GENETIC VARIABILITY OF HOST POPULATIONS OF THE EUROPEAN CORN BORER, *OSRTINIA NUBILALIS* (Hübner) (LEPIDOPTERA: CRAMBIDAE) IN IRAN

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ABSTRACT: The European corn borer is one of the most important economic pests that could find in all parts of Iran. To study the genetic diversity of *O. nubilalis* during summer 2013-2014 from first generation in wheat and in second generation from other hosts, 15 individuals of each sex were collected for preliminary analysis. The genomic DNA from European corn borer larvae extracted from host populations of corn, wheat, okra, barley, melon, sugar beet and cocklebur from Ardabil province of Iran using polymerase chain reaction (PCR) and 10 different SSR primers produced 36 polymorphic bands. Nine of the SSR primers showed high variability across the distinct populations with 100 percent polymorphism. Within populations, genetic diversity based on Nei's gene index ranged from 0.214 to 0.600 at wheat and cocklebur in host populations, respectively. Mean and standard deviation of observed number of alleles were 3.600 and 1.265. Mean of observed hetorozygosity and gene flow for all loci were 0.0857 and 0.020, respectively. Molecular variance analysis showed significant differences within and among populations with groups variance accounted for 13.08 and 97.73, respectively. Using the un-weighted pair-group method analysis, cocklebur population grouped as unique in one cluster while the other populations grouped separately.

KEY WORDS: Genetic diversity, *Ostrinia nubilalis*, SSR, Hetorozygosity

It is important to develop a better understanding of the insect's genetic structure, genetic variation, and gene flow that can provide the basis for improvement and changes in current management strategies for insect control and resistance management (Alstand, 1995). During the early stages of diversification, incipient species often maintain high levels of gene flow, such that introgression occurs in regions of the genome not linked to genes directly involved in speciation (Wu, 2001; Lassance et al., 2010). Molecular genetic markers within or linked to genes affected by a recent selective sweep can be associated with divergent traits, and thus used to predict individual phenotypes in natural environments (Schulze & McMahon, 2004). Correspondence of phenotype with genotypes (mutations) has been established for insecticide resistance traits (French-Constant et al., 1993), but population associations can be complicated by effects of inbreeding, population structure or selection (Berlocher & McPheron, 1996; Baxter et al., 2010).

The European corn borer (ECB), *O. nubilalis* (Lep.: Crambidae) is one of the key pests causing severe yield losses, infesting several crops such as cereals, potato, cotton, pulses, tomato, vegetables and fruit crops as well as wild hosts. It is known as the most important pest of maize (*Zea mays* L.), causing worldwide crop losses. Apart from maize, there are more than 200 plants which can serve as hosts for ECB, e.g.: mugwort (*Artemisia vulgaris*) and hop (*Humulus lupulus*) (Lewis,1975). ECB is native to Europa, North and West of Africa, and Western Asia (Mutuura &Monroe, 1970). Understanding the genetic variation between the

Helicoverpa armigera Hubner (Lep.: Noctuidae), populations occurring on host plants has become necessary to find the variation in their susceptibility to distinct insecticides and suitable management (Subramanian & Mohankumar, 2006). An appropriate resistance management, however, can only be developed based on an understanding of the genetic basis and the modes of action of pest adaption (Hawthorne, 2001). Therefore it is crucial to consider information on the genetic background of the respective insect population, and on its reaction and degree of susceptibility towards the toxin of the genetically modified crop. Furthermore, there is a need for more information on the dispersal and migration behavior, the levels of gene flow between populations and alternative host plants of ECB since these data contribute to the adoption of Insect Resistance Management (IRM) plans.

Genetic Variability of Geographical Populations of the Bollworm, *H. armigera* (Lep.: Noctuidae) was evaluated using SSR (Simple Sequence Repeats) molecular marker in Iran. Molecular variance analysis stated significant within and among population variance. The maximum and minimum genetic distances were observed among Gorgan- Mughan & Kermanshah- Shahindej (Khiaban et al., 2010). ISSR (Inter Simple Sequence Repeats) as genetic markers was used in studying intra-specific variation in Noctuids. In India genetic variability of *H. armigera* (Lep.: Noctuidae) populations from six different host plants was studied using 10 microsatellite SSR marker. Finally nine of the SSR primers indicated high variability across the different host associated populations with polymorphism ranging from 75% to 100%. Cotton population grouped as unique in one cluster while all other hosts grouped separately. Microsatellite markers are highly polymorphic and co-dominant, and useful for population genetic and genome mapping studies (Goldstein & Schlotterer, 1999). SNP markers consist of base substitutions at a single genomic locus, where individual mutations are generally bi-allelic and have lower allele diversities and provide less statistical power to discriminate unique genotypes compared to microsatellite loci (Xing et al., 2005). Some studies have focused on population genetics of the ECB (Harrison & Vawter, 1977; Cardé et al., 1978; Willet & Harrison, 1999; Bourguet et al., 2000a,b). Allozyme polymorphism is well suited for population studies and has been used to investigate the genetic population structure in several migrant Noctuidae species (Daly & Gregg, 1985; Pashley et al., 1985; Korman et al., 1993; Bues et al., 1994).

Allelic distinction between pairs of populations and hierarchical decompositions of pools of examples from each host plant demonstrate that the group of populations feeding on maize differed from the group of populations feeding on mugwort (Martel et al., 2003). Phenological, phytochemical and morphological distinctions between host plants may extend genetic isolation following host changes a first step toward speciation (Bush, 1994).

Genetic variability of *O. nubilalis* was studied for 18 sub-populations in the upper Midwestern United States using AFLP (Amplified fragment length polymorphism). The result indicate that more variation exists within populations than between populations (Jeffrey et al., 2008). Pornkulwat et al. (1998) used RAPD marker that were able to distinguish univoltine from bivoltine and multivoltine ecotypes. Geographical Variation in Pheromone Response of the ECB *O. nubilalis* (Lep.: Crambidae), in North Carolina was studied. The results cleared, the distribution of the two pheromone races (97Z: 3E) seemed to remain basically unchanged from that observed in the late 1980s, and no proof of a continued westward expansion of E responsive moths was detected (Sorenson et al., 2005). The studying in different regions observed that may be explained by the voltinism patterns (univoltine vs. multivoltine, respectively) of *O. nubilalis*: multivoltine populations have opportunities for multiple matings for the duration of the year (Jeffrey et al., 2008). Saldanha (2000) taked RAPD marker

to discriminate between local populations of *O. nubilalis* and found a large genetic group consisting of univoltine, bivoltine, and multivoltine ecotypes in all parts of Nebraska. However, disadvantages of RAPD marker are apparent and therefore results can be arguable. Molecular Diversity of Cotton Bollworm *H. armigera* in India was assessed Using RAPD Marker. The level of genetic difference detected among the *H. armigera* populations with analysis suggested that RAPD marker an efficient marker technology for delineating genetic relationships amongst populations and estimating genetic diversity, thus gaining insight into genetic structure of populations and its further use in formulation of appropriate area extensive management strategies for this pest (Yenagi et al., 2012). The two pheromone races of *O. nubilalis* show partial reproductive isolation when in sympatry, and may represent incipient species in the early stages of divergence (Dopman et al., 2010; Lassance et al., 2010).

Estimates of hybridization between the E- and Z-races are important for understanding the dynamics involved in maintaining race integrity, and are consistent with previous estimates of low levels of genetic divergence between E- and Z-races and the presence of weak prezygotic mating barriers (Coates et al., 2005). Populations of ECB differ in situations of pheromone blends (E vs Z) and voltinism (univoltine vs bivoltine) (Hudon et al., 1989). Coates et al. (2005) showed that ten polymorphic dinucleotide (CA / GT and GA / CT) microsatellite loci are suitable for population genetic screening from enriched partial ECB genomic libraries. In North America, ECB consists of several morphologically indistinguishable races with different sex pheromone communication systems (Roelofs et al., 1985). Only a few studies have focused on the genetic relationships between these races. Harrison & Vawter (1977) and Carde et al. (1978) found that two sympatric pheromonal races displayed slight differences in their allelic frequencies. Recent studies have revealed that, at least in France, ECB comprises two sympatric host-associated species: a maize and mugwort associated species. The mugwort associated species infests mostly mugwort and hop, while the maize-associated species infests mostly maize, and occasionally other plants such as bird pepper, sunflower, cocklebur, and sorghum (Leniaud et al., 2006). These two host differentiated species are genetically differentiated from each other (Bourguet et al., 2000; Martel et al., 2003; Leniaud et al., 2006) and show assortative mating in the field and in cages (Malausa et al., 2005; Bethenod et al., 2004). Bourguet et al. (2000) also assayed gene flow of French populations of ECB and discriminated a great and homogenous gene flow. Finally Coates et al. (2004) significant genetic differentiation found among Atlantic coast and Midwestern United States samples.

MATERIALS AND METHODS

This study was devoted to assessing genetic differentiation between samples of ECB collected over a restricted area from Ardabil province in Iran on seven different host plants: maize, corn, wheat, okra, barley, melon, sugar beet and cocklebur (*Xanthium strumarium* L.) from family Asteraceae (Table 1). Larvae of ECB were collected during summer 2013-2014. Larvae of *O. nubilalis* were selected for the isolation of genomic DNA and stored at −70° C.

DNA Extraction

The larvae were washed thoroughly in double distilled water and the genomic DNA was extracted from the larvae using by modified protocol (Zimmerman et al., 2000). Briefly, the cleaned larvae were ground liquid nitrogen and then 500 μl buffer containing 100 mMTris-HCI (pH 8.0), 0.1 M sodium chloride, 20 mM EDTA, 0.1% of SDS and suspended in the same buffer. The suspension was incubated at 60°C for 3 hours and then the same volume of chloroform: isoamylalcohol (24:1) was added. The suspension was centrifuged at 13000g for 5

min at 4° C. The upper liquid blanket was transmited to a fresh micro centrifuge tube taking care not to eliminate the middle protein interface. Then, was added 15 μlNacl 5M and shacked by hand slowly. DNA was precipitated by adding equal volume of ice-cold isopropanol. The tube was kept for 20 min at -20°C. The precipitated DNA was spun at 13000g for 5 min and the supernatant was deleted and the DNA pellet was finally washed twice using ethanol 70% and dissolved in 200 μl TE (Tris EDTA, 100 mM). Extracted DNA was further cleaned of RNA contaminants by addition of 10 μl/100 μl of RNase. The intact genomic DNA was visualized in a 1% agarose gel. Depending upon the concentration, the DNA examples diluted by sterile water to get a working solution of 20–25 ng/μl.

PCR amplification

The genomic DNA from ECB larvae gathered from seven different hosts were prepared to PCR using 10 different SSR primers (Tan et al., 2001;Ji et al., 2003) (Table 1) obtained from Sigma-Aldrich. PCR was carried out in 20 μl reaction mixture containing 50 ng DNA the same as the template. Genomic DNA 2.0 μl (25 ng), dNTPs 0.8 μl (2.5 mM), assay buffer 1 μl (10X), SSR forward primer 2 μl (20 μM), SSR reverse primer 2 μl (20 μM), Taq DNA polymerase 0.15 μl (3 units), magnesium chloride 0.15 μl (25mM), sterile distilled water 3.7 μl, were added and PCR was performed in thermal cycler programmed for 5 min at 94° C for initial denaturation. Following the Preliminary denaturation the thermal cycler programmed for 35 cycles of 1 min at 94° C for denaturation , 1 min for annealing belong on primers and 50 second at 72° C for extension and additional cycle of 10 min at 72° C for final extension.

Elecrtophoresis of PCR products

PCR products were analyzed by electrophoresis in 3% metaphor gel electrophoresis run at 70 W for 30 min in 1x TBE buffer. The bands was visualized using the Ethidium bromide method.

Data Analysis

The molecular size of the amplified outputs was evaluated using a 100bp DNA marker (Fermentas Inc., www.fermentas.com.) The samples were analyzed all 10 primers to check the producing of bands.

According to log molecular weight of the movement 100bp DNA marker (Fermentas Inc., USA) and their migration distances scatter plots were established and trend lines with best fit was fitted. Based on the mathematical expression of the trend lines the molecular weight of the fragment corresponding to their migration distances was computed. The individual DNA bands were scored in the amplification profile of each one sample. Only apparent bands with fine resolution were scored. The percentage of polymorphism was computed as the proportion of the polymorphic markers to the all numbers of markers. The polymorphism information content value was also examined (Smith et al., 1997). After cluster analysis of the similarity coefficients by the un-weighted pair-group technique analysis, UPGMA, dendrogram drawed (Sneath & Sockal, 1973) using NTSYS Pc-2.0.

Analysis of molecular variance (AMOVA, Excoffier et al., 1992) was conducted with ARLEQUIN 2.0 (Schneider et al., 2000).We put wheat, barley and corn in one group therefore 7 host populations changed to 5 groups. In this analysis, variance of the SSR data set was partitioned at three hierarchical levels: (1) between-population component (2) a regional or 5 sub- population component (3) and a within-population component. Unlike the calculations used for Nei's GST values. The significance of the three variance components was checked using 1000 random permutations. A two-part AMOVA analysis was conducted to check genetic divergence (FST) as a factor of variation between individuals within a given population and between populations. An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) consensus cluster was analyzed as outlined by Sneath & Skoal (1973) was conducted using NTSYSpc ver. 2.1 on all 7 populations

of ECB to illustrate genetic similarity. Data were analyzed using POPGENE version 1.32 (Yeh & Boyle, 1997). Applying a Co-dominant marker data set 36 markers assuming Hardy-Weinberg equilibrium. The percent (%) polymorphism, genetic diversity or heterozygosity (H), GST, and gene flow determination (Nm) was then assessed within and among every one of populations. Individual populations were analyzed for genetic diversity (H) for every sub-population as per Nei (1973).

RESULTS

The genetic variability of 7 populations of ECB collected from different hosts was investigated by PCR analysis of DNA from one larvae randomly selected from each of these populations using 10 SSR primers. All 10 primers listed produced scorable markers in each DNA sample. A total of 36 markers from 10 primers were available for analysis across the different populations. The highest numbers of 6 markers were produced by the primer OS6, followed by 5 markers by OS4 with high degree of polymorphism 75–100%.

The ECB populations occurring on corn, wheat, okra, barley, melon, sugar beet were found to be closely related, while the population occurring on cocklebur was found to differ widely (Figure 1).

However, this study suggests that, although ECB populations are found on several different types of host plant, the ECB populations on non-maize plants may constitute separate subpopulations and, therefore, cannot necessarily be viewed as alternative refuges as proposed by Gould (1998) and Alstad & Andow (1995).

Our results show that populations may also be distinguished on the basis of the type of host plant colonized (maize vs cocklebur). Molecular variance analysis showed significant within and among population with groups variance accounted for 13.08 and 97.73, respectively (Table 2). Computed FST values for these comparisons were low to moderate, from 0.72727 to 1.0000 with a mean of 0.13797 (Table 3). Cluster analysis based on molecular data in host populations assigned the studied ECB populations into two groups.

The maximum and minimum genetic distance matrix was observed between melon- cocklebur (1.8281) and barley -corn (0.5108), respectively (Table 4). In the present study the grouping of the ECB populations indicated high similarity among populations, while the population collected from the cocklebur was found to be more variable. This phenomenon indicates a strong genetic variability among ECB populations collected from different host plants. Reddy & Kumar (2004) found differences in susceptibility to different insecticides among *H. armigera* populations collected from three hosts tomato, chickpea and grapes, they suggested that this difference might be due to the variation in plant factors. The results of the present study also suggest that genetic variation among populations collected from different host plants might be due to host characteristics.

Cunningham (1999) by studying the genetic diversity between bollworm populations in different host crops, suggested that polyphagous insects tend to be monophagic at the micro ecological level. The reason this tendency might be due to the migration of populations from different locations (Subramanian & Mohankumar, 2006).

The result of the relative abundance studying of Bollworm on different host plants proved a multicrop situations can be as an important natural refuge in central and southern India (Subramanian & Mohankumar, 2006).

DISCUSSION

The results of studying explained importance in a multi crop ecosystem such as in India where a polyphagous insect has many of its hosts in the vicinity which may lead to interbreeding among isolated populations. Such an interbreeding phenomenon between varying host associated populations indicates the presence of natural refugee in multi crop environments. Detailed field level investigations on the polyphagy of individual ECB and the mating behavior of such individual populations combined with evaluation of their genetic diversity remains to be done (Subramanian & Mohankumar, 2006). The results of genetically studying support that the identification of genetic variation in host populations is very essential for pest management.

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Table 1. Characteristics of samples of *O. nubilalis* collected from different host species using SSR markers.

Location	Population	Family	Latitude	Longitude	Date (month/year)
Mogan	Okra	Malvaceae	39.28"N	48.38"E	07/2013
Mogan	Melon	Cucurbitaceae	39.42"N	47.41"E	07/2013
Mogan	Sugar beet	Chenopodiaceae	39.28"N	47.41"E	07/2013
Mogan	Cocklebur	Asteraceae	39.35"N	47.34"E	07/2013
Mogan	Corn	Poaceae	39.49"N	47.81"E	06/2013
Mogan	Wheat	Poaceae	39.28"N	47.41"E	06/2013
Mogan	Barley	Poaceae	39.33"N	47.38"E	06/2013

Table 2. AMOVA of 7 tested host populations of *O.nubilalis* using 10 SSR primers.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	4	23.690	-0.35417 Va	-10.80836
Among population within groups	2	13.667	3.20238 Vb	97.72934
within populations	7	3.000	0.42857 Vc	13.07902
Total	13	40.357	3.27679	

Fixation Indices: F_{ST}: 0.86921, F_{SC}: 0.88197, F_{CT}: -0.10808

Table3. Population pairwise FST of 7 tested host populations comparisons using 10 SSR primers.

	Melon	Okra	Barely	Corn	Cocklebur	Sugar beet	Wheat
Melon	0.0000						
Okra	0.85714	0.0000					
Barely	0.93750	0.91667	0.0000				
Corn	0.75000	0.72727	0.85714	0.0000			
Cocklebur	0.88889	0.87500	0.91667	0.80000	0.0000		
Sugar beet	0.87500	0.80000	0.93750	0.78571	0.85185	0.0000	
Wheat	0.92857	0.90000	1.0000	0.85714	0.91667	0.92857	0.0000

Table 4. Distance genetic matrix for *O.nubilalis* populations collected from different host species using SSR markers.

	Melon	Okra	Barely	Corn	Cocklebur	Sugar beet	Wheat
Melon	0.0000						
Okra	1.5404	0.0000					
Barely	0.8818	0.8818	0.0000				
Corn	0.8102	0.9438	0.6216	0.0000			
Cocklebur	1.8281	1.5404	1.1695	1.2802	0.0000		
Sugar beet	1.5404	0.8473	1.1695	0.9438	0.9808	0.0000	
Wheat	1.1695	0.8818	0.5108	1.1324	1.1695	1.1695	0.0000

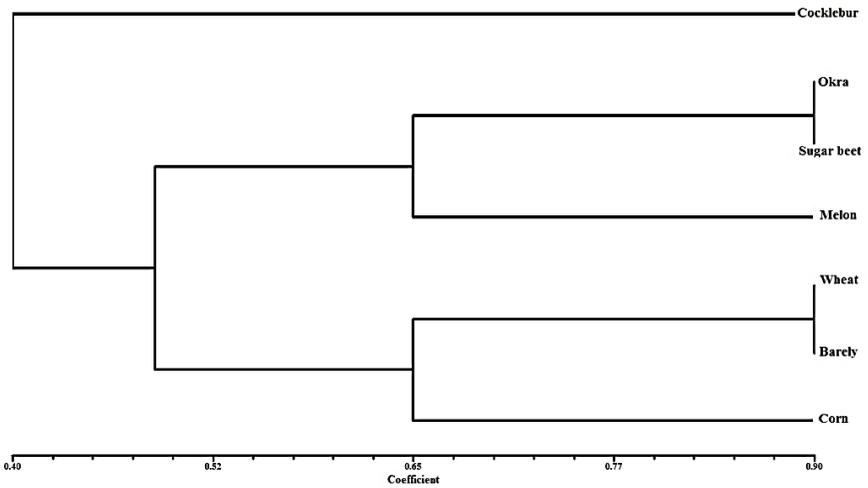Fixation Indices, FST: 0.86921,FSC: 0.88197,FCT: -0.10808

Figure 1. Cluster analysis of *Ostrinia nubilalis* in different hosts Dendrogram Based Neis (1972) Genetic distance: Method UPGMA Modified from NEIGHBOR procedure of PHYLIP Version 3.5.