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RAPD-PCR studies on bumble bees (*Bombus haemorrhoidalis* and *Bombus rufofasciatus*) and their important pests (*Physocephala tibialis and Aphomia sociella*)

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ABSTRACT

Bumble bees (*Bombus* spp.) are important pollinators attacked by different pests. Present studies were conducted to know the genetic relatedness among bumble bee species *Bombus haemorrhoidalis*; *Bombus rufofaciatus* and their important pests i.e. conopid fly, (*Physocephala tibialis*) and bee moth (*Aphomia sociella*). DNA was isolated from all the insects under study and quantified. PCR was carried out using 14 random OPA primers. In all the primers bands were observed ranging from 300-700 bp in *B. haemorrhoidalis* whereas in *B. rufofaciatus* they were recorded between 300-900 bp regions. In case of *Physocephalatibialis* amplified DNA bands were observed between 200-700 bp region whereas in *A. sociella* bands were obtained between 300-1000 bp. Based on these studies 62% genome similarity was found between *B. haemorrhoidalis* and *B. rufofaciatus* whereas very less similarity (51%) was recorded between *A. sociella*, *P. tibialis*, *B. haemorrhoidalis* and *B. rufofaciatus*. In future such studies can be used for finding the relation between different insect species which are parasitically or socially related to each other.

Key words :

Introduction

Bumble bees belong to order hymenoptera, family apidae and genus bombus. These have more than 250 species spread throughout the temperate regions of the globe. Few species also found in tropical regions. A global decline in pollinator including bumble bees abundance and diversity has demanded more research attention on their ecology and genetics. According to Mandal *et al*. (2010), genetic studies of insects depend on the ability to extract DNA of sufficient quantity and quality for PCR amplification and sequencing. Different methods have been tried by Deenihan (2011) for extracting DNA from 20 to 38 years old specimens of queen and worker bumble bees. According to Duennes *et al*. (2012), the relationships between the geographical isolation of two taxa (*B. ephippiatus* and *B. wilmattae*) and a third species, *B. impatiens* (only in north of Mexico) was remained controversial for long. The phylogenetic analysis of DNA sequences, resolved the phylogeny of these three taxa. These lineages were not only correspond to geographic and habitat variations across their range but also to distinct color pattern groups present in the species. Pests complex of bumble bees were also studied using molecular techniques (molecular markers) of identification. According to Kissinger *et al.* (2011), pathogens have

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been implicated as potential factors in the recent declining of some North American bumble bee (*Bombus*) species. But little information was available about the natural enemy complex of bumble bees in the United States. Different researchers (Collins and Weigmann, 2002; Kutty *et al*., 2008; Peterson *et al*., 2007; Winterton *et al*., 2007) utilized the DNA sequences in characterization of phylogenetic analysis of dipteran families and subfamilies. It was known that the nuclear ribosomal gene 28S, the only gene present universally in the genome of all dipterans, have a great importance in phylogenetic studies. Gibson and Skevington (2011) successfully amplified and sequenced the PGD, 12S and 28S regions of the genome. It was found that the analyses region lies between 374 and 3824bp in the conopid fly. According to Jones (2014), *B. hypnorum* had a lower functional genetic diversity at the sex-determining locus than native species. That's why this species is more susceptible to the parasitic infections. Keeping in view the importance of relationship between the insects and their associations, present studies were conducted to know the genetic relationship between bumble bees and their pests.

Material and Method

The different procedures used in the molecular studies of bumble beesand their pests were as follow:

The DNA was extracted from all the insects using the following methodology

DNA Extraction

The insect material (bumble bees, wax moth and conopid fly) were isolated from their cultures and after killing them in the killing bottles were stored at -20^{0}C in small glass vials. Total genomic DNA from young larvae (bumble bee, conopid fly and wax moth) and adults (bumble bees) of four insect species was extracted as followed by Roux *et al*. (2006) with some modifications as:

Ground 200-300 mg insect material in liquid nitrogen in pre-chilled pestle mortar and transfer the ground material to 750 mL preheated (68^{o}C) extraction buffer in eppendorf tubes. Mix it by swirling and incubated at 68^{o}C for 2 hours. Add 750 µL of chloroform: isoamyl alcohol (24: 1), stir thoroughly and spin it at 13000 rpm for 15 minutes at 4^o C. Then, transfer the supernatant into new eppendorf tubes. After this, DNA is precipitated by addition of 500 µl ice-cold isopropanol in each tube. Keep it at -20^o C for one hour, then spin at 13000 rpm for 15 minutes at 4^{o}C and the pellet was taken out. Wash the DNA pellet with 70% ethanol for 20 minutes and spin it at 10000 rpm for 5 minutes at 4^o C. Dry the pellet overnight and dissolve in 100 µl TE buffer (pH 8.0). For purification, add 1 µl of RNase (10 mg/ml) to TE containing DNA, mixed gently and incubated for 1 hour at 37^{0}C. Spinn it at 11000 rpm for 15 minutes at 4^{0}C to pellet the DNA. Wash the DNA pellet with 70% ethanol. Then Dry the DNA pellet and dissolve in TE buffer.

Assessment of DNA quality and quantity

Quality of DNA was checked by running a gel on 0.8% agarose. Quantity of DNA was assessed by using picometer (eppendorf 1080) in the Department of Plant Pathology, UHF, Nauni, Solan. The ratio of absorbance at 260 and 280 nm was measured to check the contamination of protein, and the concentration of DNA solution was measured at 260 nm or 280 nm. All the samples were diluted to a concentration of 30-40ng/µl before use.

Random amplified polymorphic DNA (RAPD)

Fourteen RAPD primers used in the present studies were listed below with their sequence:

1	OPA-01	CAGGCCCTTC
2	OPA-02	TGCCGAGCTG
3	OPA-03	AGTCAGCCAC
4	OPA-04	AATCGGGCTG
5	OPA-05	AGGGGTCTTG
6	OPA-16	AGCCAGCGAA
7	OPA-17	GACCGCTTGT
8	OPA-18	AGGTGACCGT
9	OPD-01	ACCGCGAAGG
10	OPD-07	TTGGCACGGG
11	OPD-12	CACCGTATCC
12	OPO-07	CAGCACTGAC
13	OPAT-05	ACACCTGCCA
14	OPAT-20	ACATCAGCCC

PCR reaction

PCR reaction was carried out in a thermocycler (eppendorf Mastercycler gradient) in a 25 µL reaction volume containing the following components:- 1U of Taq DNA polymerase, 1X Taq DNA polymerase buffer containing 1.5 mM $MgCl_2$, 10 p moles of primer, 2.5mM of deoxynucleotide triphosphate (dNTPs) and 30-40ng of template DNA. The method used for PCR reaction was the modification of Roux

et al. (2006).

Components of PCR master mix (200 µl):

dNTP's	30 µl
PCR buffer	50µl
Primer	20µl
Taq DNA polymerase	6µl
$MgCl_2$	6µl
Distilled water	288µl
template DNA	3µl

The following temperature profile was used for the DNA amplification

Denaturation step at 94°C for 5 minutes was followed by 40 cycles at 94° C for 1 minute. Primer annealing at 36°C for 1 minute and amplification at 72°C for 1 minute. After 40 cycles, there was a final step of 10 minutes at 72°C.

Detection of PCR products

The products of RAPD based PCR were detected by electrophoresis(BangloreGenei, IndiaLimited) on1.2 per cent agarosegelunder submerged conditions. 1X TAE buffer was used as gel and tray buffer with 3µl ethidium bromide. Added 2 µl of 6X loading dye (bromophenol blue) to each PCR amplified sample and loaded into the gel. 100 base pair to 3000 base pair ladders (GeNei™) were used as size marker. The gel was run at 80 V and 110 Ampere until the loading dye reached the gel front and viewed the amplified DNA under the UV transilluminator and the images were taken through Biovis gel documentation system.

Scoring of bands

The amplified bands after separation were visualized using Gel Documentation system. Further the bands were scored for percentage polymorphism for each set of primer amplified product using NTSYS 2.2.

Analysis of RAPD data

The scored bands were analyzed in the form of binary system to prepare the similarity index. The bands with same molecular weight and mobility were treated as identical fragments. Data matrices were prepared in which the presence of a band was coded as 1 whereas the absence as 0. The data matrices were analyzed by the SIMQUAL Program of NTSYS-PC (Version 2.2) and similarities between genotypes were estimated using Jaccard similarity coefficient, calculated as J = A /(N-D), where A is the number of positive matches (i.e. presence of band in both samples), D is the number of negative matches (i.e. absence of band in both samples) and N is the total sample size including both the number of matches and unmatches. Dendrogram was produced from the resultant similarity matrices using the UPGMA method.

Results and Discussions

DNA of *B. haemorrhoidalis*, *Bombus rufofaciatus*, *Physocephala tibialis* and *Aphomia sociella* swas isolated which was detected through Gel electrophoresis. Studies on RAPD-PCR of respected insectswere conducted by using fourteen random decamer primers designed by Operon Technologies (Alameda, CA).

PCR studies of bumble bee, *B. haemorrhoidalis* and *B. rufofaciatus*

Out of 14 OPA, OPD, OPO and OPAT primers used, 11 primers gave DNA amplification with the present DNA. Three primers did not show any amplification. Present results showed a banding pattern between 300-700bp (Fig. a) for indigenous bumble bee, *B. haemorrhoidalis*. The DNA of *B. rufofaciatus* showed amplification with twelve primers out of total fourteen primers used. The DNA bands were formed between 300- 900bp region. One unique band was found very close to 900bp region (Fig b) which was absent in *B. haemorrhoidalis*. Two primers did not bind with the DNA of *B. rufofaciatus.* Earlier, Gadau *et al*. (2001) calculated the haploid

Table 1. Similarity Coefficient values of RAPD data using Jaccard's similarity correlation coefficient

Insect	*Bombus haemorrhoidalis* (BHS)	*Bombus rufofaciatus* (BRS)	*Physocephala tibialis* (PT)	*Aphomia sociella* (AS)
Bombus haemorrhoidalis (BHS)	1.000			
Bombus rufofaciatus (BRS)	0.615	1.000		
Physocephala tibialis (PT)	0.515	0.517	1.000	
Aphomia sociella (AS)	0.571	0.452	0.517	1.000

genome of *B. terrestris* to be 274 Mb. In RAPD PCR, the bands found were in the range of 250-1100bp. Earlier, different researchers have found generegion of 650bp by DNA barcoding. This region was bordered by conserved sequence regions which allowed high confidence in species level identification (Hebert *et al*., 2003; Folmer *et al*., 1994; Simmons and Weller, 2001).

PCR studies of bee moth, *Aphomia sociella*

In bee moth, *A. sociella*, eleven markers showed band formation between 300-1000bp. Three primers did not amplified the DNA as no banding was observed (Fig. c). Murthy *et a*l. (2014) reported the band formation of amplified DNA between 250-1500bp in *Plutellaxylostella* (Yponomeutidae; Lepidoptera).

PCR studies of conopid fly, *Physocephala tibialis*

On gel electrophoresis conopid fly DNA displayed banding pattern between 200-700bp (Fig d) with twelve primers. Two primers did not displayed any band formation. Gibson and Skevington (2011) successfully amplified and sequenced the PGD, 12S and 28S regions of the genome of conopid fly. It was found that the analyses region lies between 374 and 3824bp.

Similarity studies

Data in the similarity matrix (Table 1) showed that *B. haemorrhoidalis* (BHS) has maximun per cent similarity (62%) with *B. rufofasciatus* (BRS). Whereas, minimum similarity was 45.2% between *B. rufofaciatus* and *Aphomia sociella* (AS) followed by 51.5% in *B. haemorrhoidalis* and *Physocephala tibialis* (PT). However, 57% similarity was observed between *B. haemorrhoidalis* and *Aphomia sociella*. Similarity between *Physocephala tibialis* and *Aphomia sociella* was found to be 51.7%.

Dendrogram based on RAPD banding pattern

Dendrogram was created using the similarity coefficient and unweighted pair group mean average (UPGMA) method in order to visualize genetic differentiation among bumble bees and their pests (Fig. e). It revealed 61.50% similarity between bumble bees. While the pests (*P. tibialis* and *A. sociella*) were found to be 51% related with respect to their genome.

Conclusions

Bumble bees (*B. haemorrhoidalis* and *B. rufofaciatus*) showed a close relationship with each other. RAPD amplification revealed distinct banding pattern for both the bombus species. Thus, signify their geographical and species level distinction. Conopid fly and bee moth showed a banding pattern lies between 200-700bp and 300-1000bp region of the genome. Both these pests have specific and distinct genome profile. These studies will help in revealing more genomic information of bumble bees and their pests relationships.

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