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Phylogenetic Analysis of *Noctuoid moths* (Lepidoptera: Noctuoidea) by RAPD-PCR Technique

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ABSTRACT: Noctuoidea is one of the most diverse families within the Lepidoptera. In present study we examined the phylogeny of the Noctuoidea between four species of moths and constructed Phylogenetic tree based on the data from the RAPD technique. Random amplified polymorphic DNA technique has the potential to become a powerful technique for studying genetic relationship and genetic diversity in Lepidopteron. Two primers namely OPA-9 and OPA-13 were used for RAPD analysis. A total of 31 bands were scored with the two decamer primer of them all are monomorphic and percentage of polymorphism is 0%. On the basis of banding pattern of RAPD bands dendrogram constructed with Neighbor joining method which shows Phylogenetic evolution history within four species of Noctuoidea. There is significant difference between branching pattern between the four species which signifies the need of using of RAPD molecular technique for taxonomic classification and to plot the evolutionary relationship between the species.

Keywords: Noctuoidea; Genetic Diversity; Phylogenetic; Cluster analysis and RAPD-PCR.

INTRODUCTION: Molecular studies provide discrete data for Phylogenetic analysis of highly diverse groups of organisms. Lepidopteron constitute one of the largest orders of class Insecta and are of great economic importance as they destroy plants, timber, stored food grains and manufactured goods. Some of them are beneficial as they bring about pollination. RAPD-PCR method employs single random primers and results are used for the differentiation of species and the reconstruction of phylogeny. Christen, 2015 discussed DNA from excised markers generated by RAPD-PCR used as the basis to generate 2 species-specific sequence-characterized amplified region molecular markers. Resulting primer pairs, named CR92A1 and J1A2 (each with forward and reverse components), produced amplicons of 852 and 848 base pairs, respectively. These primers may become important tools for veterinary laboratories and the screwworm eradication and exclusion program for rapid identification or verification of suspicious larval samples in presumed outbreaks. Germplasm characterization is important for conservation and utilization of genetic resources. DNA markers allow researchers to identify accessions at taxonomic level access the relative diversity within and among species. Since genetic differentiation is correlated with geographic isolation it may be appropriate to analyses accessions that represent a wide range of geographic regions in order to estimate the genetic diversity within the breeding stock (Skroch *et al.,* 1998). Yazidi *et al*., 2015 described RAPD-PCR reveals genetic polymorphism among Leishmania major strains from Tunisian patients. Numerous classifications of the superfamily groups of Noctuoidea have been proposed. Fibiger and Lafontaine (2005) proposed a new classification with ten families: Oenosandridae, Doidae, Notodotidae, Strepsimanidae, Nolidae, Lymantridae, Arctidae, Erebidae, Micronoctuidae and Noctuidae. Several molecular studies have examined higher level relationship within the Noctuidae. Weller *et al.,* (1994), used partial sequences of nuclear 28S rRNA (300 bp) and mitochondrial NDI (320 bp) from 26 Noctuoid species, including 10 Noctuoidea to differentiate each other. Two recent molecular studies on Ditrysian Lepidoptera analyze members of controversial family Noctuidae and superfamily Noctuoidea (Mitchell *et al*., 2006; Zahiri *et al*., 2011) to find distinct characters. DNA-based techniques have been widely used for authentication of plant and animal species. This is especially useful in case of those that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable. Various types of DNA based molecular techniques are utilized to evaluate DNA polymorphism. This method has been widely used in determination of population structure without prior knowledge of DNA sequences e.g. on the basis of RAPDs, genetic polymorphism in natural

populations (Haag *et al.*, 1993), strain differentiation (Gill *et al.*, 2006; Heckel and Gahan 1995; Pfeifer and Humble 1995; Schreiber 1997) and geographically isolated populations (Zhou *et al.*, 2000) have been studied. These are hybridization-based methods, polymerase chain reaction (PCR) based methods and sequencing based methods. PCR-based markers involve in vitro amplification of particular DNA sequences or loci, with the help of specific or arbitrary oligonucleotide primers and the thermostable DNA polymerase enzyme. PCR-based techniques where random primers are used include random amplified polymorphic DNA (RAPD). Different taxa often may not permit optimal DNA yields from one isolation protocol. For example, some closely related species of the same genus require different isolation protocols. Thus, an efficient protocol for isolation of DNA as well as the optimization of the PCR conditions is required. Kannan, 2015 illustrated an efficient method for extraction of genomic DNA from insect gut bacteria - culture dependent.

Two species of butterflies belonging to family Pieridae have been characterized at molecular level by Sharma *et al.*, (2003), by RAPD-PCR analysis. The other workers namely Avise (1994); Forister *et al.*, (2008); Sperling (1995); Sperling *et al.*, (1996, 1999); Kingman (2000); Bogdanowicz *et al.*, (2000); Aagard *et al.*, (2002); Hebert *et al.*, (2003); Kato and Yagi (2004); Vandewoestijne (2004); Zakharov (2004) and Kaila *et al.*, (2006) are engaged in molecular taxonomy and Phylogenetic study of insects including Lepidoptera. The results have clearly indicated that RAPD is a reliable method to differentiate the species of Lepidoptera. It is because of this that RAPD technique has been extended to characterize more species of Lepidoptera. (Garner and Slavicek 1996) identified and characterized RAPD-PCR marker and distinguished Asian and North American gypsy moths RAPD-PCR used as DNA length polymorphism and differentiated the two moth strains. The moths share many common features like morphology, inheritance of similar habitat and nocturnal habit. They exhibit similar emergent behavior towards food and feed. Thus to overcome the difficulty to identify the species on the basis of morphology, occurrence and behavior and feed biology, the molecular approach is an appropriate option. Keeping in view the utility and significance of RAPD-PCR technique in differentiating cell lines as well as the advantage of studies done for identification of species on the basis of RAPD-PCR, thus the present work emphasizes on the RAPD-PCR marker approach for genetic differentiation of four moth species of Superfamily Noctuoidea.

MATERIAL AND METHODS:

Collection of Moths: Four species of moths belonging to superfamily Noctuoidea were collected from conifer forests of Himachal Pradesh, India. The species are listed in Table (1). The DNA was isolated using the DNA extraction kit Axygen with minor modifications.

Preparation of Genomic DNA: Insect tissue (from the head, thoracic and legs) of approximately 10–20 mg was transferred to a mortar, pre-chilled on ice. Grind rapidly and vigorously to form a homogenate. Add 350 µl of PBS and 0.9 µl of RNase A. Gently grind for 30 seconds to homogenously mix the PBS with the ground tissue. Collect 350 µl of the homogenate and transfer to a 2 ml microfuge tube. If the volume of the homogenate is less than 350 µl, make it up to 350 µl with PBS. Add 20 µl Proteinase K and 150 µl Buffer C-L. Mix immediately by vortexing for 1 minute. Incubate at 56°C for 15 minutes. Briefly centrifuge to remove drops from inside the lid. Add 350 µl Buffer P-D to the sample and mix by vortexing at top speed for 30 seconds. Centrifuge at 12,000xg for 10 minutes at ambient temperature to pellet cellular debris. Place a Miniprep column into a 2 ml microfuge tube. Pipette the clarified supernatant obtained into the Miniprep column. Centrifuge for 1 minute at 12,000xg. Discard the filtrate from the 2 ml microfuge tube. Place the Miniprep column back into the 2 ml microfuge tube. Pipette 500 µl of Buffer W1 to the Miniprep column and centrifuge at 12,000xg for 1 minute. Discard the filtrate and place the Miniprep column back into the 2 ml microfuge tube. Add 700 µl of Buffer W2 and centrifuge for 1 minute at 12,000xg**.** Discard the filtrate from the 2 ml microfuge tube and repeat this wash step with a second 700 µl aliquot of Buffer W2. Discard filtrate from the 2 ml microfuge tube. Place the Miniprep column back into the 2 ml microfuge tube and centrifuge for 1 minute at 12,000xg. Transfer the Miniprep column into a clean 1.5 ml microfuge tube. To elute the genomic DNA, add 100-200 µl of Eluent to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge for 1 minute at 12,000xg.

DNA quantization: Absorbance ratio of DNA extracted from the four moth species ranged between 1.2-1.6 as determined by spectrophotometric method.

RAPD – PCR amplification: Polymerase chain reactions for random amplified polymorphic DNA (RAPD) analysis were carried out in 25 µl volume. Each reaction tube contained 20 ng of genomic DNA, 1.0 U of Taq DNA polymerase, 0.2 mM of each dNTP, 2.5 mM MgCl2, and 10 pmol of a decanucleotide primer (OPA-09: 5'GGGTAACGCC3', and OPA-13:

5'CAGCACCCAC3'). The amplifications were carried out by using a thermal cycler programmed at 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, a final extension step at 72°C for 5 min and stored at 4°C. The amplified PCR products were run on a 1.4 % agarose gel using 1x TBE buffer and stained with Ethidium bromide. The gels were photographed under UV illumination.

Table 1: List of Noctuid Spp. And type of Forest habitat.

S. No.	Name	Super-family	Family	Sub-family	Forest
1	*Chryodexis acuta* Walker (sample – A)	Noctuoidea	Noctuidae	Plusiinae	Chilgoza and Chirpine forest
2	*Asota caricae* Fabricius (sample –B)	Noctuoidea	Noctuidae	Aganainae	Chilgoza and Chirpine forest
3	*Pseudaletia separata* Walker (sample –C)	Noctuoidea	Noctuidae	Hadeninae	Chilgoza and Deodar forest
4	*Cerura liturata* Walker (sample –D)	Noctuoidea	Notodontidae	Notodontinae	Deodar and Kail forest

Dendrogram Plot: Dendrogram were constructed for the data obtained with each of the two primers, using nearest neighbor analysis of hierarchical clustering. A bootstrap process was used to assess the reliability of the dendrogram.

RESULTS AND DISCUSSION:

RAPD-PCR analysis:

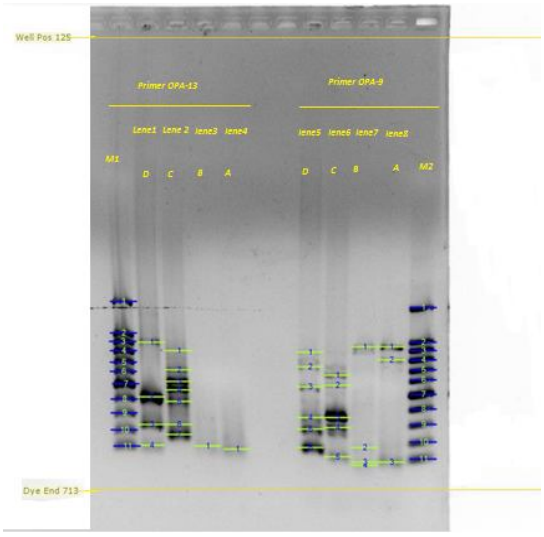

Figure 1: Gel Doc image showing various band of RAPD-PCR.

Table 1: Analysis Information of Various band with Rf values.

Markers				
Lane	**Band**	**Position**	**Base pair size**	**Rf**
1	1	469	1500	0.585
1	2	510	1000	0.655
1	3	520	900	0.672
1	4	533	800	0.694
1	5	549	700	0.721
1	6	560	600	0.740
1	7	576	500	0.767
1	8	597	400	0.803
1	9	618	300	0.838
1	10	637	200	0.871
1	11	664	100	0.917
2	1	468	1500	0.583
2	2	509	1000	0.653
2	3	520	900	0.672
2	4	532	800	0.692
2	5	547	700	0.718
2	6	559	600	0.738
2	7	575	500	0.765
2	8	595	400	0.799
2	9	613	300	0.830
2	10	635	200	0.867
2	11	656	100	0.903
RAPD BANDS				
Lane	**Band**	**Position**	**Base pair Size**	**Rf**
1	1	521	1000	0.673
1	2	592	424	0.794
1	3	628	300	0.855
1	4	655	133	0.901
2	1	532	900	0.692
2	2	557	700	0.735
2	3	572	525	0.760
2	4	583	467	0.779
2	5	598	395	0.804
2	6	628	300	0.855
2	7	641	185	0.878
3	1	656	100	0.903
4	1	660	115	0.910
5	1	534	794	0.696
5	2	553	664	0.728
5	3	579	486	0.772
5	4	619	295	0.840
5	5	634	216	0.866
5	6	658	122	0.906
6	1	564	575	0.747
6	2	577	495	0.769
6	3	619	295	0.840
6	4	632	226	0.862
6	5	670	100	0.927
7	1	527	846	0.684
7	2	658	122	0.906
7	3	677	0.939	-
7	4	681	0.946	-
8	1	527	846	0.684
8	2	544	800	0.713
8	3	677	0.939	

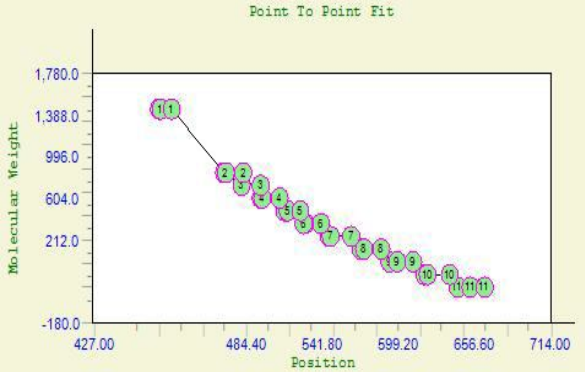

Figure 2: Molecular marker Weight vs. Position Graph.

Table 2: Number of bands with various primers.

Sample	A	B	C	D	Total Number of bands
OPA-9	3	4	5	6	18
OPA-13	1	1	7	4	13

The RAPD-PCR patterns were assessed using two random primers namely, OPA 9 and OPA 13 with the genomic DNA isolated from four species of moths. For each primer, a multiple band profile comprising one to five major amplification products, a varying number of weak products and a faintly smeared region, were observed. The number and size of amplified products varied depending upon the sequence of random primers and genotype used. A total of 31 bands were produced (Table 2). The size of the amplified products ranged from 100bp to 1500bp having different intensities. Different primers produced different banding patterns. Both primers OPA 9, and OPA 13 produced distinct and highly reproducible bands; those were selected for analysis and comparison. The total numbers of bands obtained from each selected primer were 31. Primer OPA 9 generated maximum 18 bands, while primer OPA 13 generated 13 bands. RAPD amplification patterns are shown in figures 1.

Primer OPA 9: A total of 0% polymorphic bands and 18 monomorphic bands were scored; the size ranged between 500 bp to 1500 bp. The number of bands generated in different species ranged from 3 bands in *Chryodexis acuta* Walker, 4 bands in *Asota caricae* Fabricius, 5 bands in *Pseudaletia separata* Walker and 6 bands *in Cerura liturata* Walker. The monomorphic bands were 18. The average number of bands generated in four different species was 4.5. This OPA 9 primer produced 0% polymorphic bands.

Primer OPA 13: In this primer 0% polymorphic bands and 13 monomorphic bands were generated ranging from 500 bp to 1500bp. 1bands in *Chryodexis acuta* Walker , 1 bands in *Asota caricae* Fabricius, 7 bands in *Pseudaletia separata* Walker and 4 bands *in Cerura liturata* Walker. Monomorphic bands were 13. The average number of bands generated in four different species was 3.25. This primer produced 0% polymorphic bands.

Phylogenetic relationship among the four species: The dendrogram based on neighbor joining cluster analysis of bands obtained from RAPD-PCR is shown in Figure 3. The moths dendrogram constructed using NJ divided the superfamily Noctuoidea into two clades. Cluster-1 comprises of one species namely *Pseudaletia separata* Walker and cluster 2 comprises of 3 species namely *Cerura liturata* Walker, *Chryodexis acuta* Walker, *Asota caricae* Fabricius. Cluster 2 is subdivided into two subcluster 1 consiting of *Cerura liturata* Walker and subcluster 2 further divided into 2 comprises of *Chryodexis acuta* Walker *and Asota caricae.*

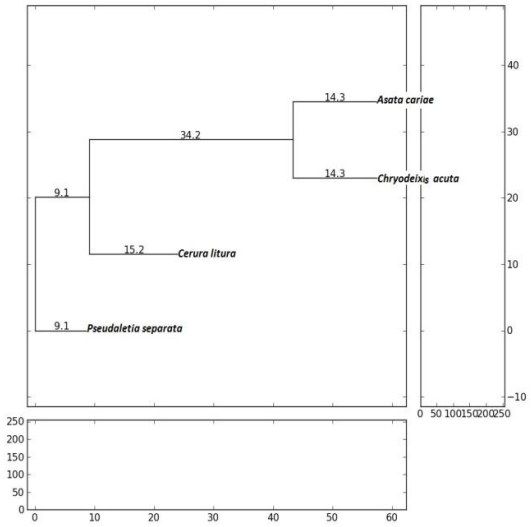

Figure 3: Neighbor joining plot showing Phylogenetic relationship among various spp.

From NJ dendrogram it concluded that *Pseudaletia separata* Walker is oldest evolved species and *Chryodexis acuta* Walker and *Asota caricae* Fabricius is closely related as compared to *Cerura liturata* Walker. *Cerura liturata* Walker is from family Notodontidae showing that it is closely related to family Noctuidae. These results signify the importance of RAPD-PCR molecular biology technique in taxonomy and evolutionary biological studies of insect species. The present study on protocol optimization for genomic DNA isolation of high purity and RAPD PCR is the first report in moths in Himachal Pradesh. This powerful approach will serve as a rapid molecular tool for accurate identification of moth species in the field conditions and their effective control subsequently.

The polymerase chain reaction-randomly amplified polymorphic DNAs (PCR-RAPD) has been particularly used for genetic and molecular studies as it is a simple and rapid method for determining genetic diversity and similarity in various organisms. It also has the advantage that no prior knowledge of the genome under research is necessary (Fischer and Husi 2000; Klinbunga and Ampayup 2000). In taxonomic studies the classification of species is based on Linnaean hierarchical system. Most of the researchers still prefer to use this hierarchical system. However quite a few problems are encountered in the correct identification and classification due to similarities. To overcome this problem, the advanced molecular technique, namely randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has been used in assessing insect genetic diversity (A. *et al.,* 2003; Black *et al.,* 1992; Perumal*et al.,* 2009; Qiu*et al.,* 2009). RAPD markers have become the most common touchstones for evaluating species similarities and differences between two individuals at genetic level (Jain *et al.,* 2010). Based on the number of amplified bands shared in common, two species show resemblances. Dissimilarity has also been ascertained as a result of certain fragment being present in one species and being absent in another. Earlier scientists have used RAPD to discriminate insect species (Black *et al.,* 1992; Cenis *et al.,* 1993; Vanlerberghe-Masutti 1994; Wilkerson *et al.,* 1993). The five primers used in RAPD analysis showed polymorphism within and between all the Erebidae moths. These primers did not produce specific banding patterns. Banding patterns produced by each primer were highly variable and most amplified bands were polymorphic, indicating genetic variation among all the moths. The large set of markers obtained confirmed the ability of the RAPD technique to distinguish organisms at the species level. Out of the five random primers of R series used for RAPD analysis, amplification was observed in three primers. Standardization of RAPD protocol for noctuid moth species was done with 20 primers namely OPA-1 to OPA-20 and OPA-13 showed better results (Kumar *et. al.,* 2014). Primers R5, R7 and R11 producing highest number of bands. Similar results have been obtained in butterflies (Sharma *et al.,* 2003; Sharma *et al.,* 2006) and moth *Cydiapomonella* (Khaghaninia 2011). High level of polymorphism between species was observed. None of the amplified fragment was present in all the species. Cluster analysis of RAPD and bands showed that the species grouped themselves into two major clusters. Such cluster analysis has also been reported in *Hirsutella*species from eriophyid mite, *Aceriaguerreronis* infesting on coconut palm by (Amritha *et al.,* 2010). RAPD can find extensive use in the identification and segregation of cryptic specie and population within the species.

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