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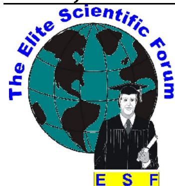

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RESEARCH ARTICLE

Molecular Characterization of Scarab Beetles (Scarabaeidae: Coleoptera) using RAPD Markers

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ABSTRACT

The Scarab beetles belonging to family Scarabaeidae were collected from different areas of Faisalabad, Pakistan during 2012 and analyzed for the assessment of genetic polymorphism by using random amplified polymorphic DNA (RAPD) markers. The amplified fragments using a total of 15 short oligonucleotide primers of arbitrary sequence showed a polymorphic banding pattern for all the species. The data generated through these bands were analyzed to construct a dendrogram by the UPGMA method. Two main clusters were obtained (A & B); the cluster B was further subdivided into three sub-clusters. The similarity coefficient of cluster "A" and cluster "B" was found to be 57.2%. The genetic similarities of the scarab beetle species ranged from 44% to 71.0%. The *Scarabaeous pithecius* and *Onthophagus atroplitus* had the greatest similarity (71%). The similarity coefficient of *Schizonycha rufficollis* and *Onthophagus atroplitus* was the lowest one (44%). Since DNA polymorphisms among the collected species were high as revealed through RAPD analysis, therefore, it is concluded that the genetic diversity in scarab beetle species is high in Faisalabad, Pakistan.

INTRODUCTION

The superfamily Scarabaeoidea is one of the largest subdivisions of beetles with an estimated 35,000 species worldwide. Unlike the majority of beetles, Scarabaeoids are well known due to their relatively large size, often bright colouration, significant economic importance and to the association of the genus *Scarabaeus* Linneaus, 1758 with sacred symbols of ancient Egypt (Anonymous, 1999; Zahoor et al., 2003; Grebennikov and Scholtz, 2004). Heart scarabs had human heads, and were placed with a mummy; this would ensure the rebirth of the deceased. In Classic Greek mythology, a scarab alone had the right to fly to Zeus on Mount Olympus. In Christian animal symbolism of medieval times, the scarab was considered as a symbol of the sinner (Anonymous, 1999; Zahoor et al., 2003).

The family Scarabaeidae is by far the largest and most diverse group within the super family Scarabaeoidea. It consists of several well defined subfamilies and several groups of uncertain status. There are ~1600 genera and 27,000 species known in the family. Thirteen subfamilies are currently recognized (Grebennikov and Scholtz, 2004). Scarab beetles or the dung beetles play their role as scavengers and bury dung pats into the soil. Larvae may consume more than check values again their dry body weight each day. These beetles are the nature's way of recycling carbon and minerals back into the soil to be further broken down into humus for plants (Fincher, 1997; Vulinec, 2000). These beetles are rounded, usually dark in color, although some have a metallic sheen, and vary in size from a fraction of an inch to several inches long. Nevertheless, as much as 90% of seeds defecated onto the surface of the soil may be destroyed by rodents or other seed predators unless buried; this burial is accomplished almost entirely by dung beetles (Estrada and Coates, 1991).

With respect to the use of classical approaches to the species identification, recently molecular genetics has been emerged with the development of DNA based techniques in a variety of animal species (Cabrero-Sanudo, 2004; Faith et al., 2004; Caterino et al., 2000).

Thanks to these DNA markers; few body-parts or tissues may suffice for DNA extraction; and also the specimens can be kept alive for further experiments or preserved as morphological vouchers (Paskewitz and Collins, 1997). Among the several DNA based techniques, Random Amplified Polymorphic DNA (RAPD) gained importance due to its simplicity, efficiency and non-requirement of sequence information (Jain et al., 2010; Embrapa and Rural, 1997), and hence, it has been successfully used for the generation of genetic similarities and phylogenetic analysis (Jain et al., 2010; Cabrero-Sanudo, 2004; Faith et al., 2004; Krzywinski and Besanki see in the reference, 2003; Caterino et al., 2001; Cognato and Sperling, 2000). RAPD markers are successfully used for the identification of different insect species (Wells et al., 2001; Wells and Sperling, 2001). Moreover, it provides an opportunity to estimate relatedness within and among various species based on DNA variation.

Particularly with the family Scarabaeidae, the phylogeny is still not very well defined. The scarabaeid lineage has been reported to include only the family Scarabaeidae (Bell et al., 2004; Philips et al., 2004). To date, there is very few information available which provides a classification of the superfamily based on a formal cladistic procedure (Browne and Scholtz, 1995). Therefore, a dire need is required to explore the genetic diversity of Scarabaeidae. With regard to genetic variations among scarab beetles or the dung beetles in Pakistan, there have been no references for such kind of scientific study. In order to decipher the phylogenetic relationship within the family Scarabaeidae, the Scarab beetle species were collected and molecularly characterized to reveal their genetic variations by using RAPD markers in the present study.

MATERIALS AND METHODS

Sampling and Identification of Scarab Beetles

Collection of Scarab beetles was made randomly by netting, hand picking, pitfall trapping and light trapping (Zahoor et al., 2003). The collected samples of scarab beetles were identified to species levels using available taxonomic keys. Some of the specimens were identified by comparing specimens against collection in the Museum of CABI Regional Biosciences Center, Daata Ganj Bakhsh Road, Rawalpindi, Pakistan. The experimental work was conducted in the Department of Zoology, Wildlife and Fisheries, GC University, Faisalabad, Pakistan and few PCR reactions were also performed at the Center of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture Faisalabad, Pakistan.

DNA extraction

The collected beetles for molecular study were immediately transferred to 70% ethanol and stored at -20°C (Robert, 2002). DNA extraction was made by using CTAB buffer method (Clark et al., 2001) and TNE buffer method (Aljanabi and Martinez, 1997). The concentration of total genomic DNA was measured by Spectrophotometer (CECIL, CE 2021, 2000 series) at 260 nm wavelength. The genomic DNA was diluted 100 times with a cuvette volume of 500µl. Quality of DNA was checked by running 5-7µl DNA on 1.0% agarose gel prepared in 0.5X TBE buffer.

Polymerase Chain Reaction (PCR) Analysis

For Random Amplified Polymorphic DNA analysis, concentration of genomic DNA, 10X PCR buffer with $(NH_4)_2SO_4$, $MgCl_2$, dNTPs (dATP, dCTP, dGTP, dTTP), 10-mer random primer (Genelink Company) and *Taq* DNA polymerase were optimized. PCR was carried in 25 µl reaction mixture containing 3 mM $MgCl_2$, 2.5 mM each of dATP, dCTP, dGTP, dTTP, 0.2µM primer, 15 ng of genomic DNA, and 1 unit of *Taq* polymerase throughprogrammable thermal cycler (Eppendorf Mastercycler, USA). A total of 15 primers were used for the analysis (Table 1). The thermal cycler was programmed for 5 minutes initial denaturation at 95^{0}C, followed by 1 minute denaturation at 95^{0}C, 1 minute primer annealing at 37^{0}C and 2 minutes extension at 72^{0}C for 40 cycles and then final extension at 72^{0}C for 10 minutes. The PCR products were electrophoresed at 80 V in 1.0% agarose gel for approximately 2 hours using 0.5X Tris Boric acid (TBE) buffer containing Ethidium Bromide (0.5 µg/ml) along with a DNA molecular size marker.

Table 1: Gene Link RAPD Primers and their sequences

Sr. No.	Primer Code	Sequence (5´to 3´)
1	1 GL Decamer C-01	5´- TTCGAGCCAG-3´
2	2 GL Decamer C-02	5´- GTGAGGCGTC-3´
3	4 GL Decamer C-04	5´- CCGCATCTAC-3´
4	5 GL Decamer C-05	5´- GATGACCGCC-3´
5	6 GL Decamer C-06	5´- GAACGGACTC-3´
6	8 GL Decamer C-08	5´- TGGACCGGTG-3´
7	9 GL Decamer C-09	5´- CTCACCGTCC-3´
8	10 GL Decamer C-10	5´- TGTCTGGGTG-3´
9	11 GL Decamer C-11	5´- AAAGCTGCGG-3´
10	1 GL Decamer A-01	5´- CAGGCCCTTC-3´
11	2 GL Decamer A-02	5´- TGCCGAGCTG-3´
12	3 GL Decamer A-03	5´- AGTCAGCCAC-3´
13	4 GL Decamer A-04	5´- AATCGGGCTG-3´
14	5 GL Decamer A-05	5´- AGGGGTCTTG-3´
15	7 GL Decamer A-07	5´- GAAACGGGTG-3´

Scoring and Analysis of Data

The fingerprints were examined under ultra violet Transilluminator and photographed using Gel documentation system (WEALTEC, Dolphin-DOC). Amplicons were scored by starting from top of the lane to its bottom. All visible and unambiguously scorable fragments amplified by the primers were scored under the heading of total scorable fragments. Amplification

profiles were compared with each other and to molecular size marker and bands of DNA fragments were scored as present (1) or absent (0). Those ambiguous bands which could not be evidently discriminated were not counted. The data of the primers were used to estimate genetic similarity on the basis of number of shared amplification products (Nei and Li, 1979). The coefficients were calculated and the similarity coefficients were utilized to generate a dendrogram by using unweighted pair group method of arithmetic means (UPGMA) (Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

Random amplified polymorphic DNA analysis

The genetic variability of scarab beetles collected from different sites of Faisalabad, Pakistan was investigated. Legs of beetle specimens were used for DNA extraction so that there might be no chances of contamination of micro-flora present in the gut of insects (Clark *et al*, 2001). The average yield of DNA was found to be 54µg/ml for all the samples and the maximum concentration was 250 µg/ml. A total of 135 DNA fragments were generated by 15 primers, with an average of about 9.0 bands per primer. All of the primers listed produced scorable markers in each DNA sample. Bands that a primer yielded in this study ranged from two to thirteen. The amplified fragments from the scarab beetles were in the same range as already defined by Richards et al. (1997) not in references.

Reactions were duplicated from time to time to check the consistency of amplified products. Only easily resolved bright DNA bands were considered and scored. All the species showed diversity with each other on their amplification profile bases. Of these molecularly characterized 135 amplified DNA bands, almost all the bands showed polymorphism among the collected scarab beetle species. The level of polymorphism was different with different primers among these beetles. Sample gels resulting from the C-04, C-06 primers across the populations collected are presented in Fig. 1. These results indicated a high level of DNA variation among these scarab beetle species.

Cluster Analysis

The genetic similarity matrix of RAPD data for the ten scarab beetle species was constructed based on Nei and Li's (1979) coefficient of similarity as shown in Table 2. The genetic similarities of these beetle species ranged from 44% to 71.0%. *Scarabaeous pithecius* and *Onthophagus atroplitus* had the greatest similarity (71%). The number of amplified fragments from both *Catharsius pithecius* and *Onthophagus atroplitus* with 15 primers were 81 and 86 respectively (data not shown). The genetic similarity between *Schizonycha*

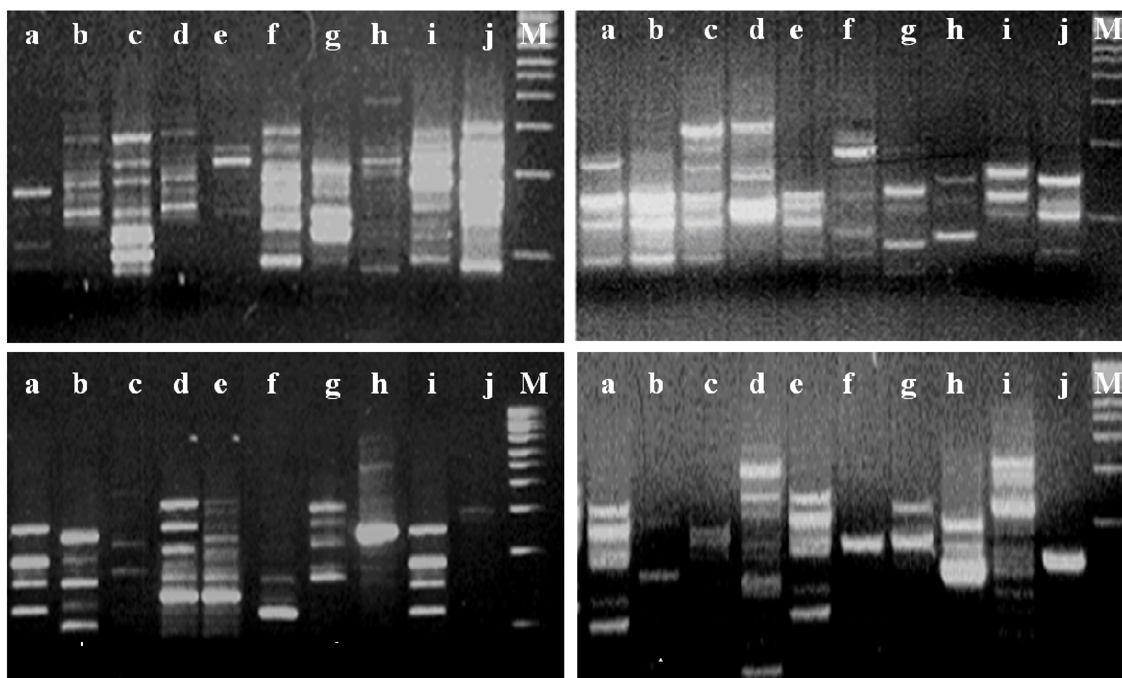

Fig. 1: Amplification profile of ten scarab beetle species with primers
Upper left panel: C-04; Upper right pane: A-04; Lower left pale: C-06; Lower right panel: A-07
Lane a= *Adoretus bimarginatus*, Lane b= *Schizonycha rufficollis,* Lane c=*Anomala lineatopennis,* Lane d= *Lepidista mansucta*, Lane e= *Onthophagus nilgirensis*, Lane f= *Scarabaeous pithecius*, Lane g= *Anomal varicolor*, Lane h= *Anomala bengalensis*, Lane i= *Pentadon bispinosis*, Lane j= *Onthophagus atroplitus,* Lane M= Size marker (1Kb)

Table 2: Similarity matrixes of Scarab beetle species obtained from RAPD markers

Name	*Adoretus bimarginatus*	*Schizonycha rufficollis*	*Anomala lineatopennis*	*Lepidista mansucta*	*Onthophagus nilgirensis*	*Scarabaeus pithecius*	*Anomala varicolor*	*Anomala bengalensis*	*Pentadon bispinosis*	*Onthophagus Atroplitus*
Anomala varicolor	1									
Schizonycha rufficollis	0.682	1								
Anomala lineatopennis	0.676	0.669	1							
Lepidista mansucta	0.658	0.709	0.659	1						
Onthophagusnilgirensis	0.604	0.544	0.536	0.63	1					
Scarabaeus pithecius	0.622	0.515	0.537	0.566	0.508	1				
Adoretus bimarginatus	0.678	0.572	0.582	0.595	0.543	0.691	1			
Anomala bengalensis	0.647	0.626	0.624	0.668	0.516	0.656	0.629	1		
Onthophagus atroplitus	0.651	0.634	0.589	0.612	0.689	0.524	0.584	0.545	1	
Pentadon bispinesus	0.613	0.448	0.564	0.612	0.545	0.71	0.685	0.636	0.52	1

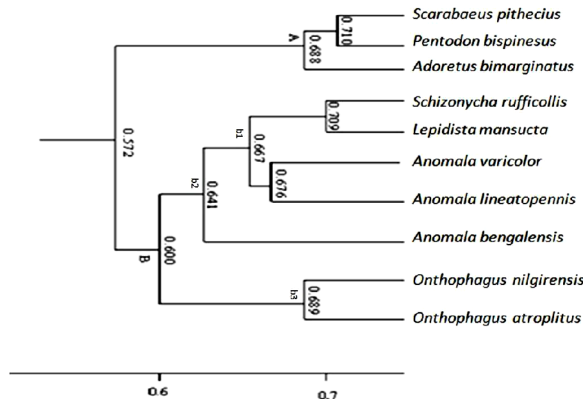

Fig. 2: Dendrogram among ten scarab beetle species generated through RAPD data using UPGMA method

rufficollis and *Lepidista mansucta* was the second highest (70%). The similarity coefficient of *Schizonycha rufficollis* and *Onthophagus atroplitus* was the lowest (44%).

The genetic similarity matrix of RAPD data were utilized for the construction of a dendrogram (Fig. 2) by the UPGMA method as described by Jae Sam et al. (1997), Pronkulwat et al. (1998) and Szalanski et al. (2000). Since DNA polymorphisms among the collected species were high as revealed by the RAPD analysis, the genetic diversity of these species seems to be high. The dendrogram obtained from the cluster analysis of nine similarity matrices of scarab beetles further revealed interesting patterns with the identification of two main clusters corresponding to ten scarab beetle species. Cluster 'A' consisted of three species *Scarabaeous pithecius, Pentadon bispinosis* and *Adoretus bimarginatus.* The maximum closely related pair was of sacarb beetles, *Scarabaeous pithecius* and *Pentadon bispinosis*. The cluster 'B' was further divided into three sub-clusters, 'b_1', 'b_2' and 'b_3'. The sub-cluster b_1 comprised of *Schizonycha rufficollis, Lepidista mansucta, Anomala varicolor* and *Anomala lineatopennis.* The sub-cluster b_2 consisted of five scarab beetle species, *Schizonycha rufficollis, Lepidista mansucta, Anomala varicolor, Anomala lineatopennis* and *Anomala bengalensis,* while the sub-cluster b_3 had two scarab beetle species, *Onthophagus nilgirensis and Onthophagus atroplitus*.The maximum closely related scarab beetle species, *Anomala varicolorAnomala lineatopennis* and *Anomala bengalensis* were found in sub-clusters, 'b_1' and 'b_2. The similarity coefficient of cluster "A" and Cluster "B" was found to be 57.2% (Fig. 2).

Molecular Characterization

In the present study, the bands that a primer yielded ranged from two to thirteen. The faint bands were also observed but were not scored. The RAPD methodology produced consistent results with optimized conditions and therefore, has a potential to be employed for phylogenetic relationships and taxonomic classification (Jain et al., 2010). The polymorphism revealed by RAPD could be a result of nucleotide changes at the primer annealing site or due to addition or deletion between two priming sites that may have resulted in different lengths of the amplification products (Williams et al., 1990). RADP markers are considered to be inherited in a Mendelian manner, but unlike other DNA level markers they are co-dominant, and have complete dominant phenotypes (Caterino et al., 2000). The bands of the same size amplified from different species are considered as the same *loci* and bands of different size are considered as different *loci*. In addition, the amplification size not clear from different species might have homologous sequences as described by Jain et al. (2010).

Generally, the size and number of the fragments produced strictly depend upon the nucleotide sequence of the primer used and upon the source of the template DNA. The phylogenetic relationships of the major lineages of beetles are relatively poorly known (Browne and Scholtz, 1995). Only recently has some of the morphological data been examined phylogenetically. Browne and Scholtz (1995) brought together all of the available data in the final paper of the series and proposed a phylogenetic classification of the Scarabaeoidea. Moreover, the molecular sequence information is being gathered now (Villalba et al., 2002; Bell et al., 2004; Grebennikov and Scholtz, 2004; Philips et al., 2004). In the present research, the

relatedness found in all the collected species were accurate as revealed by their morphological characters and in accordance to the dendrogram of similarity for dung beetles in 1 ha and 10 ha forest fragments as defined by Klein (1989).

Conclusion

All of the primers listed produced scorable markers in each DNA sample. Of these amplified DNA bands, almost all the bands showed polymorphism among scarab beetle species. The RAPD methodology produced consistent results, and it is concluded from the current findings that scarab beetles have high level of DNA polymorphism as well as high genetic diversity. There was a single primer (C-04) that can identify more than one species without the aid of other primers. Therefore, based on the current RAPD profile data, it is concluded that the scarab beetle species can be identified by using one RAPD marker or a combination of two or more markers for the molecular characterization of Scarab beetles contrary to above statement of one maeker is sufficient.

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