Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(3): 1117-1121
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Received: 01-03-2018
Accepted: 02-04-2018

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Analyzing the genetic variability of rice weevil, *Sitophilus oryzae* (Coleoptra; Curculionidae) in different geographic locations of district Charsadda 2018, Khyber Pakhtunkhwa (KP), Pakistan

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Abstract
The rice weevil (*Sitophilus oryzae*) is a grain feeding specie belonging to family Curculionidae, order Coleoptra. *S. oryzae* is ranked as the first important pest causing the highest damage of 50 tested countries. The present research work was focused on the molecular and genetic analysis of *S. oryzae* from district Charsadda Khyber Pakhtunkhwa (KP). The Sieve method was randomly used to capture the adult *S. oryzae*. To separate the red flour beetle from wheat flour mills we used sieves with 4mm mesh. DNA extraction was made from individual adult *S. oryzae* using TNE salt extraction protocol. RAPD markers were used for PCR amplification during the first step to find out genetic variability, gene flow and rate of migration in different *S. oryzae* populations. The PCR products were analyzed using gel electrophoresis. The data generated through RAPD markers were analyzed through POPGENE software. The current study can be taken as a starting point for future researchers aimed at defining the level of intra and inter species genetic diversity. Based on the results, it is suggested that polymorphic markers out of RAPD method be used for understanding genetic variability in *S. oryzae* population in Pakistan and could be helpful in future vector control programs.

Keywords: Khyber Pakhtunkhwa, PCR, RAPD, Charsadda, rice weevil

1. Introduction

The rice weevil (*Sitophilus oryzae*) is a grain feeding species belonging to family Curculionidae, order Coleoptra. Studies on various strains of C. oryzae showed that the species actually included two distinct weevils, S. *oryzae* (L) and Sitophilus zeamais [26]. The rice weevil can be distinguished from two other weevils in the Sitophilus genus (S. zeamais and S. granarius) by morphological characters of the adults, shape of male genitalia and body size [34]. The species can be distinguished based on biological differences including life cycle, flying ability and distribution. These Sitophilus species can also be identified by molecular markers [17, 26], karyotype (2n=22 in S. oryzae and S. zeamais, 2n=24 in S. granarius) as well as some biochemical differences [6, 7]. S. oryzae female inserts eggs into a seed on which she has fed, sealing that oviposition hole with a biogenic material. The adult can mate immediately upon emergence or up to two days after emergence [4]. One mature adult develops per grain although more than one egg may have been deposited inside the grain [35]. The life cycle of rice weevil is from 37 to 40 days at 25 ºC and 70% relative humidity in which the development time for each stage: egg, larvae, pupae and adult occur within 4-7 days. The duration of immature stages of development is dependent on the moisture content of the grain, the temperature and relative humidity [11, 12]. The conditions for optimum development and oviposition of the rice weevil are 28-30 ºC and 75-90% relative humidity. The lifespan of the rice weevil extends up to eight months and they can survive starvation for one month [4]. Males and females of this species can be identified by external characters [15]. The female lays 300- 400 eggs during their life span. S. oryzae is one of the most serious insect pests of stored grain, and is therefore of huge concern to grain handling and export industries. Rice weevil attacks a wide range of cereals (wheat, barley, oat, corn, sorghum, rice, millet, buckwheat, rye) [30]. According to a global survey carried out in 85 countries, S. oryzae is ranked as the first important pest causing

the highest damage and was found in 67.7% of grain samples, destroyed 45% total world grain production (1056x106 million tons) and 55% total exporting grain (39x106 million tons) of 50 tested countries [12].

1.1 Life cycle
The egg period of rice weevil occupy an average of 4.41 days [3]. Rice weevils were reared in laboratory on unpolished rice at 27 to 28 ℃ and 90 to 95 per cent relative humidity [33]. According to him, the egg period occupied 6 to 7 days. An average of 2.65 days of incubation period was noted in laboratory [22]. The egg period lasted for 5 to 10 days whereas recorded 3 to 10 days of incubation period in Formosa and the hatchability of egg of S. oryzae were influenced by the female age [19]. Incubation period of rice weevil on maize at 23 to 30 ℃ and 79 to 87 per cent relative humidity was also recorded [13]. Larval period of 30 and 23 days were recorded on maize and up to four larvae in a grain which occupied 18 to 20 days to complete larval stage in rice [8, 23]. Pupal period occupied six to nine days with an average of eight days and pre-pupal period occupied one to two days [2]. The life cycle was completed in about a month or may be prolonged for several months under favorable conditions [27].

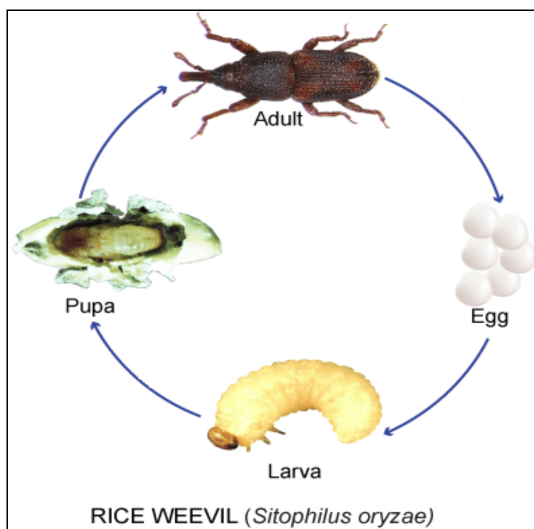

Fig 1: Life cycle of *Sitophilus oryzae*

2. Materials and Methods
2.1 Sampling and Study Area
The present study was designed to elucidate the genetic diversity of red floor beetle in different localities of district Charsadda. Sieve method was randomly used to capture the adult red floor beetles. To separate the red flour beetle from wheat flour mills we used sieves with 4mm mesh. The sampling was done from June to November 2018. Khyber PakhtoonKhwa is the second largest province of Pakistan, situated in the northwestern region of the country. It has a famous international border in its North-West with Afghanistan known as Durand Line that's why in earlier times the province was known as North-West Frontier Province (NWFP). The total area of the province is 74, 521 km2 (28,773 sq. mi). Latitude and Longitude of province Khyber PakhtoonKhwa is 33.1462 and 71.1893 respectively, with variable climate and temperature at varied zones of province Khyber PakhtoonKhwa. Charsadda stands in the rolling flat plains of province Khyber PakhtoonKhwa lies between 34-03' and 34-38' north latitudes and 71-28' and 71-53' east longitudes. The famous river Kabul separates Charsadda from the provincial capital Peshawar.

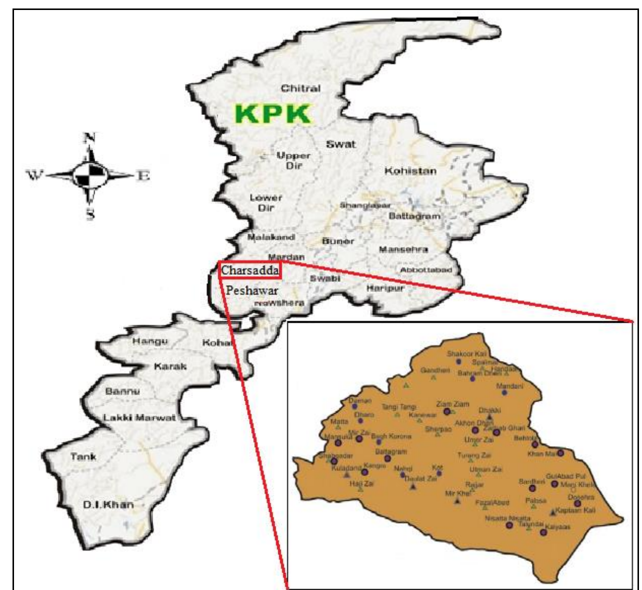

Fig 2: Map of province Khyber Pakhtunkhwa (KP) including Charsadda

2.2 Identification and preservations
Collected samples were identified by studying morphological characters of red flour beetle under stereomicroscope. After identification samples were preserved in 70% alcohol using vials with label of date, breeding area, district name, sample number and stored at 4 °C, under standard conditions temperature 27-30°C, relative humidity 65-95% and 12-h light/dark cycle [5].

2.3 Molecular study
The Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) recent developments in molecular biology have made it possible to apply DNA based technologies for genome analysis in a variety of animal species. Among the several DNA based techniques, Random Amplified Polymorphic DNA (RAPD) [28] gained importance due to its simplicity, efficiency and non-requirement of sequence information [25]. Due to the technical simplicity and speed of RAPD methodology, RAPD markers have been successfully used for the generation of genetic similarities and phylogenetic analysis [36]. A significant advantage of PCR over other approaches is the minute amount of template DNA required. A few scales or a leg segment may suffice for DNA extraction. Specimens can be kept alive for crossing experiments, submitted to other analysis, or preserved as morphological vouchers [13]. As random amplified polymorphic DNA PCR (RAPD-PCR) relies on the analysis of banding patterns generated with arbitrary decamer primers, it requires no prior knowledge of the genome and tends to target repetitive and rapidly evolving genome regions [29]. Amplification in RAPD analysis occurs anywhere in a genome that contains two complimentary sequences to the primer that are within the length-limits of the PCR [20]. It is a fast and easy method for identifying DNA polymorphism generated from several regions of the genome [21]. Polymorphisms between individuals arise through. Moreover, it provides an opportunity to estimate relatedness within and among species based on DNA variation. RAPD markers are successfully used for the identification of different insect species [31]. The development and application of methods alternative to morphological identification is neither cheap nor trivial. Morphology remains the simplest, fastest and least expensive means of identification for many of insect species

[10]. Efficient use of RAPD markers requires scrupulous standardization of all conditions of collection, extraction, and amplification; and also the appropriate data analysis [32].

2.4 Advantages of RAPD-PCR Analysis

As previously no work has been done on the biodiversity and molecular characterization of beetles in Pakistan, so it is the need to estimate to what extent man's exploitations of natural resources has imparted adverse impact on the biodiversity of insects and what kind of genetic variations in insects arises due to these activities. The present research study was conducted to estimate the biodiversity and to analyze the molecular characterization of some scarab beetles species using RAPD-PCR analysis.

2.5 DNA extraction and Polymerase chain reaction (PCR)

DNA extraction from collecting samples of *Red Flour Beetle* populations was made by using TNE buffer method [16, 18]. To assess the genetic variation in *Red Flour Beetle* populations of district Charsadda. The conditions of Polymerase chain reaction (PCR) was optimized by using Random Amplified Polymorphic DNA (RAPD) 10 bases arbitrary nucleotide primers. Gene Link-A series RAPD primers were used for the amplification of extracted DNA samples. The volume of reaction was 25 µl containing following concentration of PCR regents. The thermal PCR conditions for amplification of DNA samples were optimized by ups and down range of temperature. There were following optimized conditions. Finally the PCR products were run on 1.6% agarose gel electrophoresis at 80 voltages for one hour and gel was observed on Gel Doc apparatus and different amplified bands were observed and saved the image for results.

2.6 Data Analysis

The size of amplified bands or loci compared with bands of DNA markers. The data generated from the detection of polymorphic amplified fragments analyzed using Popgene 2018 software. All amplification products will be scored as present (1) or absent (0) for each of the samples. Ambiguous bands that could not be clearly distinguished would not be scored. The bands counted by starting from the top of the lanes to their bottom in all lanes.

3. Results

3.1 Random Amplified Polymorphic DNA Analysis

For the assessment of genetic variations random amplified polymorphic DNA (RAPD)-PCR technique was used. Many characters influence the reproducibility of RAPD-PCR technique such as template quality and quantity, sequence of primer, polymerase and the type of thermal cycler [24]. The RAPD technique offers some advantages when applied to studies within the intraspecific level [1]. Keeping in view different concentrations of $MgCl_2$, *Taq* DNA polymerase and template DNA were optimized for PCR conditions. DNA concentrations of 10, 15, and 20, 25 and 30ng/25µl in each reaction were studied. The concentration of 25 ng/25µl was found to produce the most consistent and reproducible banding patterns. The concentrations of $MgCl_2$ studied (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 mM), and found 3 mM concentration optimum for consistent results. Similarly *Taq* DNA polymerase concentrations were studied (0.5, 1.0, 1.5 and 2.0 units/25µl reaction). Two unit concentration of *Taq* was found optimum for better amplification of genomic DNA.

3.2 Genetic analysis of *Sitophilus oryzae*

To assess the genetic variations of collected samples twelve samples were analyzed through RAPD-PCR. Six decamer oligonucleotide (10bp) A-series RAPD primers designed by Gene-Link company were used for the amplifications of extracted DNA (Table 1). Each primer produced distinct, easily detectable bands of variable intensities. A total of 360 DNA fragments were generated by 6 primers in all samples with average 6.0 bands per primer for all samples; 360 samples with an average of about 17.6, 16.1 and 26.1 bands per primer in district Charsadda. The size of amplified fragments was found to be with the help of 1 kb/ 100bp DNA molecular marker. The size of the lowest band of the DNA marker was 250 base pair (bp) and highest band with 10,000 base pair. 1 kb DNA marker had 14 bands having size as; 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000 &10,000 bps from lower to higher in the range of 200 bp to 2500. The primers that produced amplification profile were only included in study.

Table 1: RAPD Primer Sequence (Genelink) and their codes

Sr. No.	Primer name (code)	Primer sequence	GC contents
1	GL Decamer A-03 (A-03)	AGTCAGCCAC	60%
2	GL Decamer A-04 (A-04)	AATCGGGCTG	60%
3	GL Decamer A-05 (A-05)	AGGGGTCTTG	60%
4	GL Decamer A-06 (A-06)	GGTCCCTGAC	70%
5	GL Decamer A-08 (A-08)	GTGACGTAGG	60%
6	GL Decamer A-10 (A-10)	GTGATCGCAG	60%

4. Discussion

To check the consistency of amplified products, reactions were duplicated from 2-3 times. Only easily resolved bright DNA bands were considered and scored. The amplified bands showed diversity of *Sitophilus oryzae* species. These molecularly characterized 134,141 and 157 DNA bands amplified by 6 primers mostly showed polymorphism among twelve samples. The level of polymorphism was different with different primers among *Sitophilus oryzae* populations. These results indicated that DNA variation was existed within populations of *Sitophilus oryzae* species of district Charsadda.

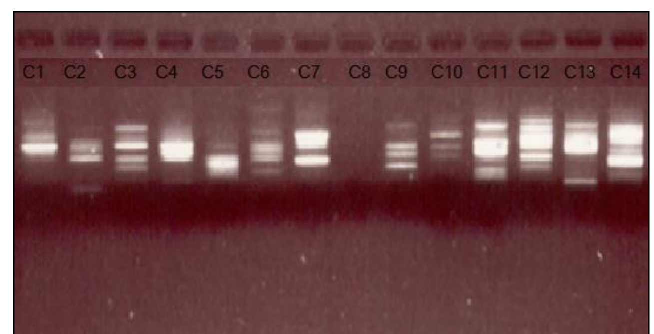

Fig 3: DNA extractions from district Charsadda samples of *Sitophilus oryzae*

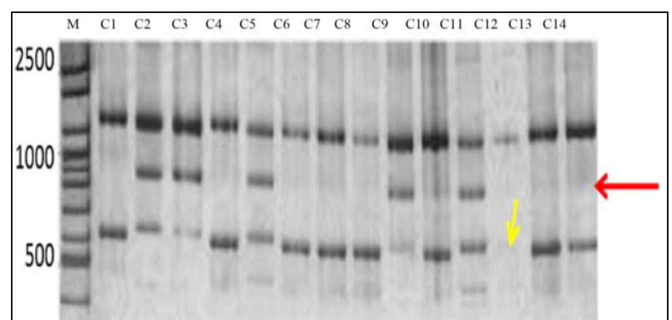

Fig 4: Amplification profile of *Sitophilus oryzae* species from district Charsadda with primers A-03.

In the current results Six RAPD markers were analyzed in fourteen samples from district Charsadda, the amplified loci were ranged from 100 to 2000 bp. A-04 amplified maximum number of loci at 700bp which all the samples showed monomorphic banding pattern except the two sample in lane1,4,,6,7,8,10,13,14 respectively (Fig 3 and 4). At the 750bp and 600 bp all the banding pattern was same that showed monomorphic loci in all the population of *Sitophilus oryzae* that were collected from the different sites of district Charsadda. It is mentioned that there are two bands can be used as markers for the identification of genetic variability in *Sitophilus oryzae* from lane 2,3,5,7,9 as shown in figure 4, At 460bp size of bands showed polymorphic patterns of loci which is absent in four samples population of *Sitophilus oryzae*. At 200 base pair, the polymorhpy band were observed and similarly at 100bp size of the population showed polymorhpy banding pattern in lane 4 and 5 (Fig 4).

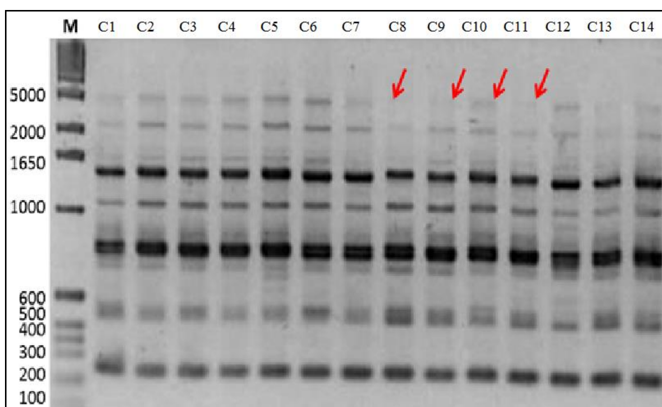

Fig 5: Amplification profile of *Sitophilus oryzae* species from district Charsadda population with primers A-04.

5. Conclusions

This study is the first report using RAPD analysis to analyze the genetic diversity of *Sitophilus oryzae* species of Pakistan. Based on the results, it is suggested that polymorphic markers out of RAPD method be used for understanding genetic variability in *Sitophilus oryzae* population in Pakistan and could be helpful in future vector control programs. According to [5], this high differentiation may reflect important differences in competence, susceptibility to the parasite, and resistance to insecticides, so that populations from different areas of Charsadda must be treated as independent epidemiological units. The RAPD patterns of the primers used in the present study could be a source of additional DNA markers that may be used in the development of genome maps for *Sitophilus oryzae* species. In addition it is suggested that RAPD markers can be used for the identification and molecular characterization of different species in the future. Producing much polymorphic information of target genomes with more primers can compensate the shortcoming of this method. However, more detailed experiments that can deduce phylogenetic configuration should follow to prove the genetic differentiation based on polymorphic markers. These results can be taken as a starting point for future researchers aimed at defining the level of intra and inter species genetic diversity. For this purpose, a large number of insect species or any predator or pest species should be sampled and analyzed using additional primers. Furthermore, in order to detect genetic discrimination among different species distinct polymorphic bands can be cloned and sequenced. The RAPD patterns of the primers used in the present study could be a source of additional DNA markers that may be used in the development of genome maps for different insect species.

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