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RESEARCH ARTICLE

Detection of Genetic Diversity among Populations of *Ectomyelois ceratoniae* Using RAPD Markers

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Abstract

Ectomyelois ceratoniae (carob moth) (Lepidoptera: Pyralidae) is a serious pest of stored products and fruit trees throughout the world. It is the most important pest of pomegranate, *Punica granatum L.*, in Taif governorate, the larva of which feed inside the fruit and highly affects fruit quality. The high yield and quality of pomegranates in Taif has made it an important export commodity. Hence the objective of the present study is to investigate the genetic variation among three geographic populations of carob moth, *Ectomyelois ceratoniae* in Taif using the Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) technique. We estimated the genetic diversity among three carob moth populations from three different regions; North, South and middle Taif. Initially, ten primers were screened among all the populations of which five primers amplified the genomic DNA. Three primers generated reproducible and distinct RAPD profiles and were used for further analysis. RAPD profiles exhibited bands between 174 to 1432 bp in length. Average genetic distances among populations ranged from 0.1257 to 0.6657. The estimated average Gst value across all loci was 0.1974, suggesting (very) low gene flow among the different localities. The phylogenetic tree constructed by unweighted pair-group method of analysis (UPGMA) shows the North, middle and South populations, respectively, seems to be approximately as closely linked to each other from the dendrogram. The North population is more related to the middle populations. We conclude that there is a high level of genetic variation and population differentiation indicated dynamic evolution in these populations as revealed by variation at RAPD loci. This information had a significant impact on the development and delivery of the effective control program against the carob moth.

Introduction

The diversity of phytophagous insect assemblages is set both by plant and insect attributes. Insect-plant evolution can accordingly be approached either from a phytocentric or from an entomocentric standpoint. Pomegranate, Punica granatum L. (Myrtales: Ponicaceae), is the main host of the carob moth, *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae) in Taif province. The high yield and quality of pomegranates in Taif has made it an important export commodity. The most important pest on this fruit is carob moth, *Ectomyelois ceratoniae* **(Zeller, 1839)** (Lepidoptera: Pyralidae), the larva of which feed inside the fruit and highly affects fruit quality. This moth is commonly found in pomegranate orchards in Taif, but there are only a few records of its damage on other host plants such as fig **(Shakeri 1993)** and pistachio **(Mehrnejad 2002);** it is a major pest on citrus, date, almond and etc. in the other countries **(Morton 1987; Alrubeai 1987; Warner et al. 1990; Van den Berg 1995; Tous and Ferguson 1996; Mesbah et al. 1998; Bouka et al. 2000).** The most recommended control method for this pest is by

collecting and destroying infected pomegranates at the end of growth season that eliminates over-wintering sites **(Behdad 1991).** This control method has also been used for controlling the pest on other fruit such as macadamia **(Van den Berg 1995).** Biological control **(Nasrollahi et al. 1998),** staffing the pomegranate fruit neck **(Mirkarimi 1996),** and removing flags **(Shakeri 2004)** are other methods that have been described. The two latter methods also remove hatching sites.

Larvae feed on inner parts of the fruit and highly reduce its quality indices. Several other host plants of *E. ceratoniae*, such as citrus, date and almond have been recorded **(Morton 1987; Alrubeai 1987; Warner et al. 1990; Van den Berg 1995; Tous and Ferguson 1996; Mesbah et al. 1998; Bouka et al. 2000).** The most recommended control method is collecting and destroying infected pomegranates that eliminate over-wintering sites at the end of growth season **(Behdad 1991).** This control method has also been used for macadamia **(Van den Berg 1995).** Biological control was used by **Nasrollahi et al. (1998).** Two other methods, staffing the pomegranate fruit neck **(Mirkarimi 1996)** and removing its flags **(Shakeri 2004)** were suggested to eliminate the sites that the moth uses to lay eggs. These methods make these places inconvenient for laying eggs. In spite of the important role of the genetic structure of insect populations in pest management, there is no documented information on genetic aspects of different *E. ceratoniae* populations.

Larvae are attractive targets and can be easily eradicated at their breeding sites for which an accurate identification of the species is required to determine whether it belongs to a species group that poses a potential risk. Molecular markers such as random amplified polymorphic DNA (RAPD), single strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) are providing new perspectives in the field of medical entomology for the genetic characterization of cryptic species of disease vectors. In this study RAPD-PCR markers were used to determine the differences and/or similarity among several geographic populations of *E. ceratoniae.* Among different data sets, genetic data are basic to the design and successful application of any pest management strategy **(Sluss and Graham 1979).** Estimating genetic variation and genetic structure of natural populations relies on genotyping individual specimens **(Yan et al. 1999).** The analysis of genetic variation using DNA markers has become an important approach for assessing the population genetics of a variety of insect species **(Reineke et al. 1998).** To analyze the genetic data, several techniques can be used.

The Random amplified polymorphic DNA markers or RAPD technology is a powerful method for population genetic studies that was described by **Vos et al. (1995).** It has provided a quick and efficient screen for DNA sequence-based polymorphisms at a very large number of loci. The major advantage is that no prior DNA sequence information is required. The vast range of potential primers that can be used gives the technique great diagnostic power. Reproducible RAPD bands can be found by a careful selection of primers, optimization of PCR conditions for the target species and replication. This method has been applied in many aspects of insect population studies such as distinguishing different geographic populations of gypsy moth, Lymantria dispar L. **(Reineke 1998),** host-associated strains of fall army worm, Spodoptera frugiperda **(McMichael and Powell 1999)** and indicating host-associated lineages of the snakeweed grasshopper, Hesperotettix viridis **(Sword et al. 2005).** Hence, the present study aimed to evaluate RAPD-PCR markers for population-level studies in crab moth to assess the genetic diversity of the tropical *E.ceratoniae* inhabits the Taif in King Saudi Arabia. To our knowledge, the present study is the first one to show the genetic diversity among *E.ceratoniae* in K.S.A.

MATERIALS and METHODS

1. Samples collections

A total of thirteen *E.ceratoniae* larvae were collected from three regions, North, South and middle, (Sased, Bany Saad and Wadi-Wag respectively) in Taif province of Western K.S.A on host of pomegranate, P. During sampling, some similar larvae belonging to other Lepidoptera could also be found inside collected fruits. These larvae were reared in their natural infected host to adulthood and species identification was performed on adults. Adults of *E. ceratoniae* were separated and kept at −40° C after emergence.

2. DNA Extraction

Genomic DNA was extracted according to the protocol of **Sambrook and Russell (2001).** Briefly, larvae tissues were homogenized in 1.5 ml microcentrifuge tube containing 1 ml lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1% SDS and 100 µg/ml proteinase K.) pH 8.0. Samples were incubated either for 3 hours at 55°C or for 16 h at 37°C. DNase-free RNase was added to a final concentration of 20 µg/ml and the digest incubated for another 60 min. After precipitation of the SDS by adding potassium acetate solution to a final concentration of 0.75 M in respect of potassium and 1.25 M in respect of acetate, the DNA was precipitated with one volume of isopropanol.

The pellet was washed with 70% ethanol, dried and redissolved in 10 mM Tris-HCl (pH 8.0) and stored at -20°C until used.

3- Quantity, purity and quality of DNA

Quantitative estimation of DNA samples was done by a double beam UV-Spectrophotometer (Shimadzu, UV-2450-Japan) by measuring the DNA concentration at 260 nm and 280 nm. Purity of DNA was checked by means of absorbance ratios A260/A280 for protein contamination. Further, the samples were run on 1% agarose electrophoresis to check the quality of DNA **(Sambrook et al., 1989)** along with one kb plus DNA ladder (GeneRulerTM, Fermentas). Only high quality of DNA was used for RAPD analysis. The bands were visualized under UV light in Gel Doc XR system (Bio-Rad, USA).

4- RAPD – PCR amplification

To generate RAPD profiles from *E. ceratoniae* larvae DNA, a set of 10 oligodecamers primers (**Table1**) from the Operon Technologies (Operon Technologies Inc., Alameda, Calif.: A, B, C and D) were used. Primers were designated as useful if they yielded well-amplified, distinguishable polymorphic bands. Finally three primers (OPC20, OPA09 and OPB14) were selected and used to amplify DNA from all individuals. DNA amplification reactions were performed under conditions reported by **Williams et al. (1990) and Hassan et al., (2012).**
Polymerase chain reactions for random amplified polymorphic DNA (RAPD) analysis were carried out in 25 µl volume. Each reaction tube contained 20 ng of genomic DNA, 1.0 U of Taq DNA polymerase (Invitrogen), 0.2 mM of each dNTP (Fermentas), 2.5 mM MgCl2, and 10 pmol of a decanucleotide primer. The amplifications were carried out by using a thermal cycler (MJ-Mini, Bio-Rad, USA) programmed at 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, a final extension step at 72°C for 5 min and stored at 4°C. A volume of 5 µl of each sample was mixed with 2 µL 6× gel loading buffer and used for electrophoresis on 1.2% agarose gel. RAPD patterns were visualized and documented using the Gel Documentation system, Gel-Pro Analyzer (Media Cybernetics). A Φx174 DNA digested with HaeIII were used as known molecular size DNA markers.

Table 1: Sequence, operon codes and GC content of random primers used to study variation in *E.ceratoniae*

Primers	Sequence (5'- 3')	GC%	Primers	Sequence (5'- 3')	GC%
OPB02	**5'-TGATCCCTGG-3'**	**60**	**OPA15**	**5-TTC CGA ACC C-3**	**60**
OPB04	**5'-GGACTGGAGT-3'**	**60**	**OPC18**	**5'TGAGTGGGTG-3'**	**60**
OPB14	**5-TCC GCT CTG G-3**	**70**	**OPC20**	**5'-ACTTCGCCAC-3'**	**60**
OPA07	**5-GAA ACG GGT G-3**	**60**	**OPD01**	**5'-ACCGCGAAGG-3'**	**70**
OPA09	**5-GGG TAA CGC C-3**	**70**	**OPD06**	**5'-ACCTGAACGG-3'**	**60**

5- Recording of data and statistical analysis

RAPD patterns were scored for the presence and absence of amplicons. In a binary matrix the presence of a band was recorded as one and the absence as zero. The scores obtained using all primers in the RAPD analysis were then combined to create a single data matrix. This was used for estimating polymorphic loci, **(Nei's 1973)** gene diversity (h), allele frequencies, genetic distance (D) and genetic identity (I). All calculations were carried out using the population genetic analysis software, PopGene version 1.31 **(Yeh et al., 1999).**

RESULTS

A band was considered to be polymorphic if it was present in at least one genotype and absent in the others. A data matrix was generated in which each band was scored 1 if present and 0 if absent. The percentage of polymorphic loci, Nei's genetic diversity **(Nei, 1978),** Shannon index, average heterozygosity, and genetic distance **(Nei, 1972)** were calculated using POPGENE v. 1.31. Statistica 6.0 was used to test the difference in intrapopulation genetic distance and average heterozygosity between populations. A dendrogram of the three populations was constructed based on the unweighted pair-group method with arithmetic means (UPGMA) in POPGENE 3.2.
Selectively amplified results of 10 primer combinations were analyzed in this study. A total of 127 bands were identified in the three populations of carob moth, and the average was 12.7 bands for each primer combination. The average heterozygosity and percentage of polymorphic loci for each population are summarized in **Table (2)** and **Figurs (1, 2 & 3).** Great variation in genetic diversity was observed among populations, as indicated by the average heterozygosity and percentage of polymorphic loci. The lowest percent polymorphism (18.34%) was that of the Bany-Saad population, while, the two other populations the percentage of polymorphism reach to 28.27% in Sased

and 27.78 % in Wadi-Wag. A GST analysis was performed to estimate differences among locations. The estimated GST value averaged over all polymorphic loci was 0.2908, indicating a strong population structure, **Table (3).**

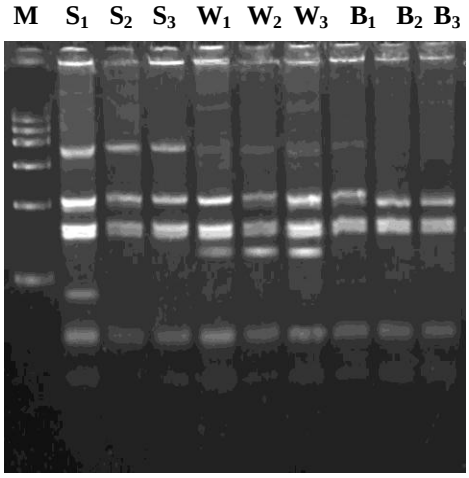

Fig.1 Random amplified polymorphic DNA (RAPD) profile generated by primer OPB14 in individual *E. ceratoniae* populations, Sased, Bany Saad and Wadi-Wag. Lane M = molecular marker (Φx174 DNA HaeIII digest). Lans S1 –S3 (Sased). Lans W1 – W3 (Wadi-Wage). Lans B1 – B3 (Bany Saad).

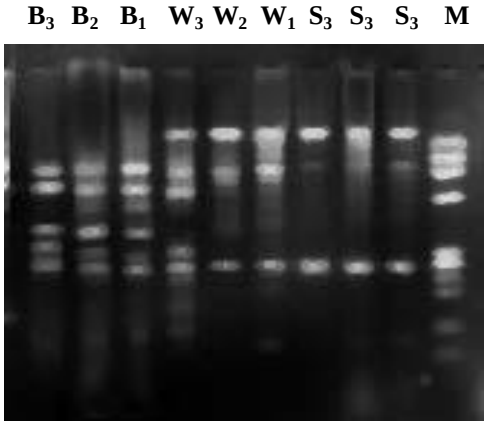

Fig.2 Random amplified polymorphic DNA (RAPD) profile generated by primer OPC20 in individual *E. ceratoniae* populations, Sased, Bany Saad and Wadi-Wag. Lane M = molecular marker (Φx174 DNA HaeIII digest). Lans S1 –S3 (Sased). Lans W1 – W3 (Wadi-Wage). Lans B1 – B3 (Bany Saad).

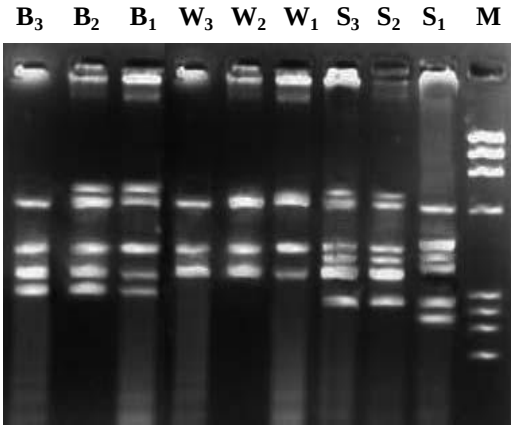

Fig.3 Random amplified polymorphic DNA (RAPD) profile generated by primer OPA09 in individual *E. ceratoniae* populations, Sased, Bany Saad and Wadi-Wag. Lane M = molecular marker (Φx174 DNA HaeIII digest). Lans S1 –S3 (Sased). Lans W1 – W3 (Wadi-Wage). Lans B1 – B3 (Bany Saad).

Table 2: Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

pop ID	Sased	Wadi-Wag	Bany-Saad
Sased	****	0.5139	0.8819
Wadi-Wag	0.1257	****	0.5745
Bany-Saad	0.6657	0.5543	****

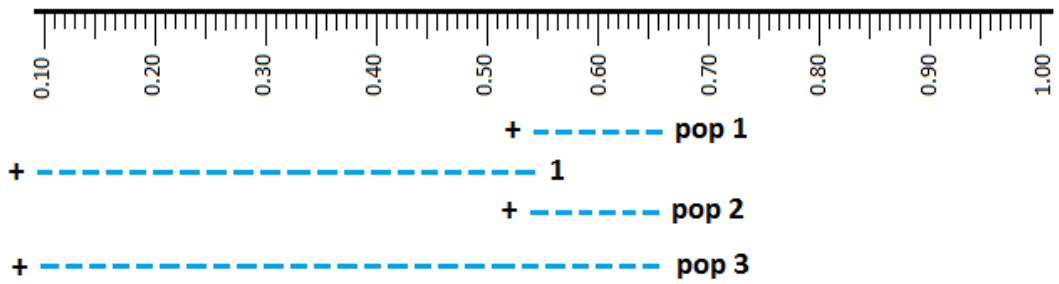

Fig 4. UPGMA dendogram generated from Nei, s genetic distances of the three populations of *E. ceratoniae*

Table 3. Genetic diversity of the three population of *E. ceratoniae*

Population	No. of alleles	Effective No. of alleles	Nei's gene diversity	Shannon's information index	Percentage polymorphic loci
Sesed	1.2747±0.4014	1.2556±0.3213	0.1256±0.178	0.1826±0.2354	28.27 %
Wadi-W	1.2578±0.4543	1.2424±0.3634	0.1235±0.193	0.1768±0.2651	27.78 %
Bany-S	1.0833±0.2803	1.1334±0.3222	0.0987±0.197	0.1076±0.2752	18.34 %

Table 4. Nei's analysis of gene diversity in *E. ceratoniae* populations.

Total gene diversity	Total gene diversity	Gene diversity within population	Coefficient of gene differentiation (GST)	Gene flow (Nm) Nm = 0.5 (1 - GST) / GST
Mean	0.2245	0.1481	0.2904	1.5643
Standard deviation	0.0573	0.0475		

The individual-based similarity trees with the highest identity values, and therefore the best fit between the distance matrix and corresponding tree, were produced through UPGMA cluster analysis (Fig 4). UPGMA was carried out to demonstrate graphically the genetic similarities among E. ceratonia populations. The genetic identity among populations ranged from a low of 0.5139 between Sased and Wadi-Wage populations to a high of 0.8819 between the Bani-Saad and Sased populations (Table 2). Nei's genetic distances for all pairwise comparisons were significantly different from zero. The genetic distance value (0.6657) between the Sased and Bany-Saad populations was the highest, whereas the value (0.1257) between the Sased and Wadi-Wage populations was the lowest (see Table 2). Therefore, UPGMA dendogram generated from Nei's genetic distance of the three E. ceratonia populations is illustrated in Figure 4. The cluster analysis shows that carob moth from the Sased and Wadi-Wage populations, which were geographically, could be closely clustered together. While, the Bany Saad population, which was located far from the other two populations, was clustered into a separate branch.

DISCUSSION

Genetic diversity information among and within populations of the carob moth in Taif indicated that application of PCR based RAPD fingerprinting using whole DNA and arbitrary primers would provide a rapid and sensitive methods for detection of genetic variations among different isolation of E ceratonia population. The RAPD assay has the potential to make useful contributions to genetic analysis of relatedness among species **(Cushwa and Medrano, 1996).** Our findings clearly indicated that some distinct differences exist among the three populations ranging from small to medium significant variations among geographic populations. The significant variation has also been shown in many other insects including Lepidoptera **(Coates et al. 2004; Koshio et al. 2002; Suinaga et al. 2004; Timm et al. 2006).** Since test geographic populations were all associated with the same host (pomegranate), population differences may have been mainly the result of geographic barriers that are defined as any terrain that prevents gene flow between populations **(Mayr and Ashlock 1991).** Sometimes distance alone can function as a barrier to genetic exchange among populations **(Ruggiero et al. 2004).** The Bany-Saad population showed the most distinct difference with the allelic and gene diversity. High genetic diversity of introduced populations can be attributed to several factors: multiple introductions **(Kolbe et al., 2004),** a single introduction of a large number of individuals from different populations **(Yue et al., 2010).** In the present study, **Figure 4** shows that distances associated with the two populations (Sased and Bany-Saad) correspond to high genetic distances in spite of rather short geographic distances. It can be concluded that geographic distances do not completely explain genetic distances associated with the two populations and there should be some other factors that isolate and decrease gene flow of this population to the others. Human transportation can play a noticeable role in creating similarity in different populations. **Failloux et al. (1997)** found significant correlation between gene flow among populations of Aedes polynesiensis and human transportation instead of geographic distances **(Chen et al. 2004; Goodisman et al. 2001).** Transportation of pomegranate fruit and young trees is presently being makes it impossible to trace movement of them. Hence, transporting pomegranate fruits and young trees to other places seems to be very possible. Whereas the value (0.1257) between the Sased and Wadi-Wage populations was the lowest (see **Table 2**). The short genetic distance and the highest gene flow **(Table 2)** between Sased and Wadi-Wage populations suggest high transportation between these places. It seems that there is enough genetic variation in natural populations of *E. ceratoniae* to show alternative phenotypes in response to control practices such as omitting places for over-wintering or sites for oviposition. Infecting other fruits, hatching on the other parts of the pomegranate fruit instead of neck such as skin and cracks of the fruit have been recorded in low density **(Shakeri 2004),** and are alternatives that may be selected against control practices. Successful invasive species are generally thought to have high genetic diversity, which allows them to escape the harmful effects of inbreeding and adapt to their new environment **(Spielman et al., 2004).**

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REFERENCES

- **Alrubeai HF. 1987.** Growth and development of Ectomyelois ceratoniae (Lepidoptera: Pyralidae). Journal of Stored Product Research 23: 133-135.
- **Behdad E. 1991.** Pests of fruit crops in Iran, 2nd edition. Markaz-e-Nashr-e-Bahman
- **Bouka H, Chemseddine M, Abbassi M, Brun J. 2000.** La pyrale des dattes dans la region de Tafilalet au Sud-Est du Maroc. Fruits 56: 189-196.
- **Chen H, Minakawa N, Beier J, Yan G. 2004.** Population genetic structure of Anopheles gambiae mosquitoes on Lake Victoria islands, west Kenya. Malaria Journal 3: 48
- **Coates BS, Sumerford DV, Hellmich RL. 2004.** Geographic and voltinism differentiation among North American Ostrinia nubilalis (European corn borer) mitochondrial cytochrome c oxidase haplotypes 9. Journal of Insect Science 4:35.
- **Cushwa WT, Medrano JF (1996).** Applications of the random amplified polymorphic DNA (RAPD) assay for genetic analysis of livestock species. Anim Biotechnol.7:11–31.
- **Failloux AB, Raymond M, Ung A, Chevillon C, Pasteur N. 1997.** Genetic differentiation associated with commercial traffic in the Polynesian mosquito, Aedes polynesiensis Marks. Biological Journal of the Linnean Society 60: 107-118.
- **Goodisman MAD, Matthews RW, Crozier R. 2001.** Hierarchical genetic structure of the introduced wasp Vespula germanica in Australia. Molecular Ecology 10: 1423-1432.
- **Hassan, A.M.; Abdel-Aziem, S.H.; Abdel-Wahhab, M.A. 2012**. Modulation ofDNA damage and alteration of gene expression during aflatoxico-sis via dietary supplementation of Spirulina (Arthrospira) and wheyprotein concentrate, Ecotoxicol. Environ. Saf. 79 (2012) 294–300.
- **Kolbe JJ, Glor RE, Rodriguez SL, Lara AC, et al. (2004).** Genetic variation increases during biological invasion by a Cuban lizard. Nature 431: 177-181.
- **Koshio Ch, Tomishima M, Shimizu K, Kim HS, Takenaka O. 2002.** Microsatellites in the gypsy moth, Lymantria dispar L. (Lepidoptera: Lymantriidae). Applied Entomology and Zoology 37 : 2309-312.
- **Mayr E, Ashlock PD. 1991.** Principles of Systematic Zoology. McGraw-Hill.
- **McMichael M, Prowell DP. 1999.** Differences in Amplified fragment-length polymorphisms in fall armyworm (Lepidoptera: Noctuidae) host strains. Annals of the Entomological Society of America 92: 175-181.
- **Mehrnejad M. 2002.** Biology of carob moth, E. ceratoniae new pest on pistachio in Rafsanjan. Applied Entomology and Phytopathology 60: 1-11.
- **Mesbah HA, El-Kady MB, El-Sayed NAA. 1998.** Survey and population studies on pests of date palm trees in Siwa oasis (Western desert, Egypt). Journal of Egyptian-German Society of Zoology 25: E99-117.
- **Mirkarimi A. 1996.** The effect of stuffing pomegranate fruit neck (calyx) on reduction of pomegranate neck worm Spectrobates (Ectomyelois) ceratoniae Zell. Available
- **Morton J. 1987.** Fruits of warm climates. Creative Resource Systems Inc.
- **Nasrollahi AA, Shojai M, Ziaii M. 1998.** Large scale production and application of Trichogramma wasps for biological control of pomegranate moth Ectomyelois ceratoniae in Yazd province. Proceeding of 13th Iranian plant protection congress. Junior College of Agriculture, Karaj 1: 167
- **Nei M (1972).** Genetic distance between populations. Am. Nat. 106: 283-292.
- **Nei M (1973).** Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci.,USA. 70: 3321-3323.
- **Nei M (1978).** Estimation of average heterozyosity and genetic distance from a small number of individuals. Genetics 89: 583-590.
- **Reineke A, Karlovsy P, Zebitz CPW. 1998.** Preparation and purification of DNA from insects for AFLP analysis. Insect Molecular Biology 7: 95-99.
- **Reineke A. 1998.** Differentiation of populations of the gypsy moth, Lymantria dispar L. (Lepidoptera, Lymantriidae) using molecular markers. PhD. thesis, Universitat Hohenheim.
- **Ruggiero LF, Aubry KB, Buskirk SW, Koehler GM, Krebs ChJ, McKelvey KS, Squires JR. 2004.** Ecology and conservation of lynx in the United States, General Technical Report RMRS-GTR-30WWWFort Collins, COU.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station
- **Sambrook J., Fritsch, EF; Maneates T. 1989:** Molecular Cloning. A laboratory manual. 3rd edn. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. NY
- **Sambrook J., Russell D.W., 2001**. Molecular cloning: a laboratory manual. Cold Spring Habor Laboratory Press, New York.

- **Shakeri M. 1993.** First report of attack of Spectrobates ceratoniae (Zell.) to figs in Iran. Applied Entomology Phytopathology 60: 93
- **Shakeri M. 2004.** A review on investigations on pomegranate neck worm in Iran. A proceeding on evaluation of finding and current problems associated with Spectrobates ceratoniae management in pomegranateMinistry of Jihad-e-agricultrue, organization of research and education, Yazd agriculture and natural resources research center18-30.
- **Sluss TP, Graham HM. 1979.** Allozyme variation in natural populations of Heliothis virescens. Annals of the Entomological Society of America 72: 317-322.
- **Spielman D, Brook BW and Frankham R (2004).** Most species are not driven to extinction before genetic factors impact them. Proc. Nat. Acad. Sci. U. S. A. 101: 15261-15264.
- **Suinaga FA, Casali VWD, Picanço M, Foster J. 2004.** Genetic divergence among tomato leafminer populations based on AFLP analysis. Pesquisa Agropecuária Brasileira 39: 7.
- **Sword G A, Joern A, Senior LB. 2005.** Host plant-associated genetic differentiation in the snakeweed grasshopper, Hesperotettix viridis (Orthoptera: Acrididae). Molecular Ecology 14: 2197- 2205.
- **Timm AE, Geertsema H, Warnich L. 2006.** Gene Flow Among Cydia pomonella (Lepidoptera: Tortricidae) Geographic and Host Populations in South Africa. Journal of Economic Entomology 99: 341-348.
- **Tous J, Ferguson L.Janick J. 1996.** Mediterranean fruits. Progress in new crop, 416-430. ASHS Press.
- **Van den Berg MA. 1995.** Pests Attacking Macadamia in South Africa. Acotanc-95, The sixth conference of the Australasian Council on Tree and Nut Crops Inc.
- **Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee Th, Homes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995.** AFLP, a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-4414.
- **Warner RL, Barnes MM, Laird EF, Lanham MD. 1990.** Chemical control of a carob moth, Ectomyelois ceratoniae (Lepidoptera: Pyralidae) and various nitidulid beetles (Coleoptera) on 'Deglet Noor' dates in California. Journal of Economic Entomology 83: 2357-2361.
- **Williams, J.K.G., Kubelik, A.R., Livak, K.J., Rafalsky, J.A., Tyngey, S.V., 1990.** DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18, 6531-6535.
- **Yan G, Romero-Severson J, Walton M, Chadee DD, Severson DW. 1999.** Population genetics of the yellow fever mosquito in Trinidad: comparisons of amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP) markers. Molecular Ecology 8: 951-963.
- **Yue GH, Li J, Bai ZY, Wang CM, et al. (2010).** Genetic diversity and population structure of the invasive alien red swamp crayfish. Biol. Inv. 12: 2697-2706.