerprints (Gupta *et al*., 1994 and Huang and Sun, 2000). Levy *et al*. (1992) reported that strain identification of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) larvae is helpful in developing effective and specific pest management plans to control these insects. Field trapped moths from various geographical locations could also be assessed by this method.

Imbalance between the production of (ROS) and antioxidant processes cause rapid increase in reactive oxygen species ROS levels in tissues causes oxidative stress (OS) and damage to DNA, lipids, or proteins (Zhang *et al*., 2015 and Matsumura *et al*., 2017). Generating ROS in the body occurs naturally as a product of many metabolic processes. ROS can be produced by plants as protection against phytophagous insects (Krishnan *et al.,* 2007). Organisms have also evolved antioxidant enzymes that catalyze the removal of ROS, thereby preventing damage to tissues (Cadenas, 1989). (Polyphenoloxidase) PPO and POD (Peroxidase) oxidoreductases are involved in the metabolism of plant phenolic compounds. They play a key role in the aphid's digestion process (Chrzanowski *et al*., 2012). Polyphenol oxidase (PPO) enzyme catalyzes the hydroxylation of monophenols to o-diphenols through monophenol oxidase activity and a subsequent oxidation of these o-diphenols to the corresponding o-quinones by a catecholase/diphenolase activity in the presence of its cosubstrate oxygen Polyphenol oxidase (1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1) In insects, it is involved in sclerotization of the insect cuticle. A remarkably large number of reactions occur, and polyphenol oxidase appears to be involved in more than just the initial oxidation of the o-diphenol to quinone methide or quinone (Sugumaran, 1988).

Peroxidases (EC number 1.11.1.7) are a large group of enzymes which play a role in various biological processes. They have antioxidant features that catalyzed the reactive oxygen species (ROS) using hydrogen peroxide (H_2O_2) generated during metabolism and are converted into harmless molecules (Foyer and Harbinson, 1994). They are named after the fact that they commonly break up peroxides (Torres *et al*., 2002) expressed and purified peroxidase isozyme C as a fusion protein in *S. frugiperda*.

According to the importance of the biochemical genetic basis it had been found that there were variabilities between isozymes activity in two populations of the *Pectinophora gossypiella* and *E. insulana* (Bartlett, 1981 and Sayed and EL-Ghobary, 2019).

Our work aimed to identify the genetic profile of *E. insulana* genome by using two techniques; SCoT and ISSR and study the isoforms of some enzymes electrophoratically detected by native gel electrophoresis of spiny bollworm strains from five Governorates compared to laboratory strain.

Material and methods

1. Insect field collection:

Field strains of *E. insulana* were collected from okra field from five different Governorates Behera, Alexandria, Menofia, Qaluobya and Sharkeya during season 2019 compared to the reared laboratory strain for isozymes study. The collected okra was dissected carefully, and the obtained larvae were kept in test tubes to perform the isozymes or DNA analysis.

2. Insect laboratory rearing:

Larvae of *E. insulana* used in this experiment was obtained from laboratory colony of Bollworm Department, Plant Protection Research Institute; Agricultural Research Center (ARC), reared for several generations under constant condition of 26 ± 2 °C and 65±5 % RH. on an artificial diet that previously described by (**Amer, 2015**).

3. DNA isolation procedure:

The bulked DNA extraction was performed using DNeasy insect Mini Kit (Biobasic). Larvae tissues were ground using liquid nitrogen to a fine powder, then, 400 µl of buffer AP1, 5 µl of proteinase k and 5 µl of

Lysozyme were added to a maximum of 100 mg of extracted larvae then vortexed vigorously. Mixture was incubated for 10 min at 65ºC and mixed 2-3 times during incubation by inverting tube. PCR was performed in 30-µl volume tubes according to Williams *et al*. (1990).

4. Polymerase chain reaction (PCR) condition for SCoT and ISSR:

The DNA amplifications were performed in an automated thermal cycle (Model Techno 512) programmed for one cycle at 94º C for 4 min followed by 45 cycles of 1 min at 94º C, 1 min at 57º C, and 2 min at 72º C. the reaction was finally stored at 72º C for 10 min and the used primers for SCoT were mentioned in Table (1) and primers used for ISSR were mentioned in Table (2).

Table (1): List of the primer names and their nucleotide sequences used in the study for SCoT procedure

No.	Name	Sequence
1	SCoT 1	5` ACG ACA TGG CGA CCA CGC 3`
2	SCoT 2	5` ACC ATG GCT ACC ACC GGC 3`
3	SCoT 4	5` ACC ATG GCT ACC ACC GCA 3`
4	SCoT 7	5` ACA ATG GCT ACC ACT GAC 3`
5	SCoT 11	5` ACA ATG GCT ACC ACT ACC 3`
6	SCoT 12	5` CAA CAA TGG CTA CCA CCG 3`
7	SCoT 15	5` CCA TGG CTA CCA CCG GCT 3`

Table (2): List of the primer names and their nucleotide sequences used in the study for ISSR procedure

No.	Name	Sequence
1	49A	5` CAC ACA CAC ACA AG 3`
2	49B	5` CAC ACA CAC ACA GG 3`
3	HB-10	5` GAG AGA GAG AGA CC 3`
4	HB-11	5` GTG TGT GTG TGT TGT CC 3`
5	HB-12	5`CAC CAC CAC GC 3`

Isozymes electrophoresis

Analysis of samples for isozyme expression by native gel electrophoresis. Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations among studied strains according to Stegemann *et al*. (1985).

5. Extraction of isozymes:

Isozymes extraction from larvae of the tested five Governorates homogenizing 0.5 g fresh leaves samples in 1 ml extraction buffer (10% glycrol) using a mortar and pestle. 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 –20ºC until use for electrophoretic analysis. *E. insulana* larvae samples were used separately for isozymes extraction. The utilized isozymes are polyphenyl oxidase (PPO) and peroxidase (Px).

6. Data analysis:

Gels were photographed scanned, analyzed using Gel Doc VILBER LOURMAT system (Yang and Quiros, 1993).

Results and discussion

1. SCoT analysis:

Seven SCoT primers and their nucleotide sequences (SCoT1,SCoT2, SCoT4, SCoT7, SCoT11, SCoT12 and SCoT15) illustrated in Table (1) were used in this technique to identify *E. insulana* genome profile, the laboratory strain showed different bands ranged between 4 to 9 bands, varying from 165 to 975 base pairs for each primer showed in Table (3) and Figure (1).

Table (3): Numbers and DNA bands molecular weight by the seven SCOT primers for *Earias insulana* larvae.

Band No	M.W Bp	SCOT Primers						
		SCOT 1	SCOT 2	SCOT 4	SCOT 7	SCOT 11	SCOT 12	SCOT 15
1	975	-	-	-	-	-	1	1
2	845	-	-	-	-	1	-	1
3	800	-	-	-	-		1	-
4	730	1	1	-	-	1	-	1
5	600	-	1	1	-	-	-	1
6	530	-	1	1	1	1	-	1
7	500	1	1	1	1	1	1	-
8	435	-	1	1	-	-	1	-
9	345	-	1	1	-	-	1	1
10	280	1	1	1	1	1	1	1
11	220	1	-	1	1	1	1	1
12	165	1	-	-	-	-	1	1
Total		5	7	7	4	6	8	9

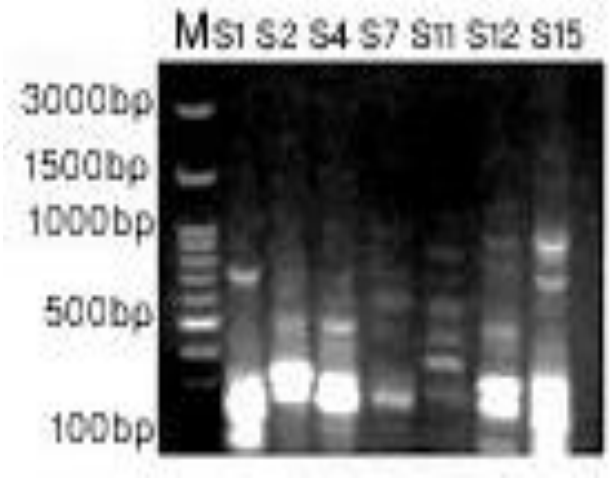


Figure (1): SCOT patterns of the *Earias insulana* larvae revealed by seven primers.

There were five bands in primer SCoT1 by molecular weight ranged between 730 to 165bp. SCoT2 and SCoT4 primers had seven bands with molecular weight ranged between 730 to280bp and from 600 to 220bp. whereas, SCoT7 had four bands varied from 530 to 220bp. while, SCoT11 had six bands with molecular weight varied between 845 to 220bp., also, SCoT12 and 15 were eight and nine bands with molecular weight varied between 165 to 975bp., a unique band appeared in SCoT12 primer with 800bp, while, two monomorphic bands were appeared with primers (SCOT12 and SCOT15) by 975bp and (SCOT11and SCOT15) by 845bp., whereas, nine polymorphic bands between all tested primers**.**

2. ISSR analysis:

Data illustrated in Table (2) showed five ISSR primers and their nucleotide sequences (49A, 49B, HB-10, HB-11 and HB-12) used to identify the *E. insulana* genome profile, these five primers resulted in different bands between 2 and 13, varying from 1630 to 175 base pairs for each primer. There were two bands in primer 49A by molecular weight ranged between 1050 to 780bp., while, primer 49B had five bands with molecular weight ranged between 1375 to 600bp. whereas, HB-10 primer had ten bands varied from 1375 to 250bp., also, primer HB-11 had three bands varied from 940 to 175bp.**,** finally, primer HB-12 had thirteen bands ranged between 1630 to 175bp. showed in Table (4) and Figure (2).

Fifteen primers appeared in all primers between 2 and 13 different bands, varying from 175 to 1630 base pairs, were obtained by five primers screened of ISSR technique presented in Table (4).

Table (4): Number and DNA bands molecular weight by the five ISSR primers for *Earias insulana* larvae.

Band No	M.W Bp	ISSR Primers				
		49A	49B	HB-10	HB-11	HB-12
1	1630	-	-	-	-	1
2	1375	-	1	1	-	1
3	1050	1	-	1	-	1
4	940	-	1	-	1	1
5	820	-	-	1	-	1
6	780	1	1	1	-	-
7	700	-	1	1	-	1
8	600	-	1	1	-	1
9	530	-	-	1	-	1
10	460	-	-	-	-	1
11	400	-	-	1	1	-
12	320	-	-	-	-	1
13	270	-	-	1	-	1
14	250	-	-	1	-	1
15	175	-	-	-	1	1
Total		2	5	10	3	13

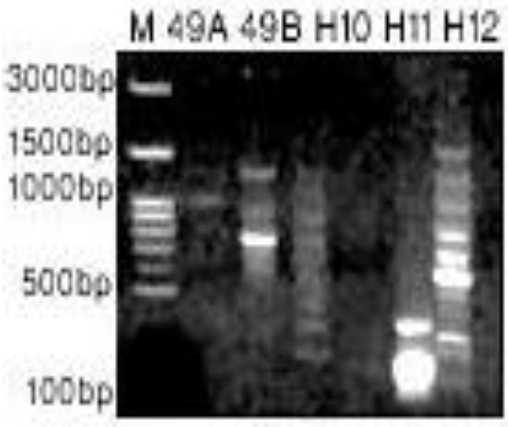


Figure (2): ISSR patterns of the *Earias insulana* larvae revealed by five primers.

A unique band appeared in ISSR (HB-12) primer with band number 1 and 10 by 1630 and 460bp., respectively, while, three monomorphic bands were appeared with primers (HB-10 and HB-12) by 820, 270 and 250 bp. respectively, and (HB-10 and HB-11) by 400bp and (HB-11 and HB-12) by 175bp. Three polymorphic bands with primers (49B, HB-10 and HB-12) by 1375, 700 and 600bp. respectively whereas, polymorphic bands with primers (49A, 49B and HB-10) by 780bp., and primers (49B, HB-11 and HB-12) by 940 bp. and polymorphic bands with primers (49A, HB-10 and HB-12) by 1050 bp.

After identification of *E. insulana* genome profile, the electrophoretic separation analysis of isozymes was done to identify the variations among studied strains. Native gel electrophoresis can detect enzymes such as polyphenyl oxidase and peroxidase.

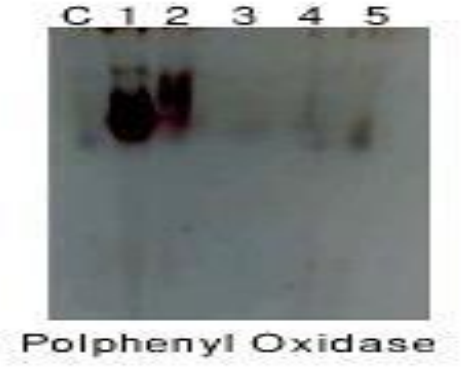
3. Polyphenyl oxidase:

Polyphenyl oxidase activity of *E. insulana* from laboratory strain and different Governorates was electrophoretically showed in Table (5) and Figure (3). All larvae showed different bands; control (laboratory) larvae strain, Behera, Alexandria and Qaluobya Governorates showed two bands with different densities, whereas, Menofia and Sharkeya Governorates have only light band.

Table (5): Distribution of polyphenyl oxidase isozyme groups of *Earias insulana* populations according to their relative mobility's and densities.

Poly Phenol Oxidase	Relative mobility	Control laboratory	Behera	Alexandria	Menofia	Qaluobya	Sharkeya
PPO1	0.216	-	++	-	-	-	-
PPO2	0.258	-	-	++	-	-	-
PPO3	0.394	-	-	++	-	-	-
PPO4	0.474	-	-	-	-	+	-
PPO5	0.484	-	+++	-	-	-	-
PPO6	0.488	-	-	-	-	-	++
PPO7	0.531	-	-	-	+	-	-
PPO8	0.577	+	-	-	-	-	-
PPO9	0.606	-	-	-	-	++	-
PPO10	0.212	++	-	-	-	-	-

+ +Moderate density Band + Low density band – No Band. +++ High density Band



C: Control (Laboratory), 1:Behera, 2: Alexandria, 3:Menofia, 4:Qaluobya, 5: Sharkeya
Figure (3): Electrophoretic separation of polyphenyl oxidase isoforms from laboratory and different Governorates strain of *Earias insulana*.

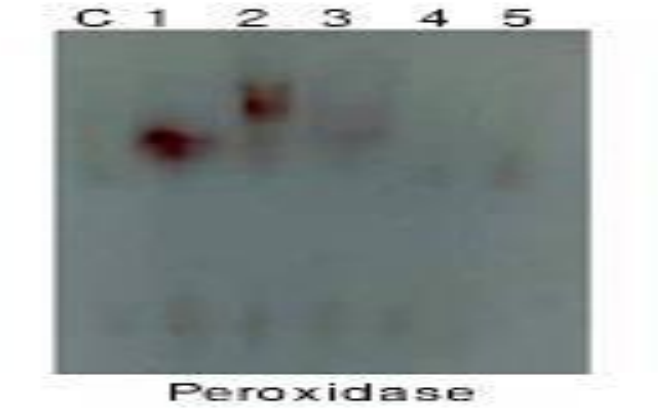
4. Peroxidase:

Peroxidase enzyme of *E. insulana* from laboratory strain and different Governorates were electrophoretically showed in Figure (4) and Table (6), two bands were appeared in Alexandria governorate larvae with heavy density, while Behera governorate have one heavy band and Menofia Governorate have one moderate band, whereas, control (Laboratory), Qaluobya and Sharkeya Governorates have only one band with low density. Two analysis methods were implemented to identify the *E. insulana* genome profile of laboratory strain for the first time in Egypt using two different techniques; SCoT and ISSR. Recognition of most parts of the *E. insulana* genome was achieved as the SCoT technique linked with genes on the other hand, ISSR technique linked among sequences.

Table (6): Distribution of peroxidase isozyme groups of *Earias insulana* populations according to their relative mobility's and densities.

Peroxidase	Relative mobility	Control Laboratory	Behera	Alexandria	Menofia	Qaluobya	Sharkeya
PX1	0.284	-	-	+++	-	-	-
PX2	0.512	-	-	-	-	-	-
PX3	0.517	-	+++	-	++	-	-
PX4	0.612	-	-	++	-	-	-
PX5	0.667	-	-	-	-	+	-
PX6	0.672	-	-	-	-	-	+
PX7	0.687	+	-	-	-	-	-

+ +Moderate density Band + Low density band – No Band. +++ High density Band



C: Control (Laboratory), 1:Behera, 2: Alexandria, 3:Menofia, 4:Qaluobya, 5: Sharkeya
Figure (4): Electrophoretic separation of peroxidase isoforms from laboratory and different Governorates strain of *Earias insulana*.

Using different techniques like SCoT, ISSR, RFLP or RAPD in genetic profile identification of an insect differs according to the purpose of the study. In this respect, many authors showed different genetic studies to identify insect genotypes or to evaluate genetic polymorphism, Levy *et al*. (1992) applied a simple method to analyze the two morphologically indistinguishable host associated strains of *S. frugiperda* using fragment length polymorphism (RFLP) marker, few nanograms of total DNA are needed to yield clear and accurate strain identification of individual insects. Also, Yuan *et al*. (2014) identified reference genes which should be used for accurate elucidation of the expression profiles of functional genes of *Nilaparvata lugens*. Whereas, Yanhong *et al*. (2018) identified some proteins might be directly correlated to the biological characteristics of the *Ericerus pela* eggs at the stage at which they were examined. In the population genetic study of *H. armigera* populations, (Endersby *et al*., 2007) excluded 3 pairs of SSR markers with the greatest null allele frequencies (i.e., 19.2%, 31.6% and 47.4%). Luque *et al*. (2002) shown that some ISSR amplifications are possible and demonstrate their applicability in studying intra- and inter-specific variation in some Noctuidae populations they found that: (i) (CA)n primer gives the most informative profiles; (ii) DNA profiles between species differ substantially; (iii) comparison of ISSR profiles can be successfully applied to study intra-specific variation.

The genetic characterization of the gypsy moth from China (*Lymantria dispar*) provided by (Fang *et al*., 2013), using five polymorphic Inter simple sequence repeat markers which produced reproducible banding patterns and revealed that ISSR markers are a highly informative and efficient tool for estimating the genetic variation and structure of the insect. Experiment of isozymes study were evaluated on spiny bollworm *E. insulana* field strains from five Governorates in Egypt (Behera, Alexandria, Menoufia, Qalyoubia and Sharkeya) compared with laboratory strain for their molecular diversity to identifying differences between field strains from different Governorates by isozymes as a molecular tool. In this respect, Sayed and El-Ghobary (2019) found that *E. insulana* populations were similar in the distribution and intensity of the peroxidase and polyphenol oxidase isoenzymes. On the other hand, many authors studied many other isozymes like, Scarpassa and Hamada (2003) found that the genetic relationships suggested by the comparison of 11 isozyme loci in four

species of the *Simulium perflavum* group indicate that these species are closely related.

It could be concluded that the identification of *E. insulana* genome with SCoT analysis technique for the first time in Egypt and ISSR analysis technique, using these two techniques had been recognized the most parts of the *E. insulana* genome, our findings may me be helpful in genetic and genomic studies.

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