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Molecular Characterization of Codling Moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) in Swat Valley Pakistan using Randomly Amplified Polymorphic DNA (RAPD) Polymerase Chain Reaction

Hayat Zada[1], Ahmad-Ur-Rahman Saljoqi[1,*], Ijaz Ali[2], Bashir Ahmad[1], Abdul Waheed Khan[2] and Shabeer Ahmad[2]

[1]*Department of Plant Protection, The University of Agriculture, Peshawar*
[2]*Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar*

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Authors' Contribution
HZ designed the experiments, collected data. AURS wrote the article. IA provided RAPD primers and helped in lab work. BA helped in sample collection and review of literature. AWK helped in lab work. SA helped in data analysis.

Key words
***Cydia pomonella*, Molecular characterization, RAPD primers, PCR.**

ABSTRACT

Codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) is the most important insect pest of apple production in Pakistan. Genomic DNA was extracted from 30 overwintering larvae of each population of Matta, Madyan and Kalam regions. Out of 30 tested primers, 21 amplified 157 polymorphic bands in three populations. The mean gene frequency (f), genetic distance (I) and Shannon's information index (h) for three populations were 1.336, 0.304 and 0.437, respectively. Nei's unbiased measures of genetic identity and genetic distance revealed that higher genetic distance was observed among the isolates from Kalam and Madyan (97.87 %) whereas low genetic distance (35.58%) was calculated for *C. pomonella* isolates from Matta and Madyan. These results revealed that molecular variations among the population of *C. pomonella* might be attributed to the climatic conditions, geographical location and pest control programs.

INTRODUCTION

Codling moth (*Cydia pomonella*) is a serious insect pest of apple fruit in different parts of the world including Swat Valley in the North West of Khyber Pakhtunkhwa, Pakistan. A high fruit damage (80%) has been observed due to the insect in temperate parts of all major continents (Barnes, 1991). While being an economically important pest of the apple worldwide, the growers have a low tolerance (<1%) for its injury (Dorn *et al*., 1999). This has necessitated the adoption of different control strategies especially frequent applications of broad-spectrum synthetic insecticides throughout the fruiting period. Besides apple, this pest attacks pear, walnut, quince and some stone fruits causing economic losses in fruit production (Ciglar, 1998). The pest was originated in Eurasia, since last two centuries it dispersed around the world with the spread of the cultivation of apples and pears (Franck *et al*., 2007). It has achieved a nearly global distribution, being one of the most successful pest insect species known today (Thaler *et al*., 2008). Currently, *Cydia pomonella* is present in North America, South America, South Africa, Australia and New Zealand, India, Pakistan and Afghanistan (Franck *et al*., 2007).

Despite its economic importance, little is known about the genetic structure and patterns of gene flow at the local and regional scale, which are important aspects for establishing an area wide control strategy (Dorn *et al*., 1999; Calkins and Faust, 2003). Pest management strategies of *C. pomonella* include regular insecticide treatments, which are known to select for resistance to several insecticide groups (Knight *et al*., 1994; Sauphanor *et al*., 1998; Dunley and Welter, 2000). Therefore, a deeper understanding of the population variation of this pest could greatly benefit management decisions for its control (Timm *et al*., 2006).

It has adapted successfully to different habitats by forming various biotypes and populations, often designated as strains, which differ among each other in several morphological, developmental and physiological features (Meraner *et al*., 2008). Knowledge of the genetic variation within *C. pomonella* populations is necessary for their efficient control and management. Molecular technologies provide new ways to study population diversity as well

* Corresponding author: drsaljoqi@yahoo.com
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as to differentiate closely related species (Williams *et al*., 1990; Deverno *et al*., 1998).

Polymerase chain reaction (PCR) strategies offer increased sensitivity and speed for the molecular characterization and variation among the populations of insects species (Deverno *et al*., 1998). Different molecular techniques have been employed, randomly amplified polymorphic DNA (RAPD) is one of them because it is efficient and easy to use for the molecular variation among the populations. This technique has been developed to detect genetic variability by PCR amplification of arbitrary segments of genomic DNA by using short, random primers and thus does not require prior knowledge of a DNA sequence. Its low cost, its efficiency in developing a large number of DNA markers in a short time and the less sophisticated equipment that it requires has made RAPD a valuable technique (Bardakci, 2001; Delaat *et al*., 2005). RAPD markers are very well suited for use in insect phylogeny areas like the detection of genetic variability among populations as well as the identification of closely related species (Benecke, 1998; Lima *et al*., 2002).

Since limited studies have been reported regarding the characterization of *C. pomonella* in this area, the current study was undertaken to ascertain the molecular variation among the population of *C. pomonella*.

MATERIALS AND METHODS

Study area and parameters

Female larvae (In the last instar male larvae well-developed purple-red testes are seen through the skin of the fifth abdominal segment, contrary to the female larvae) trapped and collected by single face cardboard fastened around the apple tree at a distance of 30 cm from the ground following the procedures of Fritsch *et al*. (2005) with some modifications. In each population, 30 overwintering female larvae were randomly selected for DNA isolation to minimize DNA contamination by endoparasites (Landry *et al*., 1999) in autumn from Matta (350 55' 19.11" of latitude North and 720 30' 37.52" of longitude East) (920.30 meters), Madyan (350 08' 0.00" of latitude North and 720 32' 0.00" of longitude East) (1333.84 meters) and Kalam (350 28' 41.66" of latitude North and 720 34' 18.61" of longitude East) (2092.30 meters). To eliminate the effect of host association in discrimination of populations, all of the specimens were collected from "Red Delicious" apple orchards. The specimens were washed and stored in 96% alcohol (Ethanol) prior to analysis in the Health laboratory of Institute of Bio-Technology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar.

Genomic DNA extraction

For genomic DNA extraction, Spinklean Genomic DNA Extraction Kit (Thermoscientific® USA) was used and Zimmerman *et al*. (2000) procedures was followed with some necessary modifications. The specimens were crushed in liquid nitrogen. DNA was extracted using manufacture's manual. Briefly, TL Buffer (250ul) was added to crushed larvae for tissue lysis. The samples were then vortexed to crash properly mix. Total of 199 units of enzyme was added and mixed thoroughly. Lysis buffer of 220 µl was added to the solutions and mixed thoroughly. A volume of 560 µl Buffer TB was then added to each eppendorf tube and mixed comprehensively through vortex to get a homogeneous solution followed by incubation for 10 minutes at 65°C. Then 200 µl absolute ethanol was added. Sample mixtures were passed through column, assembled in clean collection tube by centrifuging at 8000 xg for 1 min. Column was washed twice with 750 µl wash buffer 'PS' and centrifuged at 8000 xg for 1 min. For removing traces of ethanol the column was centrifuged again at 10000 xg for another one minute.

About 200 µl of pre heated TE buffer was added to the column membrane in new tubes, incubated at room temperature for 2 min and centrifuged at 10000xg for 1min to get DNA which was then stored at -20°C.

Polymerase chain reaction

The polymerase chain reaction (PCR) was carried out in a 25 µl volume containing genomic DNA (150 ng), 0.25 mM of RAPD primers (Genlink, USA), 200 µM of each dNTP, 50 mM of KCL, 10 mM Tris, 1.5 mM MgCl2 and 2.5 unit of Taq polymerase (Thermoscientific®, USA). The amplification was carried out in 40 cycles of 50 second at 94°C, 1 min at 28°C, 1 min at 72°C, preceded by an initial denaturation at 94°C for 4 min and final extension at 72°C for 10 min at the end in a gene amp PCR system 2700 programmable thermo cycler. The amplification products were then detected on 2% agarose gel pre-stained with ethidium bromide.

Statistical analysis

For statistical analysis of randomly amplified polymorphic DNA (RAPD), every scorable band was considered as a single locus/allele. The loci were scored as present (1) or absent (0). Bivariate 1-0 matrix was generated. Genetic distances was calculated using "Unweighted Pair Group of Arithmetic Means" (UPGMA) procedure described by Nei and Lie (1979):

$$GD = 1 - dxy / dx + dy - dxy$$

Where, GD is genetic distance between two genotypes, dxy is total No. of common loci (bands) in two genotypes, dx is total No. of loci (bands) in genotype 1 and dy is total No. of loci (bands) in genotypes.

Table I.- Name, sequence, size and molecular weight of RAPD primer used for molecular characterization of *C. pomonella.*

S. No	Primer	Sequence	Size (bp)	Tm	M. wt	% GC
1.	GL Decamer B-12	CCTTGACGCA	10	29.5°C	2987.98	60
2.	GL Decamer D-16	AGGGCGTAAG	10	29.5°C	3117.04	60
3.	GL Decamer C-04	CCGCATCTAC	10	29.5°C	2947.96	60
4.	GL Decamer C-13	AAGCCTCGTC	10	29.5°C	2987.98	60
5.	GL Decamer B-04	GGACTGGAGT	10	29.5°C	3108.04	60
6.	GL Decamer H-02	TGTAGCTGGG	10	29.5°C	3099.04	60
7.	GL Decamer E-09	CTTCACCCGA	10	29.5°C	2947.96	60
8.	GL Decamer F-01	ACGGATCCTG	10	29.5°C	3028.00	60
9.	GL Decamer A-19	CAAACGTCGG	10	29.5°C	3037.00	60
10.	GL Decamer D-08	GTGTGCCCCA	10	33.6°C	3003.99	70
11.	GL Decamer G-11	TCCCCGTCGT	10	33.6°C	2994.99	70
12.	GL Decamer F-07	CCGATATCCC	10	29.5°C	2947.96	60
13.	GL Decamer E-18	GGACTGCAGA	10	29.5°C	3077.02	60
14.	GL Decamer H-13	GACGCCACAC	10	33.6°C	2981.97	70
15.	GL Decamer B-15	GGAGGGTGTT	10	29.5°C	3139.06	60
16.	GL Decamer C-16	CACACTCCAG	10	29.5°C	2956.96	60
17.	GL Decamer C-02	GTGAGGCGTC	10	33.6°C	3084.03	70
18.	GL Decamer H-03	AGACGTCCAC	10	29.5°C	2996.98	60
19.	GL Decamer F-04	GGTGATCAGG	10	29.5°C	3108.04	60
20.	GL Decamer H-13	ACCAGGTTGG	10	29.5°C	3068.02	60
21.	GL Decamer G-02	GGCACTGAGG	10	33.6°C	3093.03	70
22.	GL Decamer* A-06	GGTCCCTGAC	10	33.6°C	3003.99	70
23.	GL Decamer* A-07	GAAACGGGTG	10	29.5°C	3117.04	60
24.	GL Decamer* B-16	TTTGCCCGGA	10	29.5°C	3019.00	60
25.	GL Decamer* D-10	GGTCTACACC	10	29.5°C	2987.98	60
26.	GL Decamer* F-11	TTGGTACCCC	10	29.5°C	2978.98	60
27.	GL Decamer* G-13	CTCTCCGCCA	10	33.6°C	2923.95	70
28.	GL Decamer* G-15	ACTGGGACTC	10	29.5°C	3028.00	60
29.	GL Decamer* H-05	AGTCGTCCCC	10	33.6°C	2963.97	70
30.	GL Decamer* H-10	CCTACGTCAG	10	29.5°C	2987.98	60

* Indicates the RAPD markers giving no results (Bands). With result (Bands), 21; Without result (Bands), 09.

RESULTS

RAPD analysis

The results pertaining to gene frequency of the RAPD primers disclosed that out of 30 RAPD primers (Tables I and II), 21 primers gave the banding pattern as depicted in Table II. Highest gene frequency on the basis of amplification pattern was observed for RAPD primer GLH-13 (3.33) followed by GLH-14 (2.77), GLE-18 (2.66), GLG-11 (2.08), GLF-07 (1.85), GLH-03 and GLB-04 (1.66) each, GLC-16 (1.55), GLC-06 (1.55), GLC-02 (1.46), GLE-09 (1.41), GLF-04 (1.42) and GLD -08 (1.03), whilst the rest of RAPD primers depict the allele frequency below 0.99. The overall mean of the genetic frequency among three population of *C. pomonella* was 1.33.

On the basis of amplification pattern, the genetic diversity of the three population of the *C. pomonella* expounded that highest genetic diversity was recorded for the RAPD primers GLC-02, GLE-18 and GLC-16 (0.44 each), followed by GLC-13 (0.38), GLH-14 (0.37), GLA-19 (0.35), GLB-15 (0.34), GLB-12 (0.33) and GLB-04 (0.32), whilst the rest of the RAPD primers explicated below 0.29 genetic diversity among three population of the *C. pomonella*. The overall mean of the genetic diversity of the three population was 0.29.

The results pertaining to Shannon's information index for each allele of the primers revealed that maximum Shannon information index was observed for GLC-04, GLE-18 and GLC-16 (0.63), followed by GLC-13 (0.55), GLH-13 (0.53), GLH-0.53) and GLA-19 (0.50), whilst the rest of the primers were less than 0.49 Shannon's information index values. The overall mean of the Shannon's information index was 0.44.

Table II.- Mean gene frequency, diversity and Shannon information index for RAPD primers used for molecular characterization of *C. pomonella* at Swat during the year 2012-13.

S. No	RAPD primers used	Total alleles amplified	Range of allele size (bp)	G. F (f)[1]	G. D (I)[2]	S. I. I. (h)[3]
1.	B-12	08	250-10000	0.4166	0.3333	0.4773
2.	D-16	08	250-10000	0.2500	0.2222	0.3182
3.	C-04	06	750-10000	0.5555	0.4444	0.6365
4.	C-13	08	250-10000	0.9166	0.3888	0.5569
5.	B-04	11	250-4500	1.6666	0.3232	0.4629
6.	H-02	10	250-4500	0.9999	0.1333	0.1909
7.	E-09	08	250-10000	1.4166	0.2777	0.3978
8.	F-01	06	250-10000	0.1111	0.1481	0.2121
9.	A-19	10	250-10000	0.8000	0.3555	0.5092
10.	D-08	09	250-3500	1.0370	0.2962	0.4243
11.	G-11	08	250-3500	2.0833	0.2777	0.3978
12.	F-07	09	250-4000	1.8518	0.2468	0.3978
13.	E-18	08	250-3000	2.6666	0.4444	0.6365
14.	H-14	06	250-3000	2.7777	0.3707	0.5304
15.	B-15	09	250-3000	0.5185	0.3456	0.4950
16.	C-16	03	750-1500	1.5555	0.4444	0.6365
17.	C-02	05	250-1500	1.4666	0.2666	0.3819
18.	H-03	04	500-1500	1.6666	0.2222	0.3182
19.	F-04	07	250-2500	1.4284	0.1904	0.2727
20.	H-13	06	250-2000	3.3333	0.4444	0.5304
21.	G-02	08	250-3000	0.5416	0.2222	0.3978
Mean		07	250-10000	1.33618	0.30467	0.4372

1, gene frequency (f); 2, genetic distance (I); 3, Shannon information index (h).

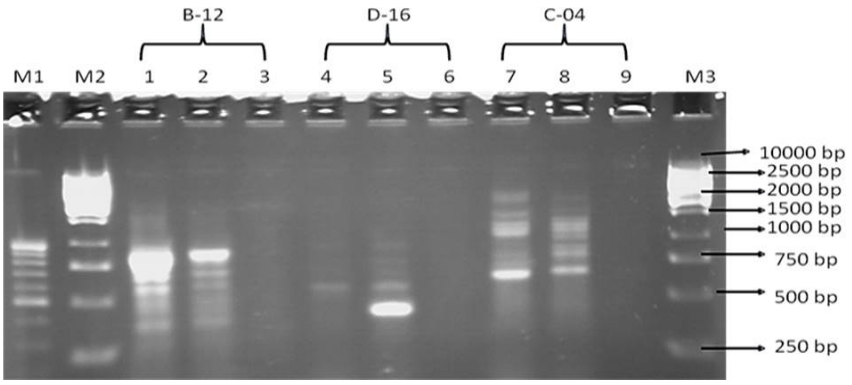

Fig. 1. Electrophoreogrm showing PCR based amplification products of Coding moth *Cydia pomonella* population collected from three regions (Matta, Kalam and Madyan) of District Swat by using RAPD primers B-12, D-16 and C-04.

Nei's unbiased measures of genetic identity and genetic distance

Table III, pertaining to the Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among the three population of *C. pomonella*. Higher genetic distance was observed among the isolates from Kalam and Madyan (97.87 %) whereas low genetic distance (35.58%) was calculated from the *C. pomonella* isolates from Matta and Madyan, which indicates that the population of *C. pomonella* in both the regions has not so variation/diversity as compared to population in Kalam. Similarly the Nei's genetic identity revealed that higher genetic similarity (70.06%) was resided by the *C. pomonella* population at Matta and Madyan while the low level of identity (37.58%) were examined in isolates from Madyan and Kalam (Fig. 3).

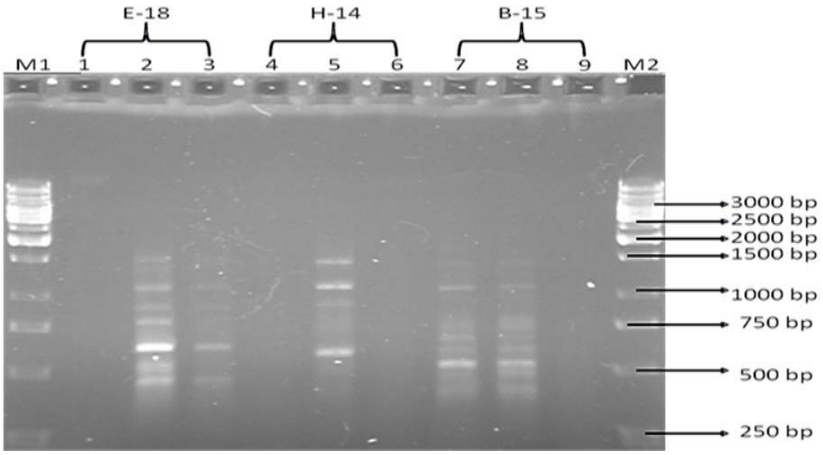

Fig. 2. Electrophoreogrm showing PCR based amplification products of Coding moth *Cydia pomonella* population collected from three regions (Matta, Kalam and Madyan) of District Swat by using RAPD primers E-18, H-14 and B-15.

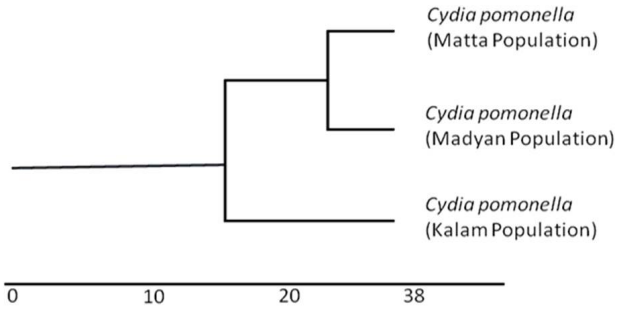

Fig. 3. Dendogram constructed on the basis of similarity index among three populations of *Cydia pomonella* (Matta, Kalam and Madyan) based on RAPD data using UPGMA and Nei's genetic index.

Table III.- Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) for *C. pomonella* populations collected from three geographically distant region Swat based on 21 RAPD primers analysis

Population	Matta	Kalam	Madyan
Matta		0.5732	0.7006
Kalam	0.5564		0.3758
Madyan	0.3558	0.9787	

DISCUSSION

On the basis of amplification pattern, highest gene frequency (f) was evaluated for RAPD primer GLH-13 (f = 3.33) followed by GLH-14 (f = 2.77), GLE-18 (f = 2.66), GLG-11 (f = 2.08), GLF-07 (f = 1.85), GLH-03 and GLB-04 (f = 1.66) each, GLC-16 (f = 1.55), GLC-06 (f = 1.55), GLC-02 (f = 1.46), GLE-09 (f = 1.41), GLF-04 (f = 1.42) and GLD-08 (f = 1.03), whereas the rest of RAPD primers depicted the allele frequency below 0.99. The overall mean of the genetic frequency among three population of *C. pomonella* observed was 1.33. These results are in close conformity with findings of Lei-Men *et al*. (2012), who reported that the mean number of alleles per locus ranged from f = 4.3 to f = 12.6 and two populations of *C. pomonella* from Heilongjiang Province in northeastern China had the largest number of alleles (f = 12.6 and f = 10.6). From populations of northwestern China, one population showed the highest value of mean number of alleles (f = 9.6), followed by the second population (f = 8.6) and third population (f = 8.4). Nevertheless, the gene frequency of null alleles ranged from 0.010 to 0.203, values typical for lepidopterans (Megle'cz *et al*., 2004; Dakin and Avise, 2004), which further confirmed these results.

On the basis of intensification pattern, the genetic diversity (I) of the three population of the *C. pomonella* disclosed that highest genetic variation was detected for the RAPD primers GLC-02, GLE-18 and GLC-16 (I = 0.44 each), followed by GLC-13 (I = 0.38), GLH-14 (I = 0.37), GLA-19 (I = 0.35), GLB-15 (I = 0.34), GLB-12 (I = 0.33) and GLB-04 (I = 0.32), while the rest of the RAPD primers elucidated below 0.29 genetic diversity among three population of the *C. pomonella*. The overall mean of the genetic diversity of the three population was 0.29. These results are in close concordance with findings of Khaghaninia *et al*. (2009), they find out that by using RAPD primers genetic diversity within population of *C. pomonella* based on Nie's gene index ranged from 0.228 to 0.281 at Shabestar and Zunuz populations, respectively. They also observed the maximum (0.14) and minimum (0.04) genetic distances between the population of *C.*

pomonella at different geographical locations in Iran. Contrary to our results regarding genetic diversity among the population of *C. pomonella*, Bues *et al*. (1995), observed low genetic differentiation between sampled populations of *C. pomonella* by using allozyme markers. These results are also in close agreement with findings of Chen and Dorn (2010) reported important genetic differentiation at local geographic scale (even less than 10 km), which they mostly attributed to the sedentary behaviour of *C. pomonella* through microsatellite study on populations from Switzerland. Bayar *et al*. (2006) through investigations on population variation of *Aeolothrips intermedius* Bagnall, found population-specific RAPD primers for their molecular differentiation.

The highest Shannon's information index (h) for each allele of the primers GLC-04, GLE-18 and GLC-16 were h = 0.63, followed by GLC-13 (h = 0.55), GLH-13 (h = 0.53) and GLA-19 (h = 0.50), whereas the rest of the primers were less than 0.49 Shannon's information index (h) values. The overall mean of the Shannon's information index (h) was 0.44. These results are closely corroborated with findings of Timm *et al*. (2006) who reported that genetic diversity was found to be high within the South African *C. pomonella* population (h = 0.18). Only three amplified fragment length polymorphism (AFLP) fragments were monomorphic at the 95% level, resulting in 98.60% of the scored loci being polymorphic. They further stated that gene diversity within *C. pomonella* populations collected from different regions varied, whereas gene diversity within the English and Canadian populations was reckoned as h = 0.046 and 0.052, respectively.

The results pertaining to the Nei's genetic identity and genetic distance among the three population of *C. pomonella* was also worked out. Higher genetic distance was resided among the isolates from Kalam and Madyan (97.87%) whereas low genetic distance (35.58%) was observed in the *C. pomonella* isolates from Matta and Madyan. It indicates that the population of *C. pomonella* in both of the regions has not showed multiplicity as compared to population at Kalam. These results are in close conformity with findings of Khaghaninia *et al*. (2009), who observed maximum and minimum genetic distances between the population of *C. pomonella* at different geographical locations in Iran and significant correlation was noticed between genetic and geographic distance matrices in the population of *C. pomonella* revealed by Mantel test. It is assumed that due to climate change and frequent insecticide treatments, *C. pomonella* populations differentiate into many ecotypes with different biological and physiological requirements related to their development (Thaler *et al*., 2008). These results contrast with those obtained for *C. pomonella* populations from France and Switzerland, in which allozyme analysis indicated a large degree of genetic similarity between geographic populations (Bue's and Toubon, 1992). Nonetheless, the molecular variability among and between populations of *C. pomonella* has been determined in Italy by using RAPD primers (Gomez *et al*., 2004, 2005).

Likewise, the Nei's genetic identity was also reckoned. The higher genetic similarity (70.06%) was dwelled by the *C. pomonella* population at Matta and Madyan whilst the low level of identity (37.58%) was examined in isolates from Madyan and Kalam. These results are supported by the study of Timm *et al*. (2006), who used AFLP markers and successfully ascertained differences among sampled *C. pomonella* populations even at small geographic distances. Besides, Timm's *et al*. (2006) study was corroborated by Thaler *et al*. (2008), who also used AFLP markers to study the molecular phylogeny and genetic diversity of *C. pomonella* population. Deverno *et al*. (1998) distinguished two closely related species of lepidopterious moths using 17 species-specific RAPD primers. RAPD primers have also been used for studying the population diversity of the Hessian fly, *Mayetiola destructor* Say, and the wheat stem sawfly, *Cephus cinctus* Fitch, in Syria and America, respectively (Lou *et al*., 1998; Naber *et al*., 2000).

CONCLUSION

RAPD markers are efficient tools for assessing the population variation in insect pests and knowledge of the genetic variation within *C. pomonella* populations is necessary for their efficient control and management, thus such studies may offer an insight on the possible resistance to insecticides. Higher genetic distances among the populations of *C. pomonella* could be attributed to climatic conditions of the studied areas, geographical locations and elevations. Nonetheless, further studies are required to assess the molecular variation among the population of this pest.

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Statement of conflict of interest

The authors declare no conflict of interest.

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