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Molecular studies of family Erebidae moths (Lepidoptera: Noctuoidea) using RAPD-PCR technique

K. Sivasankaran[1], M. Ramakrishnan[2], D. Parandhaman[1] and S. Ignacimuthu[3,*]

[1]Division of Taxonomy and Diversity, Entomology Research Institute, Loyola College, Chennai- 600 034, Tamil Nadu, India.
[2]Division of Plant Biotechnology, Entomology Research Institute, Loyola College, Chennai- 600 034, Tamil Nadu, India.
[3]Entomology Research Institute, Loyola College, Chennai- 600 034, Tamil Nadu, India.

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ABSTRACT

Experiments were conducted to assess the relatedness of different subfamilies of Erebidae family using RAPD-PCR technique. The genomic DNA of thirteen different species of Erebidae moths belonging to different subfamilies of Erebinae, Calpinae and Hypocalinae were subjected to RAPD-PCR analysis. A total of 300 bands were scored with five RAPD primers, of which 291 were polymorphic bands while 9 were monomorphic bands; the percentage of polymorphism was 97%. Dendrogram revealed that the thirteen species were grouped into three major clusters and four subclusters.

Introduction

Molecular studies provide discrete data for phylogenetic analysis of highly diverse groups of organisms. Insects presently constitute half the known species on the planet, with an estimated number of 5 million living species (Grimaldi & Engel, 2005).

Insects comprise the largest group in the animal kingdom and possess a vast undiscovered genetic diversity and gene pool that can be better explored using molecular marker techniques. Polymerase chain reaction (PCR) technique is used for the phylogenetic analysis of many insects. This method employs single random primers and results are used for the differentiation of species and the reconstruction of phylogeny.

Noctuoid species are placed in the order Lepidoptera. 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.

The family Erebidae comes under superfamily Noctuoidea, most of which was previously classified in the family Noctuidae. The family Erebidae is a predominantly tropical subfamily and is widely distributed. The Erebidae is of considerable economic importance as it contains many agricultural pests. Many of the genera included in Erebidae have been noted to feed as adults on fruits.

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 noctuid 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.*, 2010) to find distinct characters.

In the present study, 13 species belonging to three subfamilies of Calpinae Hypocalinae and Erebinae of family Erebidae i.e. *Thyas coronata*, *Hypocala violacea*, *Oxyodes scrobiculata*, *Eudocima phalonia*, *Hypocala deflorata*, *Catocala macula*, *Pindara illibata*, *Serrodes campana*, *Eudocima salaminia*, *Sphingomorpha chlorea*, *Achaea serva* and *Erebus macrops* were assessed for their relatedness using RAPD-PCR technique. No such work has been carried out on Indian species till now. This molecular information would further facilitate the rapid identification of species of Erebidae moths.

Materials and methods

Isolation of Genomic DNA

Thirteen species of moths belonging to family Erebidae were collected from various locations from Tamil Nadu part of Western Ghats, India. The species are listed in Table (1). The DNA was isolated using the method of Margam *et al.* (2010) with minor modifications. Insect tissue (from the head, thoracic and legs) of approximately 10–20 mg was homogenized. 500 µl of CTAB buffer (100 mM Tris (P^H: 9.5); 200 mM EDTA; 1.4 M NaCl; 2% CTAB; 1% PEG 8000) was added to the homogenate and thoroughly mixed; then 4 µl of Proteinase–K (20 mg / µl) was added and incubated at 55º C for 4 hours. Thereafter 500 µl of Phenol: Chloroform (1:1) was added; the tubes were gently inverted for a few seconds. The suspension was centrifuged for 10 min at 12000 rpm at 4º C. The supernatant was taken and equal volume of Chloroform: Isoamylalcohol (24:1) was added and centrifuged for 10 min. Aqueous phase was removed; 2.5 volume of ice-cold absolute alcohol and 1/10th volume of 3M Sodium Acetate were added and mixed gently by inversion. The sample was stored at -20º C for overnight and then spun (12000 rpm for 20 min). The supernatant was discarded; the DNA pellet was washed with 70 % Ethanol twice and spun down at 14000

Tele:
E-mail addresses: entolc@hotmail.com

rpm for15 min. The pellet was air dried, dissolved in 1X TE Buffer and stored at -20ºC for immediate use at -80º C for long term storage. The DNA sample was quantified and its purity was determined using UV spectrophotometer.

RAPD Analysis

Random Amplification of DNA by PCR

DNA was amplified by the RAPD-PCR technique using Random primers R2, R5, R7, R11 and R12. The details of primers are given in table 2. PCRs were programmed as follows: an initial denaturation period of 95º C for 5 min and followed by denaturation at 95º C for 30seconds; primer annealing was done at 37º C for 1 min and extension at 72º C for 2 min. 35 cycles were run. An additional 7 min at 72º C was allowed for final extension. PCR products were stored at 4º C.

Gel analysis

The amplified PCR products were run on a 1.4 % agarose gel using 1x TBE buffer and stained with Ethidium bromide. The electrophoresis was performed until good separation of RAPD bands occurred; the gels were photographed under UV illumination.

Dendrogram Plot - Dendrogram were constructed for the data obtained with each of the three primers, using nearest neighbour analysis of hierarchical clustering (UPGMA software package Version 4.0 genomes. urv.cat/UPGMA/ Garcia-Vallve *et al*. (1999). A bootstrap process was used to assess the reliability of the dendrogram using 1000. Polymorphic Informative Content (PIC) values were analyzed for each primer, and Cophenetic Correlation Coefficient (CP) calculation was using the following formula.

$$c = \frac{\sum_{i<j}(x(i,j)-x)(t(i,j)-t)}{\sqrt{[\sum_{i<j}(x(i,j)-x)^2][\sum_{i<j}(t(i,j)-t)^2]}}.$$

Data Collection and Analysis

The bands were keyed by scoring the presence (1) or absence (0) of the bands. The (AMOVA) procedure partitioned the total variation both among populations and within populations of Erebidae moths. The populations were grouped based on the subfamilies. The analysis of molecular variance (AMOVA) was executed the software (*GelAlEx 6.5*, Peakall & Smouse, 2012) using to access the variation among the population of family Erebidae moths.

Results

The concentration of DNA extracted from the thirteen species ranged between 1500-4430 ng/µl as determined by spectrophotometric method.

RAPD-PCR analysis

The RAPD-PCR patterns were assessed using five random primers namely, R2, R5, R7, R11and R12 with the genomic DNA isolated from thirteen species of Erebidae 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 300 bands were produced (Table 3). The size of the amplified products ranged from 200bp to 3500bp having different intensities. Different primers produced different banding patterns. Only primers R5, R7 and R11produced distinct and highly reproducible bands; only those were selected for analysis and comparison. The total numbers of bands obtained from each selected primer were 107 (primer R5), 132 (R7) and 61 (R11). Primer R7 generated maximum 16 bands, while primer R11 generated minimum 1 band. RAPD amplification patterns are shown in figures 1, 2, and 3.

Figure 1. M. Marker; 1. *Thyas coronata*; 2. *Hypocala violacea*; 3. *Oxyodes scrobiculata*; 4. *Eudocima phalonia*; 5. *Hypocala deflorata*; 6. *Catocala macula*; 7. *Pindara illibata*; 8. *Serrodes campana*; 9. *Eudocima salaminia*; 10. *Sphingomorpha chlorea*; 11. *Achaea serva*; 12. *Sprima retorta*; 13. *Erebus macrops*

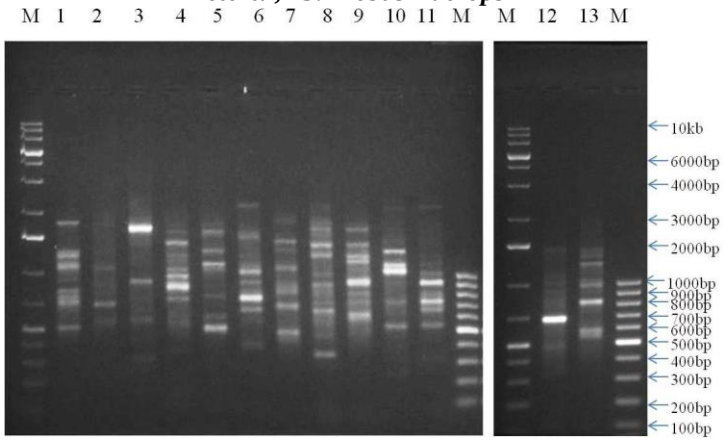


Figure 2. M. Marker; 1. *Thyas coronata*; 2. *Hypocala violacea*; 3. *Oxyodes scrobiculata*; 4. *Eudocima phalonia*; 5. *Hypocala deflorata*; 6. *Catocala macula*; 7. *Pindara illibata*; 8. *Serrodes campana*; 9. *Eudocima salaminia*; 10. *Sphingomorpha chlorea*; 11. *Achaea serva*; 12. *Sprima retorta*; 13. *Erebus macrops*.

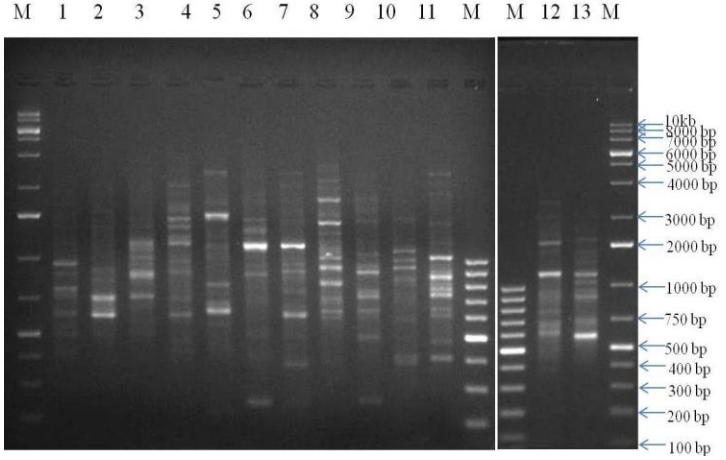


Figure 3. M. Marker; 1. *Thyas coronata*; 2. *Hypocala violacea*; 3. *Oxyodes scrobiculata*; 4. *Eudocima phalonia*; 5. *Hypocala deflorata*; 6. *Catocala macula*; 7. *Pindara illibata*; 8. *Serrodes campana*; 9. *Eudocima salaminia*; 10. *Sphingomorpha chlorea*; 11. *Achaea serva*; 12. *Sprima retorta*; 13. *Erebus macrops*

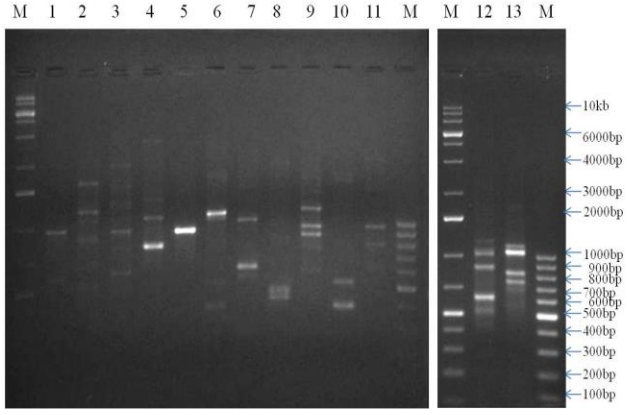


Figure 4. Dendrogram of 13 Erebidae moths based on RAPD-PCR banding patterns using R5, R7 and R11 primers

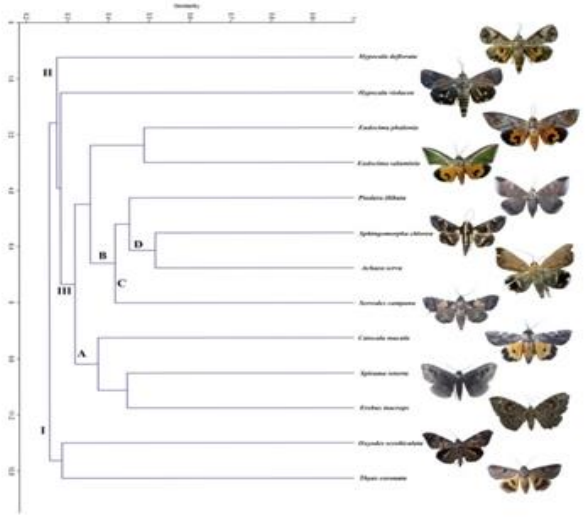


Figure 5. Percentage of Molecular variance of Family Erebidae moths

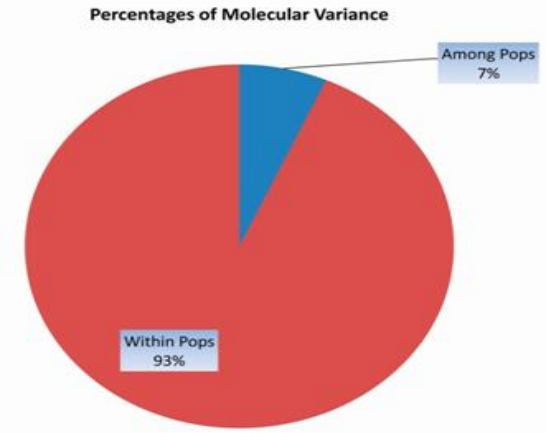
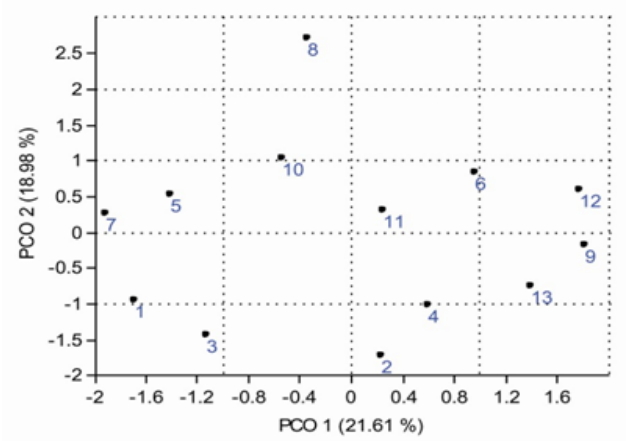


Figure 6. The principal coordinate analysis (PCO) from RAPD data for the 13 species of Erebidae moths.



Primer R5

A total of 102 polymorphic bands and five monomorphic bands were scored; the size ranged between 300bp to 3000bp. The number of bands generated in different species ranged from 12 bands in *Serrodes campana*, *Sphingomorpha chlorea* and 11 bands *Achaea serva*. 9 bands were scored in *Catocala macula*, *Eudocima phalonia*, *Eudocima salaminia*, *Pindara illibata* and 8 bands in *Hypocala deflorata*. The monomorphic bands were observed for five species: *Oxyodes scrobiculata*, *Eudocima salaminia* and *Sphingomorpha chlorea*, *Serrodes campana* and *Eudocima salaminia*. The average number of bands generated in thirteen different species was 8.23. This primer produced 95.32% polymorphic bands.

Primer R7

In this primer 128 polymorphic bands and four monomorphic bands were generated ranging from 175 bp to3100bp. 16 bands were scored in *Serrodes campana*; 15 bands in *Achaea serva* and 14 bands in *Eudocima phalonia* were scored. 12 bands were scored in *Pindara illibata*; 11bands were scored in *Hypocala deflorata*. 9 bands were scored in *Spirama retorta*, *Erebus macrops*, *Sphingomorpha chlorea* and *Catocala macula*. Monomorphic bands were scored in four species: *Spirama retorta*, *Erebus macrops*, *Catocala macula* and *Pindara illibata*. The average number of bands generated in thirteen different species was 10.15. This primer produced 94.69% polymorphic bands. Fourteen marker bands were detected from different species.

Primer R11

This primer generated 61 bands ranging from 400 bp to 3500bp. 10 bands were generated in the species *Eudocima salaminia*. 7 bands were generated in *Oxyodes scrobiculata* and *Erebus macrops*. 6 bands were scored in *Eudocima phalonia*, 5 bands were scored in *Serrodes campana* and *Spirama retorta*. The average number of bands generated in thirteen different species was 4.69. This primer amplified 100% polymorphic bands. Fourteen marker bands were detected.

Phylogenetic relationship among the thirteen species:

The dendrogram based on cluster analysis of bands obtained from RAPD-PCR is shown in Figure 4. There was overlapping of clusters. The moths were not separated into two distinct clusters but were clustered together, showing close relationship. The similarity of the dendrogram indicated three major clusters and four subclusters with similarity matrix percentage of 49%. The dendrogram was divided into three major clusters I, II and III. Subclusters were divided into four such as A, B, C and D. Most of the species fell into groups according to the locality. Cluster I had two species *Thyas coronata* and *Oxyodes scrobiculata* with similarity matrix percentage of 29%. Clusters II and III had one isolate each *Hypocala deflorata* and *Hypocala violacea* with similarity matrix percentage of 20%. Subcluster A had three species with similarity matrix percentage of 37%: *Catocala macula*, *Spirama retorta* and *Erebus macrops*. Subcluster B had two species namely *Eudocima phalonia* and *Eudocima salaminia* with similarity matrix percentage of 49%. Subcluster C had one species *Serrodes campana*. Subcluster D had three species, namely *Pindara illibata*, *Sphingomorpha chlorea* and *Achaea serva* with similarity matrix percentage of 45%. The highest similarity matrix percentage in this phylogenetic relationship was 51% between *Achaea serva* and *Sphingomorpha chlorea*, while the lowest similarity matrix percentage was found between *Pindara illibata* and *Spirama retorta* (13%). The PIC values revealed Primer R5 was 0.43, by primer R7 was 0.53 and by primer R11 was 0.44. The average of PIC value was 0.466. So, these markers are good for molecular analysis of insects. In the Cophenetic Correlation Coefficient the value was 75%. The value of the Cophenetic Correlation Coefficient is close to 100%, thus making the cluster analysis good.

The Principal Coordinate Analysis (PCO) from RAPD data for the 13 species of Erebidae moths showed that PCO1 and PCO2 constituents had 21.61% and 18.98% of the variation respectively (Figure 6). The results of PCO represent cluster analysis.

Table 1. Collection Site of Different Moths Species from Western Ghats

S.NO	Species	Locality	No. of specimens studied
1.	*Thyas coronata*	Nilgiris (Bench Mark, Botanical Garden) Coonoor Simp's Park	4
2.	*Hypocala violacea*	Kodaikanal (Moonjikkal, Bryant Park, Coaker's Walk) Nilgiris (Bench Mark, Botanical Garden, Coonoor: Simp's Park)	4
3.	*Oxyodes scrobiculata*	Kodaikanal(Moonjikkal, Bryant, Park, Coaker's Walk) Nilgiris (Bench Mark, Botanical Garden, Coonoor: Simp's Park	3
4.	*Eudocima phalonia*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal Bryant Park, Moonjikkal, Snbaganur	3
5.	*Hypocala deflorata*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal (Bryant Park, Moonjikkal)	5
6.	*Catocala macula*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal (Bryant Park, Moonjikkal)	4
7.	*Pindara illibata*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal (Bryant Park, Moonjikkal Senbaganur, Pallangi)	5
8.	*Serrodes campana*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal (Bryant Park, Moonjikkal, Bear Shola)	4
9.	*Eudocima salamina*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal (Bryant Park, Moonjikkal, Senbaganur	3
10.	*Sphingomorpha chlorea*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal (Bryant Park, Moonjikkal, Senbagnur)	5
11.	*Achaea serva*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal (Bryant Park, Moonjikkal, Pallangi)	4
12.	*Spirama retorta*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal (Bryant Park, Moonjikkal, Senbaganur, Pallangi)	4
13.	*Erebus macrops*	Nilgiris (Bench Mark, Botanical Garden), Coonoor Simp's Park; Kodaikanal (Bryant Park, Moonjikkal, Senbaganur)	5

Table 2. Sequences of various primers used.

S.No	Primers	Sequences
1	Primer 1- R 2	5' -TTCCGAACCC- 3'
2	Primer 2- R 5	5'-AGTCAGCCAC- 3'
3	Primer 3- R7	5'-GAAACGGGTG- 3'
4	Primer 4- R 11	5'-CAATCGCCGT- 3'
5	Primer 5- R12	5'-GACCGCTTGT- 3'

Table 3. Bands obtained with three different primers in the family Erebidae

Species name	Lanes	No. of Bands		
		Primer R5	Primer R7	Primer R11
Thyas coronata	1	7	7	2
Hypocala violacea	2	6	6	4
Oxyodes scrobiculata	3	6	7	7
Eudocima phalonia	4	9	14	6
Hypocala deflorata	5	8	11	1
Catocala macula	6	9	8	4
Pindara illibata	7	9	12	4
Serrodes campana	8	12	16	5
Eudocima salaminia	9	9	9	10
Sphingomorpha chlorea	10	12	9	4
Achaea serva	11	11	15	2
Spirama retorta	12	4	9	5
Erebus macrops	13	5	9	7

Table 4. Similarity Matrix for the 13 species of Erebidae computed with Jaccard coefficient

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1												
2	0.28	1											
3	0.286	0.241	1										
4	0.25	0.286	0.289	1									
5	0.286	0.2	0.212	0.289	1								
6	0.276	0.276	0.171	0.282	0.206	1							
7	0.323	0.281	0.286	0.286	0.364	0.314	1						
8	0.225	0.167	0.233	0.378	0.395	0.317	0.415	1					
9	0.216	0.286	0.289	0.487	0.225	0.389	0.317	0.409	1				
10	0.242	0.281	0.364	0.35	0.25	0.394	0.429	0.381	0.317	1			
11	0.222	0.333	0.297	0.425	0.333	0.4	0.472	0.452	0.357	0.514	1		
12	0.214	0.259	0.152	0.27	0.267	0.393	0.132	0.308	0.382	0.265	0.314	1	
13	0.233	0.37	0.323	0.316	0.206	0.355	0.278	0.286	0.389	0.278	0.4	0.444	1

Table 5. Distance matrix for the 13 species of Erebidae based on Jaccard coefficient

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	0												
2	0.72	0											
3	0.714	0.759	0										
4	0.75	0.714	0.711	0									
5	0.714	0.8	0.788	0.711	0								
6	0.724	0.724	0.829	0.718	0.794	0							
7	0.677	0.719	0.714	0.714	0.636	0.686	0						
8	0.775	0.833	0.767	0.622	0.605	0.683	0.585	0					
9	0.784	0.714	0.711	0.513	0.775	0.611	0.683	0.591	0				
10	0.758	0.719	0.636	0.65	0.75	0.606	0.571	0.619	0.683	0			
11	0.778	0.667	0.703	0.575	0.667	0.6	0.528	0.548	0.643	0.486	0		
12	0.786	0.741	0.848	0.73	0.733	0.607	0.868	0.692	0.618	0.735	0.686	0	
13	0.767	0.63	0.677	0.684	0.794	0.645	0.722	0.714	0.611	0.722	0.6	0.556	0

Table 6. Analysis of molecular variance (AMOVA) associated subdivisions in populations of Erebidae moths.

Source	df	SS	MS	Est. Var.	%
Among Pops	3	35.451	11.817	0.702	7%
Within Pops	9	88.857	9.873	93%	93%
Total	12	124.308	-	10.575	100%

Analysis of Molecular Variance (AMOVA)

An analysis of molecular variation exposed significant variation within the population and between the populations (Table 6). This variation among the populations was found to be 7% while the variation within the population was determined at 93%.

Discussion

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, viz., randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has been used in assessing insect genetic diversity (Black *et al.*, 1992; Cenis, 2003; Gobbi *et al.*, 2003; Sartor *et al.*, 2008; Sharma *et al.*, 2008; 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: Ceins *et al.*, 1993; Vanlerberghe-Masutti 1994; Wilkerson *et al*., 1993).

The five primers used in RAPD analysis in this study 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 in the present study 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 in this study (Table 1.), amplification was observed in three primers. Primers R5, R7 and R11 produced higher number of bands. Similar results have been obtained in butterflies (Sharma *et al.*, 2003; Sharma *et al.*, 2006) and moth *Cydia pomonella* (Samad Khaghaninia *et al.*, 2011). We observed high level of polymorphism between species. 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, *Aceria guerreronis* infesting coconut palm by Amritha *et al*. (2010).

In this study, a strong relatedness was observed between closely related species. The dendrogram constructed by simple matching coefficient showed some distinct differences in each cluster. In the first cluster *Oxyodes scrobiculata* and *Thyas*

coronata were more closely related to each other. *Spirama retorta* and *Erebus macrops* were more closely related to each other constituting subcluster A. *Eudocima phalonia* and *Eudocima salaminia* were more closely related to each other constituting subcluster B. *Sphingomorpha chlorea* and *Achaea serva* were more closely related to each other constituting subclusters. These three subclusters are more closely related to each other than *Serrodes campana*. Similar results have been obtained from different Egg Parasitoids of Soybean Stink Bugs Aljanabi *et al*., (1998). RAPD can find extensive use in the identification and segregation of cryptic specie and population within the species.

Conclusion

Our results suggested that the RAPD-PCR technique could provide a powerful tool to improve species identification and to better understand genetic variability of nocturnal moths.

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