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Molecular and Biochemical Diagnosis of Three Scarab Beetles (*Coleoptera: Scarabaeidae*) Commonly Infect Sugarcane Plantation in Upper Egypt

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Abstract: In the present study the estrase, protein banding pattern (SDS-PAGE) and the random amplified polymorphic DNA markers were employed to discriminate between three scarab beetles (*Coleoptera: Scarabaeidae*) namely *Catharsius sesostris , Heteronychus licas* and *Pentodon bispinosos* which are common in sugarcane field in Upper Egypt. Two estrase bands distinguish *Catharsius sesostris*. A total of 17 (80.9%) and 121 (92.4%) polymorphic bands were detected for protein and RAPD, respectively. The comparison between SDS-protein and RAPD analyses revealed that the latter gave more markers and more conclusive results. The RAPD genotype-specific markers represent 58.7% of the total markers detected. These molecular markers were sufficient to distinguish among the three-scarab species. The RAPD based dendrogram indicate that the genotypes of *Heteronychus licas* and *Pentodon bispinosos* are genetically very close. Ten out of the 131bands generated by RAPD analysis were common in the genome of the three species tested. These markers could be verified as being RAPD markers associated with the family Scarabeiadea.

Key words: Coleoptera: Scarabaeidae, protein, Esterase and RAPD analysis

INTRODUCTION

Scarabaeids, particularly the *Catharsius sesostris* (Waterhouse)*, Heteronychus licas* (Klug) and *Pentodon bispinosos* are common in sugarcane field in Upper Egypt. The larvae of scarab beetles (*Coleoptera: Scarabaeidae*), cause significant damage to many agricultural and horticultural plants. They feed during the larval stage on roots and other organic matter in the soil. Damage was most frequently noticed in late autumn and early winter and continued until early spring (Ibrahim 2004).The adult's morphological characterization of these three scarab beetles was previously described in Ibrahim and El-Torkey, (2008).

Different approaches were used to assay genetic diversity between different insect species including morphological traits, and isozyme electrophoresis. Enzyme markers have been found to be especially suitable to differentiate between individuals with respect to their genetic structure; enzymes are less changeable between individuals than other biochemical constituents of haemolymph, and other tissues (Patnaik and Datta 1995, Etebari et al. 2005). This characteristic makes them good biochemical markers (Staykova, 2008). However these techniques are insufficient to serve as accurate markers due to insufficient polymorphism produced by isozyme approach among closely related genotypes. Molecular markers have been developed to give high degree of polymorphism and stability and provide easier and faster approaches for exploring genetic variability between individuals (Gill *et al.*, 2006). A molecular marker is a readily detectable sequence of DNA or protein whose inheritance can be monitored. It is the variation in, or polymorphism of, molecular markers, which can be used in genetic diversity studies (Weising *et al.,* 1995).

Random amplified polymorphic DNA (RAPD) developed by Welsh and McCelland, (1990) and Williams *et al.,* (1990) proved to be a powerful tool in different genetic analysis. This approach detects DNA polymorphisms based on amplification using a single primer of arbitrary nucleotide sequence of genomic DNA fragments. RAPD markers are attractive because they are simple and quick, very small (nanograms) quantities of DNA are required, automation is feasible, there is no requirement for previous DNA sequence information modest coast and ability to detect relatively small amounts of genetic variation(Williams *et al*. 1990).

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Based on RAPD markers the determination the genetic polymorphisms in natural populations (Haag *et al.* 1993), strain differentiation (Heckel *et al.* 1995, Tom *et al.* 1995, Schrieber *et al.* 1997 Gill *et al.*, 2006) and geographically isolated populations (Zhou *et al.* 2000) have been studied.

The advent of molecular biological techniques clearly showed the advantages of molecular markers over morpho-biochemical markers to analyze population diversity. Recently a number of DNA marker systems such as simple sequence repeats (SSR; Prasad *et al.* 2005), random amplified polymorphic DNA (RAPD; Chatterjee and Pradeep 2003), inter-simple sequence repeats (ISSR; Kar *et al.* 2005; Pradeep *et al.* 2005), expressed sequence tag (EST; Ciolfi *et al.* 2005) have been used to study the population genetics of different organisms including insects.

The application of DNA data in taxonomy and species diagnosis has aroused a great deal of controversy, but there is general agreement that genetic information is useful for associating different developmental stages of organisms and for identifying partially preserved specimens unsuitable for morphological study (Vences *et al.*, 2005; Wheeler, 2004; Will *et al.*, 2005). A DNA-based approach has already been used to associate different developmental stages in order to identify agricultural pests and invasive species (Ball and Armstrong, 2006; Harper *et al.*, 2005; Rao *et al.*, 2006; Scheffer *et al.*, 2006), forensically important insects (Wells and Sperling, 2001), larval parasitoids (Agusti *et al.*, 2005) and endangered species in their early life stages (DeSalle and Birstein, 1996).

In the present study the genetic variation between three scarab beetles *Catharsius sesostris* (Waterhouse), *Heteronychus licas* (Klug) and *Pentodon bispinosos* was resolved using isozyme, protein and random amplified polymorphic DNA (RAPD) markers, and thereby the species-specific markers for each species were determined

MATERIAL AND METHODS

Insect samples:

The adult of white grubs were collected by using two methods: A- Digging from the soil with shovel at Girga, Al-monshaa, Negoo-bindar, Awlad Gabara (Sohag Governorate) and Sharki and Gharbi Bahgora, Nakada, Hew, Al-saiade (Qena Governorate) in 1 m around the infected Sugarcane plant during the period of adult activity (starting from June to end of September) , the depth of digging about 40 cm depth under ground.

B- Pit-fall traps. In the present study two types of Pit-fall traps was used, during the period of adult activity.

The first type is simple tin container with smooth inside walls (20 cm in diameter and 20 cm in depth) and a tin funnel which first the container to prevent the trapped insects from escaping. The dimensions of the added funnel are: upper based, 20 cm, lower base 5 cm in diameter, and high 5 cm (Willcocks, 1925).

The second type, plastic containers (each 12 - 15 cm), served as pitfall traps Archer and Musick (1977). These traps were half filled with water, and then sunk into the soil; both of the two types get buried into the soil with on their upper ends showing at ground level.

The attractant material (bait) used in trap during the evaluating study, was pieces of ripe fruits, i.e. apple, pear and peach. All trap renewed every day and captured specimens were held individually in plastic cups (10 cm diameter/ 8cm high). Each cup was filled partially with moist soil and suitable food and covered with performed plastic lids then transfer to the laboratory.

SDS-PAGE Analysis:

Protein extraction was performed using adult insect tissues. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for total storage proteins according to the method early described in Laemmli, (1970). Isozyme analysis.

Isozyme extraction was performed using individual adult insect. The sample was ground in 2 ml extraction buffer containing : 0.1% (w/v) Tris-citric acid, pH 7.5; 1% (w/v) polyvinyl pyrolidone (PVP); 0.1% (w/v) ascorbic acid and 0.1% (w/v) cysteine and centrifuged at 5333 xg (JS - 5.2 rotor), at 4 °C for 5 min. Ten ml of extracted samples were used for electrophoresis on polyacrylamide gel (SDS-PAGE) according to Stegmann *et al.* (1983) using Pharmacia electrophoresis apparatus (GE-4).

Esterase detection.

Esterase was detected by incubating the gel in darkness for one hour at 37°C in a mixture of 100 ml (0.15 M) phosphate buffer (pH 7.2) containing 20 mg 1-naphthylacetate dissolved in 2 ml acetone and 50 mg fast blue RR salt. After the incubation period the gel was rinsed in distilled water and fixed in 50% glycerol for one hour prior to scoring and photography. Rf value of each band was calculated as follow:

$$Rf = \frac{\text{Distance traveled by the band from the top of the running gel}}{\text{Distance traveled by the tracking dye}}$$

DNA Extraction:

Total genomic DNA was isolated using the method descried in Rogers and Bendich (1985). The insects were washed thoroughly in double distilled water, the cleaned insects were ground in liquid nitrogen to fine powder. Insect powder samples (0.25 gram) was suspended in 1.0 ml of 2% cetyltrimethyl ammonium bromide buffer (CTAB), containing 100 mM Tris-HCI (pH 8.0), 1.4 M sodium chloride, 20 mM EDTA, 0.1% of 2-mercaptoethanol (added just prior to use). The suspension was incubated at 65° C for 2 hours and then equal volume of chloroform was added. The suspension was centrifuged at 10000 rpm for 15 minutes at 4° C. The upper aqueous layer was recovered to a fresh micro centrifuge tube. DNA was precipitated by adding equal volume of ice-cold 95% ethanol. The precipitated DNA was spun at 10000 rpm and the resultant DNA pellet was washed with 70% ethanol and dissolved in 100 µl TE buffer. The genomic DNA was separated in a 1% agarose gel and visualized after staining with ethidum promide.

RAPD analysis:

The RAPD analysis was performed with twelve decamer primers (Operon Technology, Inc., Alameda, CA, USA). The reaction conditions were optimized and mixtures (20 ml total volume) were composed of 2.0 µl (25 ng) DNA, 2µl (25 mM) dNTPs (AB-gene Housse, UK), 2µl (10X) reaction buffer, 3µl (25mM) magnesium chloride, 1.5 µl (0.3 units) Taq polymerase (Fermentas Inc., www.fermentas.com.), 2 µl primer (20µM) and 7.5µl sterile distilled water. Amplification was carried out in a DNA thermal cycler (Biometra) programmed for 94 °C/ 4 min (1 cycle),then 40 cycles as fellows;:94°C /1 min., 36°C / 1 min, 72°C / 1 min followed by one additional cycle at 72°C /7 min. The amplification products were separated in 1% (w/v) agarose gel in 1xTBE buffer and visualized by staining with ethidum bromide. Reproducibility of DNA profiles was determined by replicating all RAPD reactions at least three times. Variations among three genotypes tested across the primers used in the present study were evaluated from pairwise comparison for the proportion of shared bands amplified (Nei, 1987). The primers name and sequences was as follows:

Primer name	Sequence
OPB-07	5`- GGTGACGCAG- 3`
OPC-05	5`- GATGACCGCC- 3`
OPQ-12	5`- AGTAGGGCAC- 3`
OPA-03	5`- AGTCAGCCAC- 3`
OPG-12	5`- CAGCTCACGA- 3`
OPQ-14	5`- GGACGCTTCA- 3`
OPN-04	5`- GACCGACCCA- 3`
OPM-05	5`- GGGAACGTGT- 3`
OPG-14	5`- GGATGAGACC- 3`
OPP-15	5`- GGAAGCCAAC- 3`
OPN-13	5`-AGCGTCACTC - 3`
OPA-10	5`- GTGATCGCAG- 3`

RESULTS AND DISCUSSION

Biochemical markers are proteins produced as a result of gene expression which can be separated by electrophoresis to identify the alleles. The most commonly used protein markers are isozymes of variant forms of the same enzyme (Vodenicharova, 1989). Enzyme electrophoresis, the first type of assay developed to detect parasitoid proteins, has proven in some cases to be a sensitive and reproducible method (Tomiuk *et al*., 1979; Castañera *et al*., 1985). In the present study three scarab beetles was analyzed at protein and estrase banding pattern. The data indicate that a total of 21 protein were obtained representing molecular weights (MW) ranging between 2.56 to 200.11 KDa. The highest numbers of protein bands were detected in *Catharsius sesostris* (15 bands), followed by 12 bands in *Pentodon bispinosos*, while the lowest number was 11 bands which was recorded in *Heteronychus licas* (Klug) (Fig. 1 and Table 1).

Seventeen out of the 21 protein bands detected were polymorphic (80.95%). Eight out of these seventeen bands were detected as species-specific protein markers that can distinguish the three species. The data presented in Table (2) show that, three bands with molecular weight of 165.19, 20.43 and 16.4 kDa were found to be specific to the *Catharsius sesostris,* followed by three bands with molecular weight of 45.81, 4.67 and 2.56 kDa can be considered as specific protein marker for *Pentodon bispinosos* and only two bands with molecular weight of 200.11, and 116.42 kDa were found in *Heteronychus licas* (Klug) (Table 2). The remaining bands showed positive or negative type of polymorphism among the three species tested. The common bands appeared in positions 76.23, 70.13, 48.2 and 38.27 kDa.

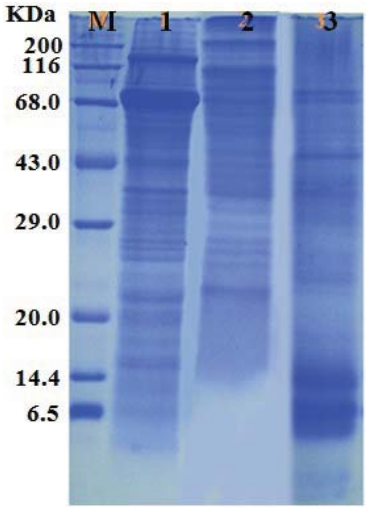

Fig. 1: The SDS-PAGE of total protein extracted from the adult insects (M: Protein marker, Lane 1-3 is Catharsius sesostris, Heteronychus licas and Pentodon bispinosos respectively

Table 1: Survey of SDS-protein markers in the three scarab beetles. Broad- range protein marker was used to detect M.W. of extracted protein (+) presence and (-)absence.

MW KDa	Catharsius sesostris	Heteronychus licas	Pentodon bispinosos
200.11	-	+	-
165.19	+	-	-
116.42	-	+	-
76.23	+	+	+
70.13	+	+	+
48.4	+	+	+
45.81	-	-	+
43.89	+	+	-
38.27	+	+	+
29.60	+	+	-
27.55	+	+	-
25.09	+	+	-
23.13	-	+	+
21.15	+	-	+
20.43	+	-	-
16.4	+	-	-
13.8	+	-	+
6.54	+	-	+
5.53	+	-	+
4.67	-	-	+
2.56	-	-	+
Total = 21			

Table 2: Genotype protein specific markers of three scarab beetles

Genotype	Band size (KDa)	Total
Catharsius sesostris	165.19, 20.43 and 16.4	3
Heteronychus licas	200.11, and 116.42	2
Pentodon bispinosos	45.81, 4.67 and 2.56	3
Total		8

The esterase banding patterns of the three scarab beetles under investigation are presented in Fig. (2). A total of four bands were detected, the electrophoretic bands showed a wide variation in their intensities among the different profiles. The highest number of bands (4 bands) was scored for *Catharsius sesostris*, while both of *Heteronychus licas* and *Pentodon bispinosos* scored one band each. The bands with the RF 0.5 appeared in two species *Catharsius sesostris* and *Heteronychus licas* and absent in the *Pentodon bispinosos*. The others one with RF 0.74 was absent in species *Pentodon bispinosos* but appeared in species *Catharsius sesostris* and *Heteronychus licas*. In contrast the two bands with RF 0.65 and 0.64 appeared in species *Catharsius sesostris* which can be considered as specific bands for this species. These results agree with Staykova, (2008) who indicate that esterase is a very suitable marker for analysis of the inter-breed and intra-breed polymorphism for the mulberry silkworm, and for determining the level of genetic variability.

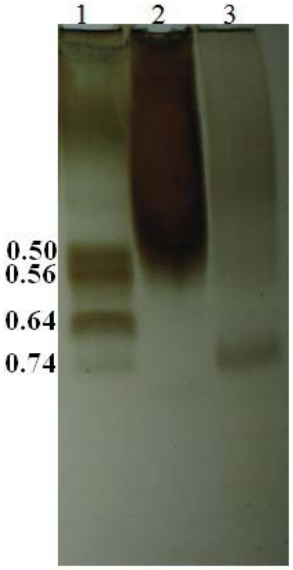

Fig. 2: Esterase banding pattern in three scarab beetles; lane 1-3 are *Catharsius sesostris (*Waterhouse*), Heteronychus licas* (Klug) and *Pentodon bispinosos* respectively.

DNA based molecular marker technique is a powerful method in genetic diversity analysis of insect pests. The major advantage of the PCR based marker systems is the generation of fragments from both single and multi loci. Since no DNA sequence information is required to perform RAPDs, these can be widely used in identification and differentiation of closely related insect species and populations of a species. RAPD-markers can be used to differentiate populations of insects (Kambhampati *et al.* 1992, Landry *et al.* 1993), species and biotypes (Black *et al.* 1992). The importance of RAPD in subspecies differentiation has been demonstrated by Ballinger-Crabtree *et al.* (1992) in mosquitoes and in aphid clone identification by Cenis *et al.* (1993).

In the present study, in order to investigate the genetic differences between the three scarab beetles used, the random amplified polymorphic DNA (RAPD) analysis was performed. All primers used in the present study resulted in the appearance of PCR products with a variable number of bands. The data shows that a total of 131 DNA markers were detected among the three scarab beetles of which, 121 bands were polymorphic (92.4%) and can be considered as useful RAPD markers for the three scarab beetles used (Fig.3 and Table 3). The largest number of RAPD bands was detected for primers OPB-07 and OPA-03 (15 bands), while the lowest was scored for OPA-10 (7 bands). Such a wide variation in the number of markers produced by these arbitrary primers may be attributed to the differences in the binding sites throughout genome of the insect species studied.

Ten bands out of 131bands were most relevant as it is common to the three species of the family *Scarabaiedea*. Three bands were the highest number of common bands revealed by OPB-07 with base pair lengths 773, 1010, and 1965 and two bands by both of OPA-03 and OPM-05 with base pair lengths 635-806 and 389-586 respectively, while a single band revealed by OPC-05, OPQ-12 and OPQ-14 with base pair lengths 172, 1143 and 557 respectively was common band between the three species (Table 3).

The species-specific RAPD markers for the different species used are listed in Table (4). Seventy seven out of the 121 polymorphic RAPD markers were found to be species-specific (63.6 %). Therefore, the three Scarabaiedea species can be identified by the RAPD markers. The largest numbers of RAPD specific markers were scored for *Catharsius sesostris* (39 markers), followed by 19 markers for both of *Heteronychus licas* and *Pentodon bispinosos* respectively. The largest numbers of RAPD specific markers (ten markers) were scored by OPG-14 and the lowest number were scored by the primers OPC-05 and OPN-13 (four markers each).

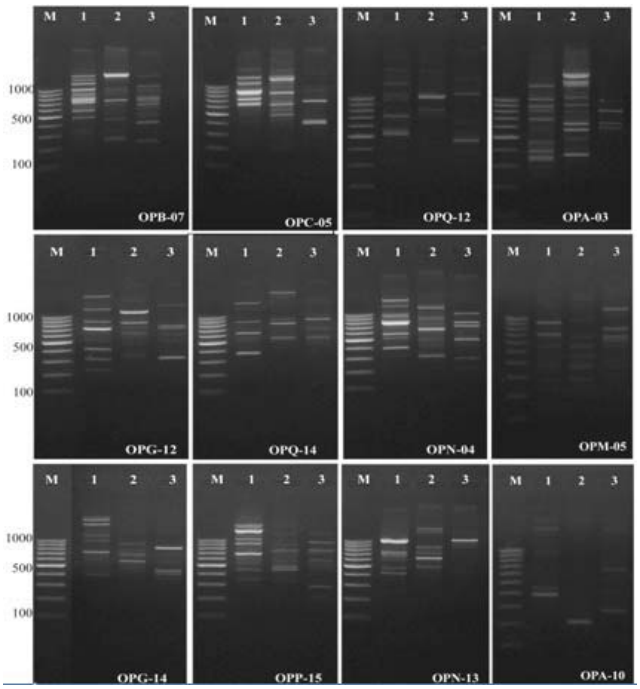

Fig. 3: RAPD banding patterns of the three Scarabaiedea species using 12 selected random primers, M: 100 bp plus DNA ladder,1- 3 are *Catharsius sesostris (*Waterhouse)*, Heteronychus licas* (Klug) and *Pentodon bispinosos* respectively.

Table 3: The numbers of total generated bands, polymorphic bands, common bands and polymorphic frequency in the three Scarabaeidae species using 12 primers.

Primer name	No of scorable bands	No of polymorphic bands %	The size of the common bands (bp)	Polymorphic frequency
OPB-07	15	12	773, 1010, 1965	80
OPC-05	10	9	172	90
OPQ-12	9	8	1143	88.8
OPA-03	15	13	635, 806	86
OPG-12	11	11	-	100
OPQ-14	8	7	557	87.5
OPN-04	13	13	-	100
OPM-05	9	7	389, 586	77.7
OPG-14	12	12	-	100
OPP-15	12	12	-	100
OPN-13	10	10	-	100
OPA-10	7	7	-	100
Total = 12	131	121	10	92.4

The RAPD-based phenogram (Fig. 4) grouped the investigated genotypes into two main clusters. The first cluster included *Catharsius sesostris*, the second cluster included *Heteronychus licas* and *Pentodon bispinosos*. This may indicate that the *Heteronychus licas* and *Pentodon bispinosos* are genetically very close.

Table 4: Species-specific RAPD markers of the three Scarabaeidae species

Primer	*Catharsius sesostris*	*Heteronychus licas*	*Pentodon bispinosos*	No of Markers
OPB-07	590, 1315, 1525	282,	256, 445	6
OPC-05	735	509, 534	410	4
OPQ-12	525, 545, 985, 2908	807	446	6
OPA-03	337, 353, 394, 455, 696	382, 504		7
OPG-12	254, 452, 1822, 3716	671, 1587	820, 2073	8
OPQ-14	392, 654, 2115	2850	753	5
OPN-04	408, 589, 2129	325, 720, 1576	390, 753	8
OPM-05	918	289, 456	692, 813, 1854	6
OPG-14	1021, 1208, 2245, 2805, 3120	552, 642, 935	433, 809	10
OPP-15	1092, 2109, 2532		216, 285, 556	6
OPN-13	425, 689, 768	2195		4
OPA-10	395, 443, 975, 2512	219	296, 689	7
Total	39	19	19	77

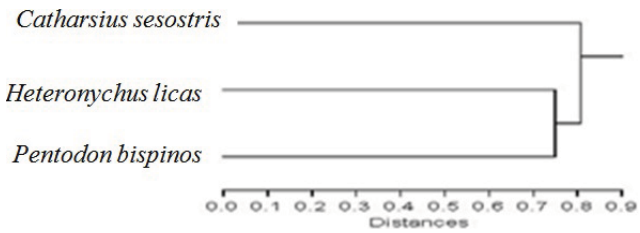

Fig. 4: RAPD based phenogram showing the relationship among three species of Scarab beetles.

Taken all the obtained results together we can conclude that, the RAPD markers are recommended in providing a quick and reliable discrimination technique, compared with the protein analyses, to identify and distinguish between the three scarab beetles. Ten bands out of 131bands were most relevant as it is common to the three species of the family *Scarabaiedea* under study and it can be regarded as the marker band of diagnostic value. These markers could be verified as being RAPD markers associated with the family Scarabaiedea. Generation of higher number of markers in the present study indicated the higher amount of genetic diversity among different populations of *Scarabaeidae*

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