AMERICAN JOURNAL OF

BIOSCIENCE AND BIOINFORMATICS (AJBB)

VOLUME 2 ISSUE 1 (2023)

PUBLISHED BY

E-PALLI PUBLISHERS, DELAWARE, USA

American Journal of Bioscience and Bioinformatics (AJBB)

Volume 2 Issue 1, Year 2023
https://journals.e-palli.com/home/index.php/ajbb

Molecular Investigation Using RAPD-PCR Marker Field Populations of *Pectinophora gossypiella* Saunders (Lepidoptera: Gelechiidae) Exposed to Some Insecticide

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Article Information

Received: May 20, 2023
Accepted: June 02, 2023
Published: June 29, 2023

Keywords

P. Gossypiella, RAPD Primers, Polymorphism, Population Differentiation, Diversity, Egyptian Governorates, Distance, Similarity

ABSTRACT

Seasonally the cotton plant cultivated in Egyptian fields is suffering from the cotton Pink Bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) attacking fruits and buds. Insecticide applications reported control failure by the most recommended conventional classes. Monitoring pest DNA changes after sublethal exposure become the new technique to investigate. Then four insecticide treatments were screened by using the molecular marker polymerase chain reaction (PCR) inspection, using four Random amplified polymorphic deoxyribonucleic acid analysis (RAPD-PCR) primers partitioned based on band reproducibility. Result of LC_{50} was 0.55, 61.1, 69.3, and 0.23 for spinoteram, novaluron, metaflumezone, and dimeuron respectively. The insecticide treatments band of gel produced detected 47 loci ranging from 87 to 63 % polymorphism. The primer efficiency value of PIC =0.361, 0.34, 0.34, 0.355, and 0.262, RP = 5.30, H = 0.473, 0.434, 0.434, 0.462 and 0.31, and MI = 0.10, 0.1386, 0.138, 0.167 and 0.0582 for the same treated samples respectively. Distance and similarity were quantified based on Nei's and genetic dissimilarity by the UPMGA method then a phylogenetic tree was constructed and grouped the entire genotypes into 2 major clusters and 6 subclusters. The RAPD primers revealed the number of alleles (Na = 0.324, 0.318, 0.318, 0.326, and 0.328), and the effective number of alleles (Ne = 0.571, 0.569, 0.558, 0.574, and 0.5784). The fixation-index (Fst) analysis narrated a very great genetic diversity (Fst = 0.626, 0.684, 0.684, 0.2, and 0.695) exists within the four treated samples respectively. The level of gene flow was (Nm = 0.239, 0.230, 0.230, 0.233, and 0.2187) respectively across the four genotypes studied. Results proved that the RAPD-PCR technique was suitable for distinguishing between insecticide treatments.

INTRODUCTION

The Egyptian cotton crop suffer seasonally infestation by a such arthropod group of bollworm complex are Pink Bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) besides Corn earworm *Helicoverpa armigera* (Hubner), *Spotted Bollworm Earias vittella* (Fab.), *Spink Bollworm E. insulana* (Boisduval), cotton leafworm *Spodoptera litura* (Fab.) and other insects (Khidr *et al*., (1996); Sandhya *et al*., (2010) and Sarwar (2017). Pink bollworm larvae fed on buds, causing fruit shedding, seed loss, and moths represent a commercial problem because their larval stage frequently enters diapause while in seed capsules, emerging after longlived for about than 50 days at different climate conditions and surviving for many months (Abd-Elhady and Abdel-Aal (2011) and Darwish *et al*., 2017). Insecticide application can control only the eggs and the first instar remains outside bulls because the larval stage enters bools and remain inside till pupal emergence in addition to the development of resistance toward most classes of insecticides represent a difficult problem fronting the effective control Tanani and Ghoneim (2018) and Salama *et al*., (2013). Insecticide exposure can organise changes to insect DNA that can be detected by molecular markers like RAPD procedures (Kimura and Crow, (1964). Several searches accompanied investigations about the different molecular markers and genome sequencing technology that progresses and is introduced in insect study development in molecular genetic identification, polymorphism, fingerprinting, and molecular diversity. Such insects were completed as predatory *coccinellids* by Roehrdanz and Flanders (1993), plant diversity by Arif, *et al*., (2010), fruitfly *Bactrocera zonata* by Al-Senosy and Badr 2018, stored insect species Jwada, *et al*., 2018, *bracon* species Bakr *et al*., 2013, and many others. The sequence of a genome and its annotation provide some biological information result like positions of protein-coding and noncoding protein and genes, regions, and their regulatory elements, including some structural and regulatory function of the gene (Singh, *et al*., 2013). The Random-amplified polymorphic DNA (RAPD) procedure was able to detect nucleotide sequence polymorphisms by using some single synthetic primer generally 10 base pairs of randomly nucleotide sequence and anneals at two different sites on complementary strands of DNA template, attend as forward and reverse primer and able to amplify fragments from 1–10 genomic sites and several discrete loci in the genome, making efficient screening of nucleotide sequence polymorphism between individuals (Kumari and Thakur (2014). The amplified products usually are between 0.5–5 kb size ranges, can separate on agarose gels including ethidium bromide, and observation under ultraviolet light for the presence or absence of band and by length differences in the amplified sequence between primer annealing sites (Dhakshanamoorthy, *et al*., 2015). Several constructed databases and many software

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computer programs designed for insect genes serve to identify gene function, and phylogenetic relationships among and within different orders in genome size. the specific algorithms of each gene structural identification within the genome provided possible comparing insect genomes, gene number, and gene order to provide data efficiencies for screening stress effect on insect that exposed and comparing organisms each other with similar or dissimilar sequences (homologous and orthologous) (Lacy, and Stevens (1999), Botstein, *et al*., 1980). In this study four insecticide treatments were screened on *P. gossypiella* larvae, investigated the changes and differences between insecticide classes and formulation by using RAPD-PCR marker extended analysis.

MATERIALS AND METHODS

Insect Sources and Maintenance

Big bulk of cotton plant bolls collected from four Egyptian fields was (Sharkia governorate) heavily infested with *Pectinophora gossybiella*, transferred to laboratory and the fourth instar larvae extracted from bolls and placed on petri dish provided with piece of cotton for maintenance till toxicity bioassay and the molecular biology procedure completion.

Insecticides and Bioassays

Toxicological studies accomplished by dipping about 10 individual's 4th instar larvae for 5 second gently and handled by forceps on each insecticide serial concentration diluted solution prepared by water; was Radient (12% SC spinoteram), technomezone (22%SC metaflumezone), Roxy (10% EC novaluron) and dimeuron (10% EC hexaflumeron). Larvae were dipped in insecticide solutions, left to dry on filter-paper, and transferred to petri dish provided with new filter paper. Control larvae dipped in water only. Three replicates for each concentration and dishes were maintained in lab chamber conditions at 25°C, 27 humidity, and 12 hours daylight, approximately. Mortality was recorded after 24 h and statistical calculation using Probit analysis to calculate LC_{50} values for the tested compounds using LeOra Software (1989), POLO-PC program according to Finney (1971) were finished. After completion of the baseline bioassay, each insecticide sub lethal treatment was performed by dipping about 50 larvae on each insecticide solution of LC_{50} ppm concentration that previously definite and data found in Table (2). After 24h, Survived larvae from the control and the treated samples were collected and sent to the laboratory of biomarker assay and stored at -20°C freezing degree.

Molecular Techniques for RAPD Procedures

1-DNA Extraction of Larvae Bodies

The cetyltrimethylammonium bromide (CTAB) DNA Extraction Protocol performed extraction procedure, according to (Doyle and Doyle, 1990). Extraction, started by adding 0.02g polyvinyl-pyrrolidone and 2.5 ul β-mercaptoethanol. About 50mg of insect body frozen tissue in an Eppendorf tube was transferred. Grinding tissue was in liquid nitrogen with blue pestles, then 500 uL of CTAB buffer was added and the tubes were mixed, incubated at 55°C for 3 h, and mixed once after 30 minutes. Then 1.5 uL RNase A was added and incubated again at 37°C for 15 minutes. Samples removed from water bath and 500 uL of chloroform added, mixed gently by shaking tubes and centrifuged for 7 minutes at 16000 xg then the aqueous phase transferred into the new-labeled tube. Then 0.1 volumes cold 7.5 M ammonium acetate and 0.6 volumes of cold isopropanol was added, mix by inverting tubes 20-30 times, incubated on ice for 30-40 minutes and centrifuge for 3 minutes at16000 xg. Supernatant discarded and 700 uL 70% EtOH added, tubes inverted 5-10 times. Centrifuge for 1 minute at 16000 xg. Discard supernatant. Hydrate pellets with 50 uL TE buffer.

2- Random Amplified Polymorphic DNA (RAPD-PCR) Amplification

All solutions were gently vortexed and centrifuged after thawing, then a thin-walled PCR tube placed on ice and for each 25 µL reaction: 12.5 µl of Dream Taq Green PCR, Master Mix (2X), 2 µl of primer, 1 µl of DNA (50ng/1µl) template and 9.5 µl of water added, nuclease-free and the total volume was 25 µl. Four primers used are shown in Table (1) and Gene Ruler 100pb DNA ladder #SM0243 thermo-fisher scientific as template. Afterward, samples were vortexed, and reactions were placed in the PCR thermal cycler, its conditions consisted of an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The amplified PCR fragments were separated on 1.5% agarose gel in Tris-acetic acid/EDTA buffer (1 × TAE) and electrophoresis was carried out with a constant voltage of 80 V for 2 h and stained with ethidium bromide (0.5 µg/mL) and photographed by gel documentation system. Procedure was repeated thrice and the bands that occurred in the three gels were used for analysis.

Table 1: The nucleotide sequence information of the primers used for RAPD-PCR analysis.

No.	Primer	Sequence (5'-3')	Frequency	Polymorphism	MEC	Tm	Length	MW	GC%
1	P13	GGAGTGCCTC	4.33	43	91530	31.9	10	3106	70
2	N8	ACCTCAGCTC	4.1	41	86940	29.3	10	3010	60
3	H5	AGTCGTCCCC	5.0	50	38610	33.5	10	3026	70
4	P8	GGAGCCCAGT	6.66	66	97470	33.7	10	3115	70

MW= Molecular weight. GC= guanine cytosine ratio Tm= melting temperature and MEC=molar extension coefficient

3-Fragment Analysis Preparations

Gel picture documents were analysed by Gelanalyzer v.19 software and get the results. Only the distinct amplified DNA fragments scored, with (1) as presence and (0) as absence, and constructing matrix of bands as binary data of similarity, distance matrices, and weak bands not included.

Data Analysis of the Gel Results

Performance of Each Primer

Performance of each primer was measured by the online program of marker efficiency analysis calculations (MEA) *iMEC* (https://irscope.shinyapps.io/*iMEC*/) by calculating different parameters. Where PIC value for each primer was calculated as proposed by Roldan-Ruiz *et al*., 2000: PICi = 2*f*i (1 – *f*i), where PIC is the polymorphism information content of the marker i, *f*i is the frequency of marker fragments that were present and 1 – *f*i is the frequency of marker fragments that were absent.

2-Genetic Differentiation

Completed by similarity indices calculations to compare patterns between populations according to (John, 2004). Dendrogram constructed by Past-software (Hammer *et al*., 2001), using UPGMA tree (Unweighted Pair Group Arithmetic Mean) under different Bootstrap 1,000 replicates of analysis (Lin *et al*., 2015).

Genetic dissimilarity coefficients among the *P.gossypiella* population individuals were estimated using a simple Euclidean distance method from FAMD software. The formula is $d_{xy} = \sqrt{\sum(x_1-y_1)^2}$ Where, d_{xy} of two signals X and Y with length k). the phylogenetic tree was obtained from unweighted pair group matrices by method with arithmetical averages (UPGMA) and bootstrap (Sneath and Sokal 1973), by FAMD and Alerquin software.

3-Genetic Relationship Achieved by Four Statistical Analyses

Firstly, cluster analysis was performed to determine the relative genetic distance and similarity between pairwise populations and to check the consistency of the genetic differentiation. Secondly, Principle Component Analysis (PCA) based on Euclidian measures. In addition to coordinate analysis, corresponding analysis and AMOVA (Analysis of Molecular Variance) were calculated using the software FAMD, Past (Schlüter and Harris (2006) by comparing results.

4-Population Genetic Information and Structures

Population structure and heterogeneity (Ht), between and within a population (Hs) completely estimated. In addition to (Gst) Nei's genetic differentiation index among populations heterozygosity calculation, gene flow between populations (Nm=0.5(1-Gst)/Gst) or = ([(1/Fst)-1]/4 were estimated. Where Gst= Ht-Hs/Ht, or=Dst/Ht and Ht the total genetic diversity =Hs+Dst, where DST is the total genetic diversity distributed among populations= Dst=Ht-Hs. The number of alleles (Na) and number of the effective alleles (Ne=1/(∑pi2)) and Fst =(HT–HS)/ HT and F= (He − Ho)/He =1 - (Ho/He) was calculated. Where n is the number of genotypes. Polymorphism information is attained where pi is the allele frequency and expected heterozygosity (He= 1-Pi^2), according to Botstein *et al*., 1980, Hedrick (2005), Chesnokov and Artemyeva (2015). H_S is calculated as the mean of gene diversity (He) values over all loci per individual (He = 1 - ∑ pi^2), where pi here is the frequency of a given allele Culley *et al*., 2002. Using POPGENE version 1.32 Raymond and Rousset (1995), and Structure software 2.2, Evanno, *et al*.,2005).

RESULTS AND DISCUSSIONS

1-Insecticide Toxicity Features

Four treatments for the collected larvae from Egyptian Sharkia governorate, toxicity results found in table (2). Data of LC_{50} and LC_{90} values in ppm and chi-square refers to the *P.gossypiella* population susceptibility levels for this insecticide. The steepest Slope ± standard error, intercept refers to the homogeneity of the population tested, and a small amount of heterozygote individuals may open the way to control this pest easily.

Many searches cited that control this pest using recommended insecticides in Egypt in experiments carried out on cotton seasons revealed that treatment of pheromones and parasitoid *Trichogramma evenecens* induced real reduction of pink bollworm infestation (90%) followed by insecticide (88–87%) according to El-Bassouiny (2021). Moreover, the biocide product as bacteria or viruses formulations revealed effective control to bollworms according to EL-Lebody (2014).

Table 2: Toxicity parameters of some insecticides against *P.gossypiella* larvae.

Strain	Slope±SE	Intercept	LC_{50} (CI95%)	LC_{90} (CI95%)	χ2	P
Spinoteram	1.56±0.125	5.4	0.55 (0.315-0.973)	3.8 (2.2-6.7)	0.319	0.001
Novaluron	1.86±0.108	1.68	61.1 (37.6-99.3)	309.4 (190.2-503.3)	0.848	0.001
Metaflumezon	1.8±0.108	2.36	69.3 (42.2-110.5)	366.8 (224.4-597.9)	0.308	0.001
Dimeuron	2.04±0.098	6.3	0.23 (0.147-0.357)	0.98 (0.63-1.53)	0.879	0.001

(CI = intervals at 95%), SE= standard error

2- Quantification of Polymorphism of RAPD Primer Fingerprinting

Insecticide treatment differentiation using gel document information displayed the total number of fragments produced by four primers was 282 with an average of 50 fragments / primer (Fig 1). Appearance and disappearance of bands detected by gel analyzer software differed from each treatment to others, also, band intensities changed

because of insecticide treatment. Each lane generated the RF value of its bands detected that defined as the migration distance of the protein through the gel divided by the migration distance of the dye front, then measure relative mobility of protein bands that lead to molecular weight determination as cited by (Hames 1998, in Biorad technical notes number 3133). PCR-RAPD technique detected low levels of polymorphism for the primers used in the four-insecticide treatment of *P. gossypiella*, ranging from 26% for dimeuron treatment and 35% for metaflumezone treatment and 36% for the control. This is because of insecticide treatment that damages the DNA of detected bands and not appears in SDS gel images that differs from the control band image and dimeuron causes the highest level of DNA damage according to band images. There are significant differences in insecticide treatments reflected on genomic DNA patterns resulted in agarose gels scanned. Some information about gel band is intensity attained by gel analyzer software. The molecular weight of unique bands of DNA oligomers amplified ranged in size from 100 to 2000 bp. Monomorphic and polymorphic bands that construct the RAPD profile fingerprinting of the amplified DNA fragments of the four treatments were ranging from 9 to 18 band for dimeuron and control, respectively (Table 3). Data of the primer information found in table (1) including melting temperature and molecular weight. From literature of this scientific field Atienzar and Jha (2006), reported that mutations are responsible for the appearance of new bands if they occurred at the same locus in a sufficient number of cells (a minimum of 10% of mutations may be required so that a new PCR product can be visible in agarose gel). The new bands are because of mutation, but the disappearance of bands are because of DNA damage due to insecticide treatment compare with the control. Similar results determined by Al-Senosy and Badr 2018, where the level of malatox resistance in peach fruit fly (*Bactrocera zonata*) by (RAPD) and (ISSR) to make fingerprinting for adults from many Egyptian governorates using five primers, detected 176 bands, with an average of 35.2 bands per primer and percentage of polymorphism was 66% to 100%.

But about the differences between PCR different marker precise data, we can find some literature illustrate differences clearly as, Costa *et al*., 2016, found fingerprinting of *Dactylis glomerata* genotypes and genetic variation detected between subspecies using (RAPD), (ISSR), (AFLP) markers were 97 bands, for RAPD, of which 40 were polymorphic (41.2%), ISSR primers amplified 91 bands, and 54 showed polymorphism (59.3%) and the AFLP showed 100 bands, of which 92 were polymorphic (92%).

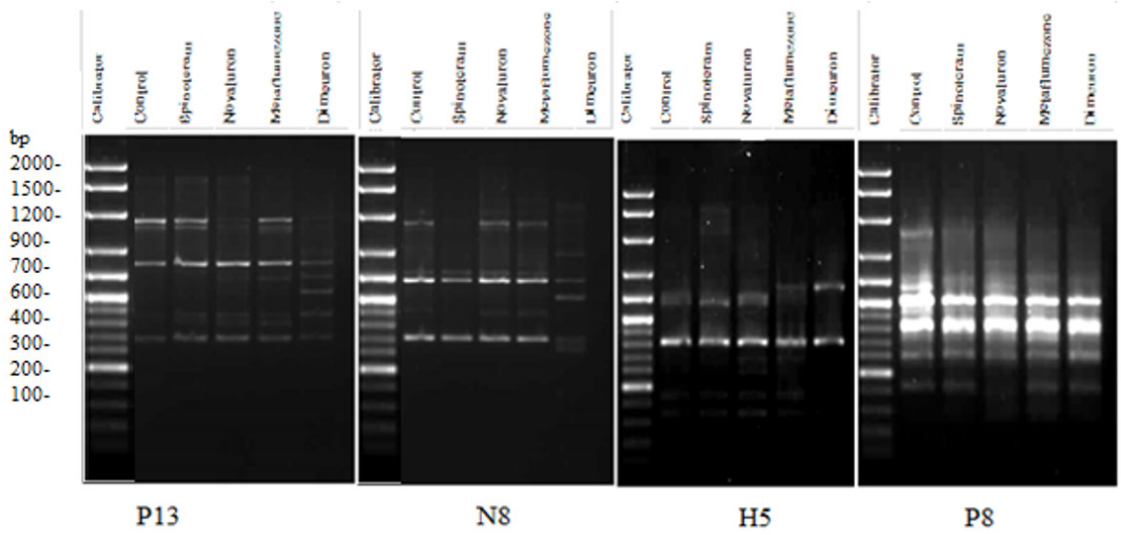

Figure 1: RAPD-PCR image profile by four primers amplification of *P. gossipiella* survived LC50 of 4-insecticide treatment and control and Molecular weight ruler on SDS polyacrelamid gel.

Table 3: Total number of monomorphic, polymorphic bands presence and polymorphism percentage of each treatment of *P. gossypiella* and control.

	Total B.	**Poly B.**	**P**	**% p**	**Fixed B.**	**F/locus**	**A.F.**	**Loci no.**
Control	18.0	5	36	87.8%	18	0.383	0.785	3
Spinoteram	15	5	34	82.9%	15	0.319	0.825	2
Novaluron	15	5	34	82.9%	15	0.319	0.825	2
Metaflumezon	17	6	35	85.36%	17	0.361	0.798	2
Dimeuron	9	3	26	63.4%	9	0.191	0.899	1
Calibrator	46	19	41		46	0.978	0.146	38
Total	**120**	**43**			**120**			**47**

B= Band, F=Frequency of presence bands, A.F. =Allele frequency and p=polymorphism=polymorphic bands/ total number of bands.

3-Performance of Each Primer

The cumulative polymorphic efficiency of all RAPD primer *iMEC* details for each insecticide treatment and control are found in table (3). Also, Details about polymorphic indices are found in Table (3). PIC is the polymorphic information content, its values is an important likelihood indices pointed on variation, diversity and number of allele frequency of all insecticide treatments and frequency distribution, where the higher PIC was 0.361 recorded for control followed by 0.35 metaflumezone treated larvae and the lowest was 0.26 for dimeuron treated larvae. The heterozygosity (H) is the second informative genomic marker of polymorphism its values varied from 0.31 for to 0.473 for control. The arithmetic means of heterozygosity (*Havp*) ranged between 0.0065 for dimeuron and 0.01 for control. The effective multiplex ratio (*EMR*) is the publicity of primer polymorphism were ranged from 18.0 to 15 for control and spinoteram or novaluron. The marker index (MI) values were 4.07, 0.181, 0.1386, 0.138, and 0.167 to 0.0583 for calibrator, control, spinoteram, novaluron, metaflumezone and dimeuron respectively. Discriminative power (D), D = 0.90 for spinoteram and novaluron and 0.858, 0.874 for control and metaflumezone but dimeuron was 0.196. The highest value in resolving power (Rp) is ranged from 90-97% per primer when analysis of primer efficiency input data, but insecticide analysis revealed with 0 Rp. Many scientific searches handled data about marker efficiency like results gained by (Khan *et al.*, 2021), where forty-four Bambara groundnut (*Vigna subterranea* L. verdc.) genotypes were sampled to explore the genetic structure, genetic inconsistency, effective multiplex ratio and fixation index were differing/differed. Also, Dhakshanamoorthy *et al.*, 2015 found similar result about primer efficiencies in *Jatropha curcas* as valuable chemical and physical properties and evaluate diversity and genetic relationship among mutants obtained from different doses of gamma rays during breeding.

Table 4: Marker efficiency analysis (MEA) for *P.gossypiella* treatments-RAPD primers.

	H	*EMR*	**H.avp**	**PIC**	**D**	**Mi**	**Rp**	**TNB**	**NPB**	**Null allele**
Calibrator	4.16	4.6	8.86	4.07	4.255	4.07	0.0	47	47	9.55
Control	0.473	18.0	0.010	0.361	0.858	0.181	0.0	18	18	8.43
Spinoteram	0.434	15.0	0.0092	0.340	0.90	0.1386	0.0	15	15	8.43
Novaluron	0.434	15.0	0.0092	0.340	0.90	0.138	0.0	15	15	7.33
Metaflumezon	0.462	17.0	0.0098	0.355	0.874	0.167	0.0	17	17	7.98
Dimeuron	0.31	9.0	0.0065	0.262	0.196	0.0583	0.0	9	9	6.25

PIC= polymorphic Information Content. Rp= Resolving Power. D= Discriminating Power. H= expected heterozygosity. Hayp= arithmetic mean heterozygosity (Havp). MI= marker index. TNB= total number of bands; EMR: effective multiplex ratio. NPB: number of polymorphic bands, TNP= total number of bands.

Some factors affect the success of RAPD band releases, which are the source of template DNA (Eisen *et al.*, 1995). The interaction of the DNA template and primer during the initial steps of PCR, (Atienzar and Jha 2006) and the best RAPD profiles obtained using kit extracted DNA followed by column purification. In addition, high DNA concentration also affects RAPD products (Quintaes *et al.*, 2004), where it could be suppressing the amplification process causing relative primer shortage (Perry *et al.*, 2003).

4-Genetic Differentiation (Distance and Similarity) Between Treatments

Data of RAPD markers of the insecticide treatment cases bring about genetic distance and similarity coefficient values pairwise data based on gel image data of absence and present bands were in table (5 and 6). The highest genetic distance value was observed between 6.083 (control) and (control), followed by 5.568 (dimeuron) and (control) also the same was between (metaflumezne and

Table 5: Genetic distances among *P. gossipiella* treatments compared to control.

Insecticides	**Control**	**Spinoteram**	**Novaluron**	**Metaflumezone**	**Dimeuron**	**Eigenvalue**	**Pi^2**	**Pi**	**Allele freq.**
Spinoteram	5.477	0	0	0	0	0.0978	1.75	3.0728	0.5
Novaluron	5.745	1.732	0	0	0	0.01761	1.756	3.081	0.5
Metaflumezon	5.568	2.646	2.449	0	0	0.01206	1.799	3.1403	0.5
Dimeuron	5.568	2.646	2.828	3.162	0	0.0071	1.799	3.1403	0.5
Control	6.083	4.123	3.742	3.742	3.464	0.0011	1.741	3.060	0.5

control), the less distance value reached 1.732 between spinoteram and novaluron and the eigenvalue ranged from 0.0011 to 0.0978 (Table 5). Data of RAPD markers lane-band scanned primers were used for the genetic similarity value co-efficient table (6). A maximum genetic reserve value was observed between (Novaluron) and (Metaflumezone), (Novaluron) and (Spinoteram) reached 0.86, but less similarity value reached 45%. While the lowest genetic distance value was observed between (Dimeuron) and (Spinoteram) reached 0.33.

Data of similarity between treatment and control reveals that control, spinoteram, novaluron, metaflumezone and dimeuron were 43, 33, 43, 33% similarity with the calibrator respectively. Subsequently all data between each insecticide and others were found in table (6). Similar results exhibited discrete DNA polymorphisms or alterations in DNA bands, varied depending on the substance being examined chlorpyrifos causes the highest level of DNA alterations (based on the appearance and disappearance of DNA bands) against *Rhyzopertha dominica* Fabricius followed by five plants extracted and evaluated by Qari and Abdel-Fattah 2017.

Table 6: Similarity and phenotypic variations among *P. gossipiella* treatment of each primer.

Insecticides	Primer	Calibrator	Control	Spinoteram	Novaluron	Metaflumezone	Dimeuron
Calibrator	P13	1	0.43	0.43	0.33	0.43	0.33
Control		0.43	1	1	0.67	1	0.33
Spinoteram		0.43	1	1	0.67	1	0.33
Novaluron		0.33	0.67	0.67	1	0.67	0.5
Metaflumezon		0.43	1	1	0.67	1	0.33
Dimeuron		0.33	0.33	0.33	0.5	0.33	1
Calibrator	N8	1	0.57	0.46	0.57	0.46	0.33
Control		0.57	1	0.86	1	0.86	0.33
Spinoteram		0.46	0.86	1	0.86	0.67	0.33
Novaluron		0.57	1	0.86	1	0.86	0.5
Metaflumezon		0.46	0.86	0.67	0.86	1	0.33
Dimeuron		0.43	0.43	0.33	0.43	0.33	0.43
Calibrator	H5	1	0.4	0.4	0.59	0.5	0.4
Control		0.4	1	1	0.75	0.57	0.33
Spinoteram		0.4	1	1	0.75	0.57	0.33
Novaluron		0.59	0.75	0.75	1	0.67	0.5
Metaflumezon		0.5	0.57	0.57	0.67	1	0.57
Dimeuron		0.4	0.33	0.33	0.5	0.57	1
Calibrator	P8	1	0.67	0.53	0.44	0.6	0.44
Control		0.67	1	0.83	0.73	0.77	0.55
Spinoteram		0.53	0.83	1	0.89	0.73	0.67
Novaluron		0.44	0.73	0.89	1	0.6	0.5
Metaflumezon		0.6	0.77	0.73	0.6	1	0.8
Dimeuron		0.44	0.55	0.67	0.5	0.8	1

5- Molecular Variance Analysis

The analysis of molecular variance (AMOVA) was performed on data of RAPD markers absence and presence of bands as molecular distance matrix. In addition to supported K-means, clustering analysis incorporated F-statistics testing (Weir and Cockerham 1984), to attain the total variance among and within population group components at different hierarchical levels, between pairs by Jacckard coefficient at Bootstrap 100 of multilocus RAPD phenotypes, calculated using FAMD software. Results of population differentiation found in table (7) showed the total variation in *P. gossyoiella* treated populations was mostly attributable to diversity within populations (77.9%), whereas the variation of diversity among populations (20.6%). while the total observed phenotypic diversity among all populations was highly significant ($P < 0.001$). These results mean that insecticide treatment gave dissimilar results that can differentiate between each other of insecticide toxicities and efficiencies. Pimentel, *et al*., (2000), Meirmans and Liu (2018), said that phenotypic and genotypic approaches must be followed to determine similarities between populations using a matrix of squared molecular distances between pairs of observations.

Table 7: Analysis of molecular variance (AMOVA) for insecticide treated populations of *P.gossypiella* using random amplified polymorphic DNA.

Source	AMOVA	Statistics	Variance components	% Variance	df	P	phiST
Among populations	SSD(AP)	0.206	-0.009Va	-5.61	3	0.001	-0.056
Within populations	SSD(WP)	7.79	0.181Vb	105.6	43	0.001	
Total	**SSD(T)**	**7.99**	**0.171Vt**	**100**	**46**	**0.001**	

4-Genetic Relationships Displayed by RAPD Markers Analysis Using Cluster Analysis

The genetic relationships among four treatments as genotypes, the scoring data used to calculate similarity matrices, cluster analysis and a phylogenetic tree dendrogram using UPGMA method Euclidean, that provided molecular taxonomy and phylogeny of the insecticide tested and differentiation between them was easy. The cluster tree (Figure 2) showed that larvae treatment divided into two main groups with genetic similarity reached 80% between pairs of populations. The group defined divided into many sub-cluster with genetic similarity reached 60% of genetic distance. Similarity or distance measures are important components based clustering algorithms to cluster similar data points into the same clusters, while dissimilar or distant data points are placed into different clusters, its distribution mostly in two or three-dimensional spaces Shirkhorshidi *et al*., 2015.

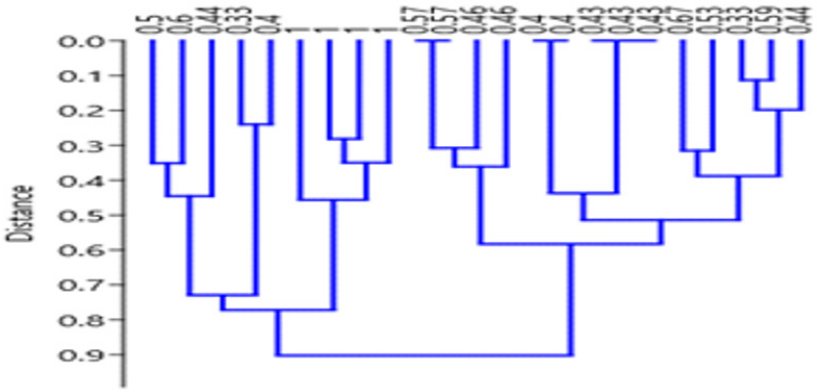

Figure 2: Dendogram of phylogenetic tree by UBGMA method attained by cluster analysis explain RAPD primer result of similarity matrices computed by past software according to Jacckard similarity coefficients of *P.gossypiella* insecticide treatments and control and showed mutation distribution of all treatments

5-Genetic Relationship Achieved by Two Statistical Analyses

Principal component analysis used for multivariate data of clustering with eigenvalues. However, the statistical options of genetic variation where groups of data plotted together based on presence versus absence of alleles in the molecular marker of data represented by figure (3 and 4), while similar individuals closed together but the dissimilar one were apart from graphical distribution. Cumulative percent of molecular genetic variation based on all axes showed the first three principal components covered 40%, of cumulative variation (Figure 3 and 4) belonged to dieumeron and spinoteram and greater than the total variation of metaflumezone, novaluron and control was 20%.

The total percentages of coordinate scores and correlation between treatments determinant of the population structure were completed and found in table (8), data revealed some score overlapping and percentage of molecular variation between insecticide treatments reached to 14.89 % of control, 9.27 of spinoteram, 6.15 of novaluron and 1.83 of dimeuron (table 8). The clustering is a technique aims to grouping a set of objects into clusters should be similar based on similarity, whereas objects in one cluster should be as dissimilar from objects in other clusters Irani *et al*., 2016.

Table 8: Pairwise genetic relationship of *P. gossypiella* insecticide treatments delivered by principle coordinate analysis.

Samples	Control	Spinoteram	Novaluron	Metaflumezone	Dimeuron	Eigien value	% of Variations
Control	0.50	0.021	0.020	0.00599	0.000117	7.54	14.89
Spinoteram	-0.121	-0.0199	0.10	-0.074	0.000117	4.69	9.27
Novaluron	-0.187	0.045	0.124	0.063	0.000116	3.11	6.15
Metaflumezon	-0.12	0.21	-0.146	-0.0068	0.000117	1.29	2.54
Dimeuron	-0.078	-0.25	-0.0104	0.011	0.000117	0.703	1.83

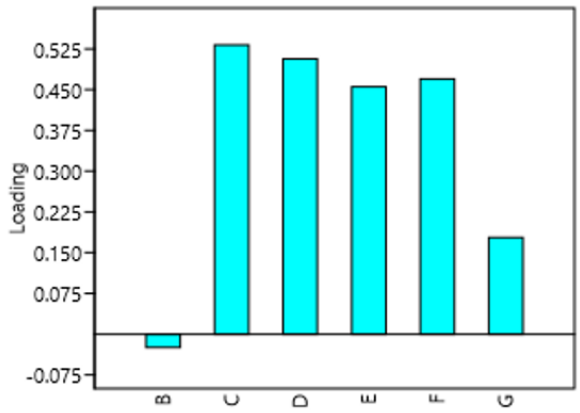

Figure 3: Differences between *P. gossypiella* insecticide treatments and control in principle component analysis showed percentage of variation arrangement calculated by Past software. Where: B=calibrator, C=control, D=spinoteram, E=novaluron, F= metflumezone and G=Dimeuron

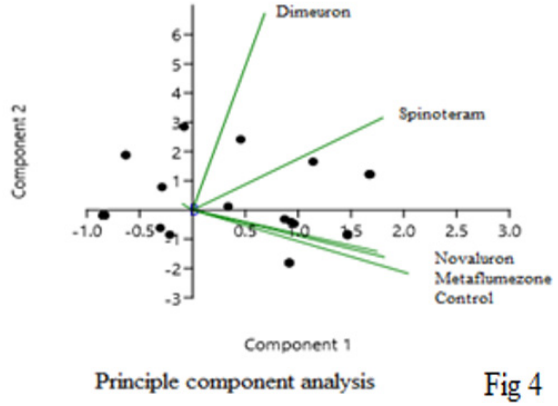

Figure 4: Principle component analysis illustrate similar and dissimilar group of treatments together by axes

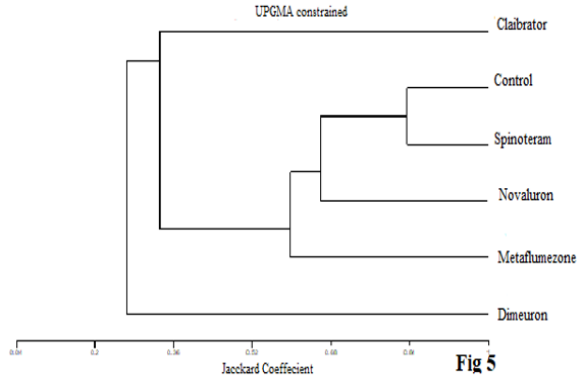

Figure 5: Phenotypic tree showed insecticide treatment of *P. gossypilella* efficiency according to UPGMA method.

Dendrogram generated by FAMD software expressing AMOVA, displaying relationships based on Dice Dissimilarity matrix to determine the genetic variability among population. Distance detected was 90, 20, 20 and 46 and Similarity was 90, 80, 80 and 53 for control, spinoteram, novaluron, metaflumezone and dimeuron.

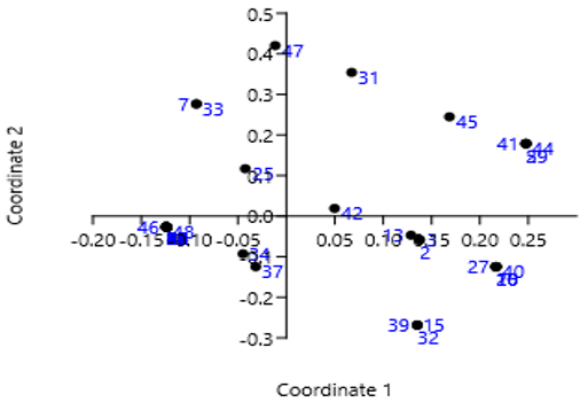

Figure 6: Coordinate analysis produced showed phylogenetic discrimination between all treatments of *P.gossypiella* via molecular variations percentage of each sample.

6- Population Polymorphism Features and Variations by Frequency Analysis

Some population structure parameters attained data and analyzed according to genetic diversity, or genetic differentiation among populations (insecticide treatments and control versus calibrator), data found in table (9). results attained was significant as FST values, gene flow using structure software for each locus, where observed homogeneity (Ho) and expected homozygosity (He) of individual clusters recorded based on conditions of H/W equilibrium, data completed and found in table (9). Between insecticide treated populations, the total genetic diversity over all subpopulations Ht was 4.4, 3.6, 3.6, 4, 1 and 2.1 and Hs is the genetic diversity intra-within subpopulations was 13.6, 11.4, 11.4, 12.9 and 6.9 for control, spinoteram, novaluron, metaflumezone and dimeuron respectively. The Nei's (1973), Wright's (1951) genetic diversity statistics FST in case of multiple alleles, where Dst is the total genetic diversity distributed among populations or inter population diversity estimated was 9.199, 7.799, 7.799, 8.799 and 4.799 respectively for the same arrangement. The genetic parameters including the number of a different allele (Na) with a frequency ≥ 0.05, the number of effective alleles (Ne) found on (Table 9). Shannon's index (I), (from FAMD software) (I) was 5.106, variance was 0.723 and Standard deviation was 0.852. The larger Shannon value means higher community diversity and differentiate between treatments. The estimated mean Fst value ranged from 0.20 to 0.695 for metaflumezone and dimeuron respectively; (F) Fixation Index was 0.316 for all treatment, (Nm) amount of gene flow ranged between, 0.2187 to 0.239 for dimeuron and spinoteram respectively. All values are significant at P< 0.001. The interpreting of all these values in brief was firstly: if all populations are completely different, then diversity will lie among populations, rather than within and (HS = 0) and GST values equal one. This can also occur when values of Ht are identical in all loci. Secondly, if the total diversity is contained within population (HS

= HT) for all loci, a GST value will be zero Culley *et al*., 2002. A wide range of GST (high values) usually resulted from a mixture of irregular allele at some loci and similar allele frequencies across populations at other loci (low GST). From literatures, the relatively low FST values refer to high-diversity in population and values of FST may depend on the PCR methods as for microsatellites may be lower than for SNPs Jakobsson *et al*., 2013.

Table 9: Genetic variations among insecticide treatments by genetic polymorphism information content according to genetic structure using fixation index (Fst) and of all primers used

Population	Ho	He	Hs	Ht	Na	Ne	F	Fst	Nm	Dst	Gst
Calibrator	1.0	0.75	11.4	34.6	0.3254	0.5714	0.333	0.670	0.2456	23.19	0.670
Control	1.0	0.756	4.4	13.6	0.3245	0.5695	0.316	0.676	0.2391	9.199	0.676
Spinoteram	1.0	0.799	3.6	11.4	0.3184	0.5559	0.316	0.684	0.2307	7.799	0.684
Novaluron	1.0	0.799	3.6	11.4	0.3184	0.5559	0.316	0.684	0.2307	7.799	0.684
Metaflumezon	1.0	0.741	4.1	12.9	0.3267	0.5744	0.316	0.20	0.2329	8.799	0.682
Demiron	1.0	0.729	2.1	6.9	0.3285	0.5784	0.316	0.695	0.2187	4.799	0.695

He= 1 − ∑pi2, F= Fixation Index= (He − Ho)/He, Fst = (H T – HS)/ H T, Fis= (Mean He − Mean Ho)/Mean He), Nm= 1/Fst/4Fst

Table 10: Correlation coefficient between insecticide treatments

Population	Calibrator	Control	Spinoteram	Novaluron	Metaflumezone	Dimeuron	Eigienvalue	% Insertia
Calibrator		0.2078	0.14602	0.49958	0.18702	0.63173		
Control	-0.18715		2.3926E-15	1.362E-07	1.286E-07	0.24551	0.0978	61.45
Spinoteram	-0.21535	0.86903		2.9213E-08	2.7118E-06	0.09443	0.0176	11.06
Novaluron	0.10095	0.68124	0.70625		0.0001295	0.09443	0.012	7.58
Metaflumezon	-0.19587	0.68222	0.62453	0.52954		0.0031771	0.007	4.46
Demiron	0.0717	0.1727	0.246	0.246	0.421	0	0.001	0.70

Some literature-cited information around genetic differentiation like Nei and Li 1979, said that there are two indexes, usually used for the polymorphism degree evaluation, are heterozygosity and homozygosity. Moreover, Hedrick (2005), Chesnokov, and Artemyeva (2015) cited that gene identification and mapping require data on genetic linkage in marker loci that possess alleles co-segregated with locus alleles and the verification process depends on the number of alleles, and their relative rates. Pimentel, *et al*., 2000, found that 19 loci were polymorphic in *T. controversa* population, whereas *T. bromi* contained 38 polymorphic loci and gene frequencies. Irregular frequencies at individual loci were largely due to fixation of different alleles and/or the loss of a common allele within a few populations, which could occur as a result of a reduction in effective population size and subsequent genetic drift Culley *et al*., 2002.

CONCLUSION

The genetic differentiation and population structure evaluated of *P.gossypiella* insecticide treated and untreated populations using RAPD primers for amplification showed high efficiency detected as molecular marker beginning from gel bands produced to all statistic accomplished. Variation of the genetic relationships attained by (UBGMA and PCA), among the evaluated insecticide treatments and divergence between them, are completed, where the low diversity refers to low genetic variation due to insecticide toxicity effect (treatments), and differs from wild genetic resources (control). Genetic structure differences detected served as genetic differentiation among populations that provide pressure forces quantifications that affect insect population genomes.

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