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Manoj Kumar
Faculty of Biotechnology, Shoolini University, Solan, Himachal Pradesh, India

Pawan Kumar
Forest Protection Division, Himalayan Forest Research Institute, Conifer Campus, Shimla, Himachal Pradesh, India

Amit Seth
Faculty of Biotechnology, Shoolini University, Solan, Himachal Pradesh, India

Correspondence
Manoj Kumar
Faculty of Biotechnology, Shoolini University, Solan, Himachal Pradesh, India

Assessment of genetic diversity and phylogenetic relationship of geometrid moth (Geometridae) in India

Manoj Kumar, Pawan Kumar and Amit Seth

Abstract
Present study was carried out to characterize the geometrid moth species at molecular level with the help of RAPD-PCR. Study was carried out in the Chirpine habitats in the subtropical region of Himachal Pradesh to access the systematic status of various geometrid groups. The study on geometrid moth systematics is very limited or negligible. Although the studies on geometrid moth have so far not been carried out by any workers in Chirpine forest in HP. PCR based methods like RAPD-PCR markers were used to find Phylogenetic relationship among the 10 geometrid moth species. RAPD decamer primers namely OPA 9 and OPA 13 showed good resolution and sufficient variation among the different moth species and produced a total of 23 polymorphic bands. The banding patterns were obtained and the dendrogram was drawn on the basis of presence and absence of bands. Jaccard's co-relation coefficient between these moth species computed from combined data from two primers. Highest correlation observed between *C. nora* and *O. marginata* while lowest correlation coefficient between *P. vararia* and *Z. apostolata*. An analysis of molecular variance revealed significant difference within and between population variance. The unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's genetic distance group 10 genotype into two main clusters with 95% bootstrap value. These results indicate that RAPD-PCR technique is a powerful tool in the study of lepidopteran systematics and molecular taxonomy of cryptic moth species.

Keywords: Geometrid moths, RAPD-PCR, phylogenetic analysis, UPGMA, systematics

1. Introduction
Lepidoptera have attracted more attention than other insects in the development of insect conservation. It is well known fact that climate have a direct impact on the biodiversity of insects as during favorable environmental condition. A sound knowledge of taxonomy is a prerequisite for assessment and understanding of biodiversity. The moth family Geometridae (inchworms or loppers), with approximately 23,000 described species, is the second most diverse family of the Lepidoptera, occurring worldwide except in the Polar Regions. Apart from a few recent attempts based on morphology and molecular studies, the phylogeny of these moths has remained largely uninvestigated. When it concerns about insects, it gets more difficult, as the difference is too minute to resolve. Morphological identification of moths is usually based on the wing patterns. Generally, it is attributed to the number and positions of spots on the wing. It is now being established that Classification of closely related lepidopteran species based on morphological features can pose several difficulties and inaccuracy on account of attributes that can change as a function of environment and prevalence of several biotypes. Recent molecular marker techniques ease the assessment of genetic diversity and facilitate genotyping, classification, inventorying and molecular phylogenetic studies. Geometridae species were collected in chirpine forest between 500 to 1500 m altitudes and subjected to RAPD analysis. The polymerase chain reaction-randomly amplified polymorphic DNA (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 [1, 2]. This method employs single random primers and results are used for the differentiation of species and the reconstruction of phylogeny. Germplasm characterization is important for conservation and utilization of genetic resources. DNA markers allow researchers to identify accessions at taxonomic level and to access the relative diversity within and among species. Since genetic differentiation is correlated with geographic isolation, it may be appropriate to

analyse accessions that represent a wide range of geographic regions in order to estimate the genetic diversity within the breeding stock [3]. 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 [4], strain differentiation [5–7] and geographically isolated populations [8] have been studied. These are based on hybridization methods, polymerase chain reaction (PCR) methods and sequencing 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 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.

Four species of Noctuid moths belonging to family Noctuoidea have been characterized at molecular level by RAPD-PCR analysis [9]. The other workers namely [10–20] and [21] 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. 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, the present work emphasizes on the RAPD-PCR marker approach for genetic differentiation of moth species of Superfamily Geometridae.

2. Materials and method

2.1 Collection of Geometridae

Species of moths belonging to Superfamily Geometridae 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.

2.2 Preparation of Genomic DNA

Insect tissue (from the head, thoracic and legs) of approximately 15 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 12,000 g 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 12,000 g. 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 12,000 g 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 12,000 g**.** 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 12,000 g. 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 12,000 g.

2.3 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 $MgCl_2$, 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 (Fig. 1 and Fig. 2).

2.4 Data analysis

The data obtained from RAPD-PCR analysis was scored in a binary form as the presence or absence (1/0) of bands for each sample with two primers. The dendrogram, based on the RAPD data was constructed for the data obtained with each of the two primers with the help of Free tree software for Windows, ver. 1.0.0.0 using UPGMA of hierarchical clustering. Jaccard's correlation coefficient was calculated and UPGMA plot was constructed on the basis of correlation data obtained. A bootstrap process was used to assess the reliability of the dendrogram.

3. Results and discussion

Random amplified polymorphic DNA markers have been used for many genetic analyses, including genome mapping, genotype fingerprinting, phylogeny reconstruction, and measuring genetic similarities. They suffer from one potential limitation, however, because the PCR that is used to produce informative amplification product often produces artefactual products as well. Methods for handling RAPD artifacts, such as deleting inconsistent or faint bands are using only those bands that the reproducible, introduce false negative into the data. All primers showed polymorphic bands with 100% polymorphism and applied to investigate the genetic diversity among geometrid moth species. The total number of amplified bands for both primers was 23. OPA-9 showed 11 amplified bands while OPA-13 showed 12 amplified bands (Table 2). All the bands were polymorphic and contributed towards genetic diversity coefficient calculation.

Table 1: Species list with lane number and Location

S. No.	Lane No. on Gel	Species	Location	Altitude
1	Lane 1(L1)	*A. hilerata*	Solan	1,502 m
2.	Lane 2(L2)	*C. nora*	Solan	1,502 m
3.	Lane 3(L3)	*Z. apospatulata*	Shimla	2,205 m
4.	Lane 4(L4)	*Z. marginata*	Shimla	2,205 m
5.	Lane 5(L5)	*H. talaca*	Bilaspur	673 m
6.	Lane 6(L6)	*P. vararia*	Bilaspur	673 m
7.	Lane 7(L7)	*O. marginata*	Shimla	2,205 m
8.	Lane 8(L8)	*A. sylvata*	Shimla	2,205 m
9.	Lane 9(L9)	*C. elanora*	Bilaspur	673 m
10.	Lane 10(L10)	*P. casta*	Solan	1,502 m

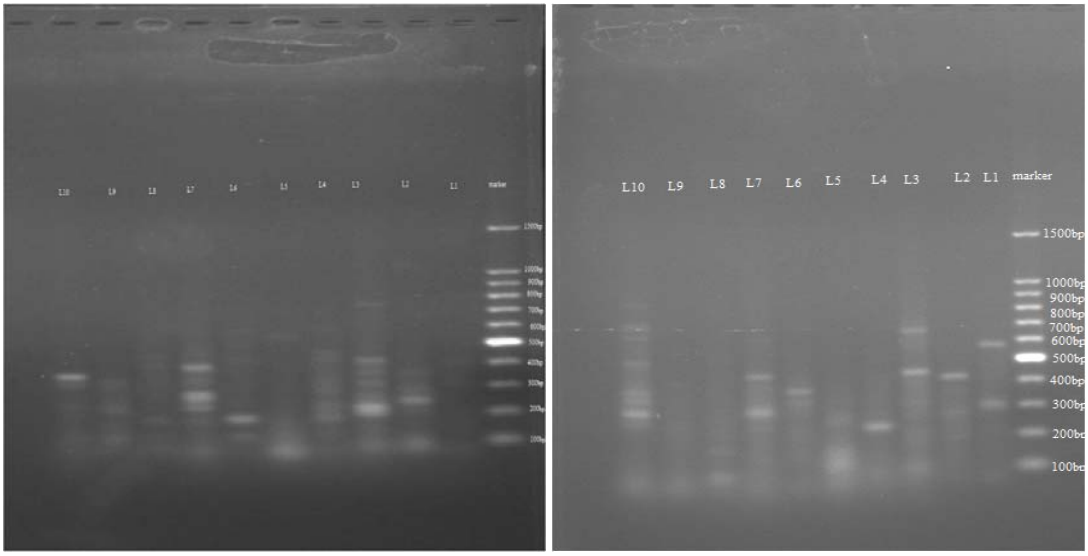

Fig 1: Photograph showing RAPD analyses of sample 1 to 10 with primer 9 Figure 2: Photograph showing RAPD analyses of sample 1 to 10 with primer 13

Table 2: RAPD primers data and the percentage of polymorphic bands

Primer Code	Nucleotide Sequences (5'-3')	Annealing Temperature Tm (˚C)	Size of Fragments(bps)	Total number of bands	Polymorphic Bands	Monomorphic bands	Percentage Polymorphism
OPA-9	GGGTAACGCC	34 ˚C	250-1600	11	11	0	100
OPA-13	CAGCACCCAC	34 ˚C	360-1700	12	12	0	100
Total				23	23	0	100

When RAPD data are used to compute genetic similarity coefficient, such artifacts can cause significant bias in the estimation. The three coefficient most widely used with RAPD data, the simple matching coefficient, Jaccard's coefficient and Nei and Li's coefficient, differ in the amount of bias produced by a given level of artefactual bands. For closely related organism, Nei and Li's coefficient display less percentage bias than the simple matching coefficient. The similarity matrix representing Jaccard's coefficient (Table-3) was used to cluster the data following the UPGMA algorithm. The gene diversity in between species was comparatively of narrow range (0.0667-0.53846) with higher mean gene diversity value. The higher gene diversity value obtained can be due to the collection of different samples from different geographic location with different altitude value. The lowest distance matrix value was found between *Pelagodes vararia* and *Protuliocnemis casta* with the value of 0.0667 while the higher genetic distance was observed between *Chiasmia nora* and *Ourapteryx marginata*. The low genetic similarity and higher genetic distance indicates the efficiency of RAPD-PCR application in discriminating between the cryptic moth species in systematic taxonomy.

Table 3: Similarity matrix of Geometrid moth species based on RAPD data

	P. casta	***C. elanora***	***A. sylvata***	***O. marginata***	***H. talaca***	***P. vararia***	***Z. marginata***	***Z. apospatulata***	***C. nora***	***A. hilerata***
P. casta		0.08333	0.17647	0.29412	0.15385	0.0667	0.0667	0.31250	0.38462	0.15385
C. elanora	0.08333		0.30303	0.07143	0.14286	0.12500	0.12500	0.07692	0.1000	0.29167
A. sylvata	0.17647	0.30303		0.14286	0.31429	0.25000	0.1111	0.07143	0.09091	0.28571
O. marginata	0.29412	0.07143	0.14286		0.21429	0.20000	0.28571	0.53333	0.53846	0.13333
H. talaca	0.15385	0.14286	0.31429	0.21429		0.22222	0.22222	0.23077	0.30000	0.11111
P. vararia	0.0667	0.12500	0.25000	0.20000	0.22222		0.09091	0.06250	0.27273	0.10000
Z. marginata	0.0667	0.12500	0.1111	0.28571	0.22222	0.09091		0.41667	0.28571	0.46429
Z. apospatulata	0.31250	0.07692	0.07143	0.53333	0.23077	0.06250	0.41667		0.18750	0.06667
C. nora	0.38462	0.1000	0.09091	0.53846	0.30000	0.27273	0.28571	0.18750		0.18182
A. hilerata	0.15385	0.29167	0.28571	0.13333	0.11111	0.10000	0.46429	0.06667	0.18182	

3.1 Phylogenetic analysis

Phylogenetic tree classifies organisms into groups of related species. If such taxonomic classifications are to reflect the course of evolution, each group should consist of species that have a common ancestor not shared by any species outside that group. A phylogeny is estimated from only part of the total data that might possibly be sampled. How much confidence should be placed in the appearance of a set of species as a monophyletic group is therefore a statistical question. Many statistical tests for phylogenies have been proposed, but most of these methods do not actually test the hypothesis that a group of species is monophyletic. Of the few methods that do construct a confidence set of phylogenies, most are only applicable or computationally feasible for just a few taxa. Furthermore, most tests can only be applied to trees estimated by a particular algorithm from a particular kind of data (DNA or protein sequences, isozyme variations, etc.).

A useful approach to statistical inference for phylogenies employs the bootstraps, which is a computer-intensive statistical technique with many applications. The bootstrap computer-intensive statistical technique is frequently applied to statistical analyses of phylogenetic trees. Bootstrapping measures how consistently the data support given taxon bipartitions [22, 23]. It is not a test to how accurate tree is, it only gives information about the stability of the tree topology (the branching order), and it helps access whether the sequence data is adequate to validate the topology [24]. The broad applicability of the bootstrap makes it a particularly valuable technique in taxonomy. Bootstrap tests for phylogenies, based on conventional statistical hypothesis tests, would therefore be desirable.

Fig 3: Dendrogram of genetic relationships among moth species based on RAPD profiling

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 [25, 26]. 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 [27–30]. Following the Unweighted Pair Group Method of Arithmetic Means (UPGMA) the 10 geometrid moth species were grouped into 2 main clusters namely Cluster A and B (Fig. 3) with 100% bootstrap. *Abraxas sylvata* was included into group A and group B further divided into two subgroups B1 and C. Subgroup B1 further divided into two subgroups included two species namely *Agathia hilerata* and *Protuliocnemis casta*. *Agathia hilerata* and is more closely related to *Protuliocnemis casta* than *Abraxas sylvata*. Although there is more morphological dissimilarity in their wing colour and shape but this similarity might be accounted because of their collection from same altitudinal location. Subgroup C further subdivided to subgroups namely C1 and D. Subgroup C1 further divided into two subgroups in which first subgroup included *Zeheba marginata* and second subgroup further divided into two groups which included more closely related species *Ourapteryx marginata* and *Zamarada apospatulata*. Subgroup D further emerges to two branching including *Chiasmia eleonora* and subgroup E. Subgroup further divides into two subgroups including *Chiasmia nora* and subgroup F. The genetically closed species *Chiasmia eleonora* and *Chiasmia nora* have similar cryptic morphological features except difference in white spots pattern on their wings. Since samples collection were done from different geographical regions thus representing genetic diversity. This difference in the relatedness indicates the important of RAPD as a tool in taxonomy of identification of cryptic species. Subgroup F includes two species *Hyposidra talaca* and *Pelagodes vararia*. RAPD-PCR technique has also been employed to study molecular characterization of morphological similar four Pieridae butterflies had been carried out by [31] using eight primers. Total 121 bands were scored, out of which 115 were polymorphic while 6 were monomorphic. Their study shows RAPD as most easy and robust technique to differentiate morphological similar species and can be easily applied to butterfly species where morphological characters are creating doubt in species identification. [32] had demonstrated the application of RAPD in decimation of two Pieridae butterflies at sex level. Population structure in the coding moth (*Cydia pomonella* L.) using RAPD markers in 13 geographical populations from northwestern Iran was studied during 2003 and 2004.Within-population genetic diversity, based on Nei's genetic index, ranged from 0.0228 to 0.281 for the *Shabester* and *Zunuz* population, respectively [33]. Strong association was observed between molecular variation and stable climatic parameters such as elevation, whereas population morphological variation was more closely related to variable parameters such as wind speed and precipitation.

4. Conclusion

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 geometrid 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. RAPD hereby proved to be useful in molecular proofing of cryptic species which are morphologically similar and hard to distinguish easily.

4.1 Conflict of interest: The authors confirm that this article has no conflict of interest.

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