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Research Article **Molecular Taxonomy**

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MOLECULAR STUDIES OF SEVEN SPECIES OF BUTTERFLIES (LEPIDOPTERA: PIERIDAE) THROUGH RAPD-PCR TECHNIQUE

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ABSTRACT

In present investigations molecular characterization of seven Pieridae butterflies was carried out using four RAPD markers. A total 57 bands were scored with four decamer primers of which 57 were polymorphic and the percentage of polymorphism was 100%. Genus specific bands have been observed i.e. a band of 818bp with primer 12 & 520bp with primer OPP-5. Dendrogram constructed using the $NTSYS_{spc2.2}$software divided the pierids into two clades. Cluster-I comprises of two species *viz*. *Pieris brassicae nepalensis* and *Pieris canidia indica.* Cluster-II comprises of 5 species *viz*. *Colias electo fieldi*, *Colias erate erate*, *Gonepteryx rahmni nepalensis, Pontia daplidice moorei* & *Terias hecabe fimbriata.* Cluster –II is subdivided into two, sub-cluster-I consisting of *Colias electo fieldi*, *Colias erate erate*, *Terias hecabe fimbriata* & *Gonepteryx rahmni nepalensis,* sub-cluster-II consists of only *Pontia daplidice moorei*.

KEY WORDS: Butterflies, Genetic diversity, Pieridae, RAPD-PCR

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INTRODUCTION

Indian butterflies consists of about 1500 species distributed over nine families and constitutes a little over 8.35% of the known species of world[2]. About 128 butterfly species of the country has been enlisted under Schedule I1(Part IV); 307 under Schedule II(Part IV) and 19 under Schedule IV(Part II) of the Wild Life Protection Act,1972. Pieridae is one of the important and most commonly seen families of Lepidoptera. This group is characterized by high chromosome number and small size of chromosomes that make differentiation of the species difficult at the cytogenetic level for high resolution genetic studies of species and populations[4]. This family is widely distributed throughout the world and contains about 1,200 species and 60 genera, out of which 109 species occur in India[1]. Although many studies on morphological, ecological and molecular attributes of several species of pieridae from world over are available, very little is known about the Indian species from this family[7].

Zakharov *et. al.*[12] demonstrated the applicability of museum specimens of butterflies through RAPD analysis. Zakharov[13] studied inter specific hybridization between *Parnassius nomion* and *Parnassius bremeri* through RAPD analysis. By testing 25 decamer primers, three and two diagnostic markers were revealed for *P.nomion* and *P. bremeri*, respectively. In 2002, Suman[8] exploited RAPD-PCR technique to reveal iterspecific genetic diversity as well as intraspecific & interspecific relatedness between the 4 species of butterflies (*Pieris brassicae nepalensis*, *Pieris canidia indica, Papilio polytes*, *Papilio demoleus*) by using two random decamer primers. Two species of butterflies belonging to family Pieridae have been characterized at molecular level by Sharma *et. al*.[3] by RAPD-PCR analysis. Sharma *et. al*.[4] used RAPD-PCR technique for molecular characterization of two species of family Pieridae. Sharma *et. al*.[5] used the same technique for five species of butterflies. Genetic relatedness of six butterflies species based on 16SrRNA have been also analyzed[7]. Tiple *et .al*.[10] study the molecular characterization of morphological similar four pieridae butterflies. Recently, in India, this technique was applied for molecular characterization of six species of *Junonia*[11]. The main aim of this study is to identify and characterize systematically the status and distribution of pierids species and molecular data and creation of a molecular database for Pieridae systematic.

MATERIALS AND METHODS

Extraction of DNA

Genomic DNA was extracted from the freshly collected each individuals of butterflies by DNeasy Blood and Tissue Kit (Quiagen/69504) following manufacturer's instructions (Westburg b. v Netherlands). The concentration of DNA was determined by Nanodrop 2000 (Thermo Scientific). Extracted DNA integrity and presence was checked by agarose gel electrophoresis (1.6% agarose).

Amplification of DNA by PCR

The six denucleotide with random sequences i.e. (P1-5'AACGCGCAAC3'; P2-5'ACCAGGGGCA3'; P3-5'TCCGAGAGGG3'; P12-5'AAGAGCCCGT3'; P17-5'TGCCGGCTTG3'; & OPP-5-5'CCCCGGTAAG3') procured from Xcleris Genomics BIONEER (USA) were used for genomic DNA amplification. PCR reaction were conducted with each reaction mixture of 25µl consisting of Taq Polymerase buffer with $MgCl_2$ 2.5µl, dNTPs 2.5µl, Primer 2.0µl, Taq DNA Polymerase0.3 µl, DNA template 4 µl & the rest milli Q water. The amplification was carried out in Thermal Cycler (Gene Amp PCR System 9700 (Applied Biosystems; Genaxy, Labnet, International Inc), with denaturation at 94°C for 10 minutes followed by 45 cycles of denaturation, annealing and extension respectively at 94°C, 32-38°C and 72 for 1 minute each and final extension at 72°C for 5 minute. The amplified products were stored at 4°C. They were run on 2% agarose gels (stained with EtBr) with mass ruler ™ DNA ladder marker (1kb, Fermentes). Gels

were photographed under UV illumination in Gel Documentation System (Bio Red; FLUOR-S^{TM} Multimage).

Data Analysis

RAPD data was used to construct the dendrogram following NTSYS-$_{pc2.0}$ software. All calculations were done using computer program NTSYS-$_{pc2.0}$ package. Pairwise similarity matrices were generated by SM (simple matching) coefficient of similarity by using the SIMQUAL format of NTSYS software. A dendrogram was constructed by using the UPGMA (unweighted pair-group method with arithmetical averages) with SAHN module of NTSYS software to show a phonetic representation of genetic relationship as revealed by the similarity coefficient

RESULTS AND DISCUSSION

In present investigations molecular characterization of seven Pieridae (*Pieris brassicae nepalensis, Pieris canidia indica, Colias electo fieldi*, *Colias erate erate*, *Gonepteryx rahmni nepalensis, Pontia daplidice moorei* & *Terias hecabe fimbriata*) butterflies was carried out using P12, P17, P3 & OPP-5 RAPD markers. The amplified fragments were scored manually for their presence (denoted as '1' or absence ('0') for each primer (Fig.1-4). Polymorphism was observed in among the seven species based on the banding patterns and base pairs. Amplification was obtained in all the four random primers tested. A total 57 bands were scored with four decamer primers of which 57 were polymorphic and the percentage of polymorphism was 100% (Table 1-6; Fig. 1-4). For the analysis and comparison of this pattern, a set of distinct, well separated bands were selected, neglecting the weak and unresolved bands. Genus specific bands have been observed i.e. a band of 818bp with primer 12 (Table 2) & 520bp with primer OPP-5 (Table 5) were amplified with species of *Pieris* & *Colias* respectively and these can be considered as marker bands of the genus (Sharma *et.al*., 2006a,b). The presence of these bands suggested the interspecific genetic relatedness between two species. Species specific bands were also observed which revealed the existence of some conserved regions within the species (Table 6). The present results were in accordance with those Sharma *et.al*.[4,5,6] & Tiple *et. al*.[10].

Table 1
RAPD primers, their sequences and % polymorphism
With seven species of Family Pieridae

Primer	Seq 5' to 3'	Total Bands	Polymorphic bands	Monomorphic bands	Polymorphism (%)
P12	AAGAGCCCGT	16	16	00	100
P17	TGCCGGCTTG	11	11	00	100
P3	TCCGAGAGGG	18	18	00	100
OPP-5	CCCCGGTAAG	12	12	00	100
Total		**57**	**57**	**00**	100

Table 2
RAPD-PCR products obtained on amplification with Primer 12

Lane →	$A/A_2/Bc$ *Pieris brassicae*	$A_1/B_1/B$ *Pieris canidia*	P_{i1} *Colias electo fieldi*	P_{i2} *Colias erate erate*	P_{i3} *Gonepteryx rhamni*	P_7 *Pontia daplidice*	P_8 *Eurame hecabe*
Band size in Base Pairs (bp) Approx.	818	818				1044	
	762					876	
	638					744	
	595						
				550			
			514			514	
		488					
	473						
	451						
						370	
	354						
		270				270	
	260	260		260			260

Table 3
RAPD-PCR products obtained on amplification with Primer 17

Lane →	$A/A_2/Bc$ *Pieris brassicae*	$A_1/B_1/B$ *Pieris canidia*	P_{i1} *Colias electo fieldi*	P_{i2} *Colias erate erate*	P_{i3} *Gonepteryx rhamni*	P_7 *Pontia daplidice*	P_8 *Eurame hecabe*
Band size		1160					
in Base Pairs	989						
(bp) Approx.				847	847		
	786						
		718					
	522		522	522			
		484					484
				414			
						319	
				258	258		
	223	223	223				

Table 4
RAPD-PCR products obtained on amplification with Primer 3

Lane →	A/A_2/Bc *Pieris brassicae*	A_1/ B_1/B *Pieris canidia*	P_{i1} *Colias electo fieldi*	P_{i2} *Colias erate erate*	P_{i3} *Gonepteryx rhamni*	P_7 *Pontia daplidice*	P_8 *Eurame hecabe*
Band size in Base Pairs (bp) Approx.	715	673	1217 1171 477	590	505	757 556 528	
		461 316 262 253	319 263	319 263 206	319		263
			149				

Table 5
RAPD-PCR products obtained on amplification with Primer OPP-5

Lane →	A/A_2/Bc *Pieris brassicae*	A_1/ B_1/B *Pieris canidia*	P_{i1} *Colias electo fieldi*	P_{i2} *Colias erate erate*	P_{i3} *Gonepteryx rhamni*	P_7 *Pontia daplidice*	P_8 *Eurame hecabe*
Band size in Base Pairs (bp) Approx.	616 352	683	520 335 197 140	520 140	876 752 544 140	982 269 140	140

Table 6
Some species specific Bands (bp) obtained with different Primers in butterfly species

Species	Primer 12	Primer17	Primer 3	Primer OPP-5
Pieris brassicae	762, 638, 595 473, 451, 354	989, 786	715	616, 352
Pieris canidia	488	1160, 718 484	673, 461, 316 262, 253	683
Colias electo fieldi	-	-	1217, 1171 477, 149	335, 197
Colias erate erate	550	414	590, 206	-
Gonepteryx rhamni	-	-	505	876, 752, 544
Pontia daplidice	1044, 876 744, 370	319	757, 556 528	982, 269
Eurame hecabe	-	-	-	-

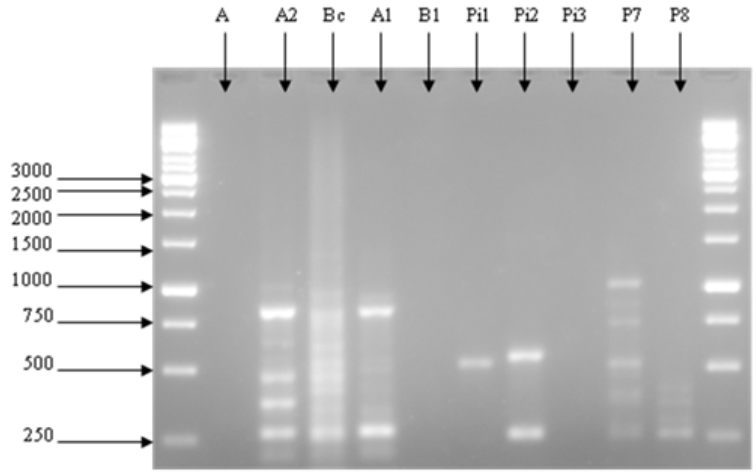


Figure 1
Photograph of agarose gel showing bands in amplified products with primer 12

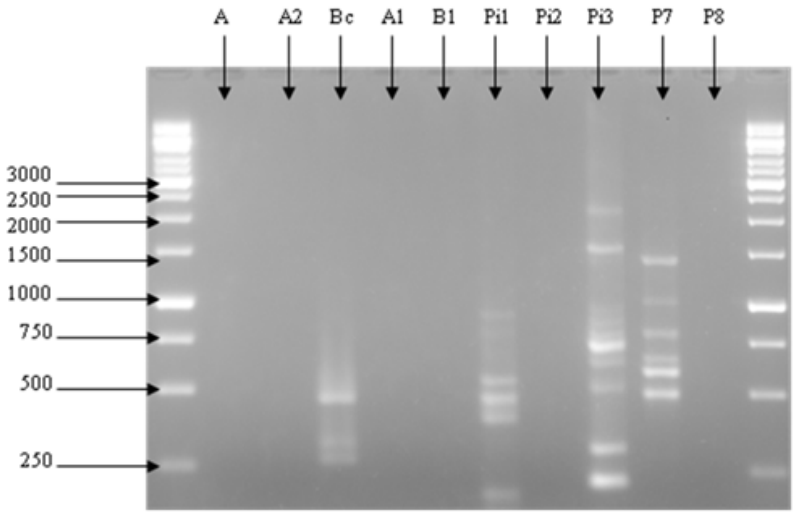


Figure 2
Photograph of agarose gel showing bands in amplified products with primer 17

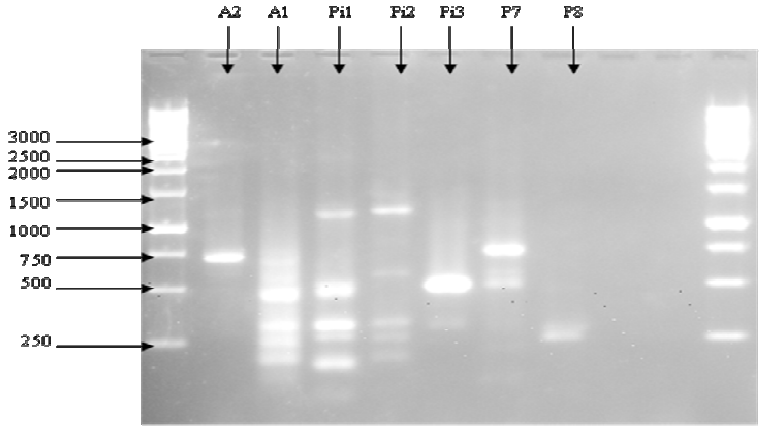
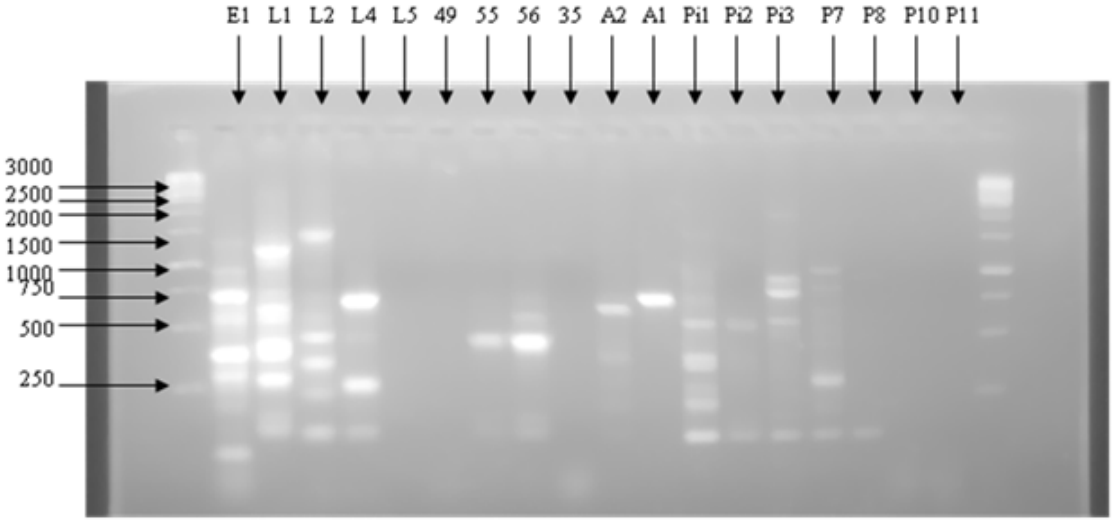


Figure 3
Photograph of agarose gel showing bands in amplified products with primer 3



Lane 0		*Gene Ruler TM 1kb DNA ladder*		
A/A$_2$/Bc	*Pieris*	*brassicae*	*Pi2*	*Colias erate erate*
A$_1$/ B$_1$/B	*Pieris canidia*		*Pi3*	*Gonepteryx rhamni*
Pi1	*Colias electo fieldi P7*			*Pontia daplidice*
P8	*Eurame hecabe*			

Figure 4
Photograph of agarose gel showing bands in amplified products with primer OPP-5

Dendrogram constructed using the NTSYS$_{spc2.2}$software divided the pierids into two clades. Cluster-I comprises of two species *viz*. *Pieris brassicae nepalensis* and *Pieris canidia indica.* Cluster-II comprises of 5 species *viz*. *Colias electo fieldi*, *Colias erate erate*, *Gonepteryx rahmni nepalensis, Pontia daplidice moorei* & *Terias hecabe fimbriata.* Cluster –II is subdivided in to two, sub-cluster-I consisting of *Colias electo fieldi*, *Colias erate erate*, *Terias hecabe fimbriata* & *Gonepteryx rahmni nepalensis,* sub-cluster-II consists of only *Pontia daplidice moorei.* Su-cluster-I is further divided in to two sub-sub-clusters, sub-sub cluster-I comprises of *Colias electo fieldi*, *Colias erate erate* & *Terias hecabe fimbriata* and sub-sub cluster-II comprises of *Gonepteryx rahmni nepalensis* (Fig5). There is a difference in the branching pattern between the molecular data, which signifies the need for using molecular tools for taxonomic classification as well as in understanding the evolutionary relationship.

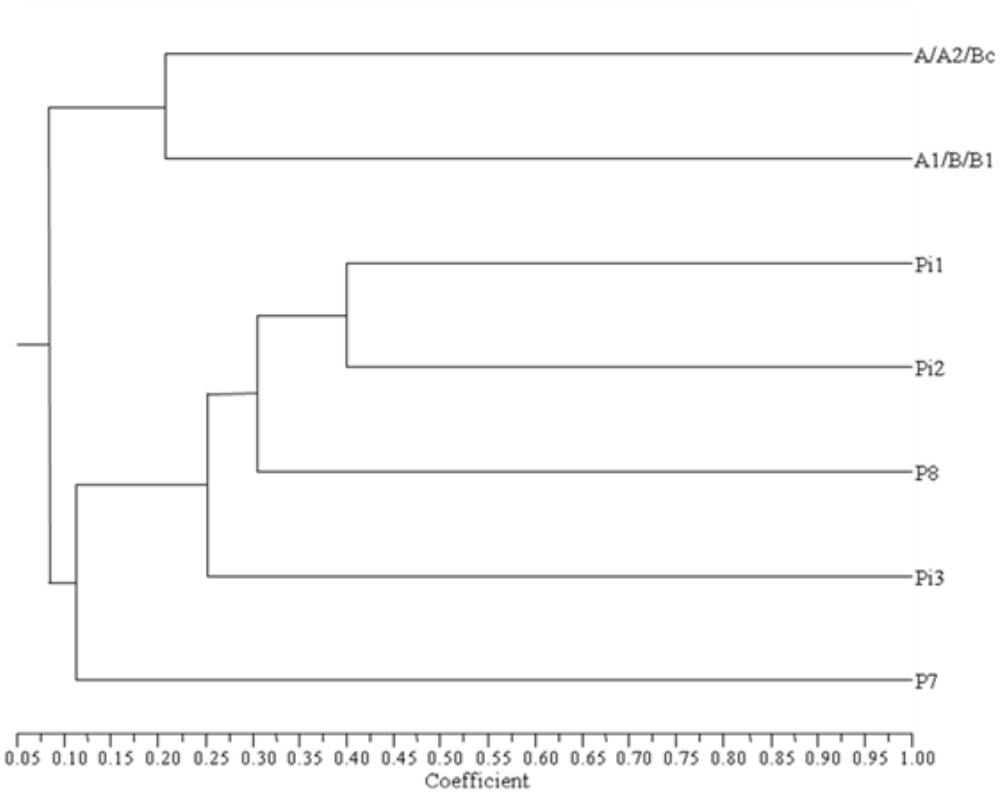


Figure 5
Dendrogram of seven Pieridae species based on 6 RAPD primer data

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