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Molecular Studies of Five Species of Butterflies (Lepidoptera: Insecta) Through RAPD-PCR Technique

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Abstract — The genomic DNA of five species of butterflies namely, *Pieris canidia, Pieris brassicae, Ixias pyrene, Ixias marianne* and *Pontia daplidice* belonging to the family Pieridae has been subjected to RAPD-PCR analysis with four decamer oligonucleotides *i.e.* P1, P2, P3 and P4. All of them produced discrete bands of various lengths revealing genetic variations as well as similarities among various species of butterflies under study. Some species specific bands were obtained with these primers. With P1, a fragment of 570 base pairs was found in both the species of the genus *Pieris*. Similarly, with P3 two bands having the base pair lengths of 310 and 160 were obtained in the same genus. These can be considered as diagnostic bands for this genus. All the primers also produced species specific bands. The results were supported by the dendrograms plotted according to nearest neighbour analysis, which further revealed that P1 and P3 were more authentic as they resulted in clustering of males and females of each species.

Key words: Dendrograms, Genetic Variation, Lepidoptera, Oligonucleotides, RAPD-PCR.

INTRODUCTION

Lepidopterans 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 and certain other species *e.g.,* member of Saturniidae, *Bombyx mori* yields silk of commercial value. This is because their correct identification and classification has become crucial. Due to the existence of closely related species, morphological attributes that change as a function of environment and the prevalence of biotypes and sibling species, their identification on the basis of morphological markers has become problematic. Molecular techniques based on DNA sequence polymorphism are now used in population genetics studies, systematics and molecular taxonomy to get an answer to systematic related problems (Nagaraja and Nagaraju 1995; Tom *et al.* 1995; Weng *et al.* 1996; Zhou *et al*. 2000; Zakharov 2001; Sharma *et al.* 2003).

In the present project, five species belonging to subfamily Pierinae of the family Pieridae, namely, *Pieris canidia; Pieris brassicae*; *Ixias pyrene, Ixias marianne* and *Pontia daplidice,* have been studied. Subtle morphological variations are present among the species, which could be traced by expert taxonomists only and still anomalies may exist. We have therefore, attempted to use RAPD-PCR technique to characterize these species, as no such work has been carried out on Indian species, with an aim to have molecular information which would further facilitate the rapid identification of species of butterflies. Such studies are in fact required to explore the correct designation of these species and to unravel the species complex if any.

We have used RAPD-PCR technique because it is cost effective, take less time, the results can be directly inferred from the gel and it reveals large amount of genetic variations, so it finds various entomological applications (Hunt and Page 1992; Heckel *et al.* 1995; Dowdy and Mcgaughey 1996).

MATERIAL AND METHODS

Five species of butterflies belonging to family Pieridae were collected from various locations at Chandigarh (India).The collection sites have been given in Table 1.

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Table 1 — Collection Site of Various Butterfly Species

S. No	Species	Locality	Time	No. of specimens studied
1.	*Pieris brassicae*	Botanical garden, Panjab university, Chandigarh, India	March, 2003	15
2.	*Pieris canidia*	Botanical garden, Panjab university, Chandigarh, India	November, 2002	15
3.	*Ixias pyrene*	Sukhna lake, Chandigarh, India	July, 2003	12
4.	*Ixias marianne*	Sukhna lake, Chandigarh, India	July, 2003	12
5.	*Pontia daplidice*	Sukhna lake, Chandigarh, India	May, 2003	12

Isolation of genomic DNA - The DNA was isolated by following the techniques of AUSBEL *et al.* (1999). For this purpose adult individuals of both the sexes of each species were used. Single adult individual (in replicates) was homogenized in 500µl of chilled TE buffer (pH-8). 70µl of 5% SDS along with 6µl of Proteinase K(25 mg/ml) was added, mixed well and incubated at 55ºC for 30m. 100µl of chilled NaCl (5M) was added and again incubated in ice for 30 minutes. Samples were centrifuged at 10,000 rpm for 10 minutes in cooling centrifuge. The upper layer was carefully transferred to another tube and an equal volume of saturated phenol was added followed by centrifugation at 10,000 rpm for 10 m. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by vortexing and the solutions were centrifuged at 10,000 rpm for 10 minutes. The upper aqueous layer was carefully transferred to another eppendorf tube and an equal volume of chilled absolute ethanol was added. An overnight incubation was carried out for overnight at 4ºC followed by centrifugation at 12,000 rpm for 10 minutes. After discarding the supernatant, the pellet was washed with two volumes of 70% ethanol and dried. It was dissolved in 40µl of TE buffer and stored at -20ºC for further use. The concentration of DNA was determined by spectrophotometric method using UV visible scanning spectrophotometer.

DNA Amplification by PCR - The DNA was amplified by using four decanucleotide primers with random sequences procured from Genetix (USA) sequences as given in Table 2.

Table 2 — Sequences of the various primers used

Primers	Sequences
Primer-1 (P1)	5'-AACGCGCAAC-3'
Primer-2 (P2)	5'-ACCAGGGGCA-3'
Primer-3 (P3)	5'-TCCGAGAGGG-3'
Primer-4 (P4)	5'-TCTGCCGTGA- 3'.

The 25 µl of reaction mixture contained 2.5µl of 10X PCR buffer,0.5µl $MgCl_2$ (2.5Mm), 2.5µl dNTPs (2mM), 1µl of Taq Polymerase(1U/µl), 2.5µl of BSA (100pM/µl), 5µl of DNA and the rest distilled water. The amplification was carried out in thermal-cycler (MJ Research, USA) under the PCR conditions summarized in Table 3.

Table 3 — Cycling conditions of Polymerase Chain Reaction

Steps	Temperature	Time
1 Cycle		
Denaturation	94ºC	5 minutes
35 Cycles		
Denaturation	94ºC	1 minute
Annealing	P1 & P4- 39.5ºC	1 minute
	P2 & P3- 43.6ºC	1 minute
Extension	72ºC	
1 Cycle		
Final Extension	72ºC	5 minutes

The amplified products were run on 2% agarose gel (stained with ethidium bromide) with DNA ladder (80-1031 bp). Gels were photographed under UV illumination.

Dendrogram Plot - Dendrograms were plotted for the data obtained with each of the four primers, using nearest neighbour analysis (Single linkage cluster analysis) of hierarchical clustering.

RESULTS AND DISCUSSION

RAPD patterns were visually analysed and scored from photographs. A series of discrete bands were obtained on amplification of DNA samples of five species of butterflies with four primers *i.e.* P1, P2, P3 and P4 (Figs. 1-4). All the primers produced a large number of bands with different intensities suggesting that the amplified fragments were repeated in the genome in varying degrees. The different primers resulted in different banding patterns. For the analysis and com-

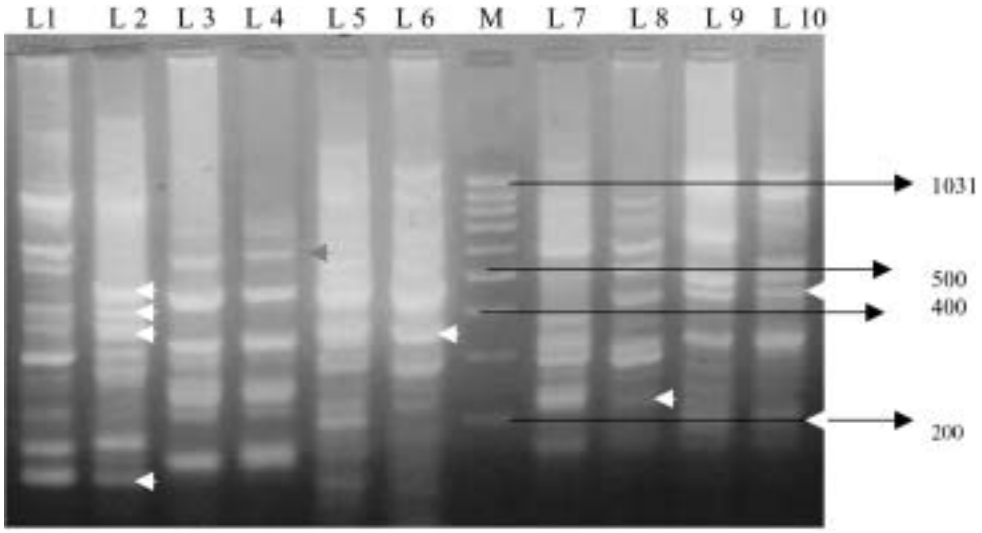

Fig. 1 — Photograph of agarose gel showing bands in amplified products with primer 1.
Lane M Gene Ruler TM 100 bp DNA ladder.
Lane L1 and 2 *Pieris canidia* ♂ and ♀
Lane L3 and L4 *Pieris brassicae* ♂ and ♀
Lane L5 and L6 *Ixias pyrene* ♂ and ♀
Lane 7 and L 8 *Ixias marianne* ♂ and ♀
Lane 9 and L10 *Pontia daplidice* ♂and ♀
- Black arrows represents base pare lengths of ladder
- White arrows represents base pare lengths of species specific bands
- Grey arrows represents base pare lengths of genus specific band

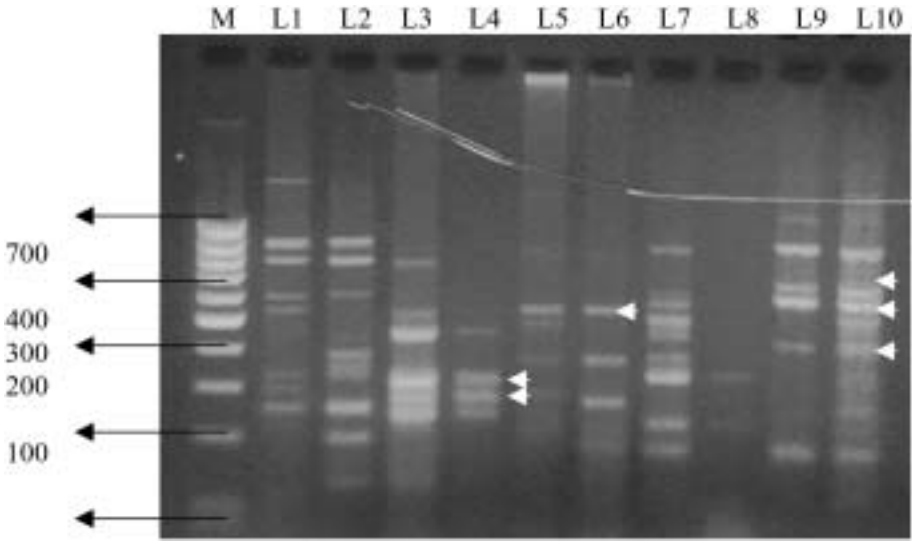

Fig. 2 — Photograph of agarose gel showing bands in amplified products with primer 2.
Lane M Gene Ruler TM 100 bp DNA ladder.
Lane L1 and 2 *Pieris canidia* ♂ and ♀
Lane L3 and L4 *Pieris brassicae* ♂ and ♀
Lane L5 and L6 *Ixias pyrene* ♂ and ♀
Lane 7 and L 8 *Ixias marianne* ♂ and ♀
Lane 9 and L10 *Pontia daplidice* ♂and ♀
- Black arrows represents base pare lengths of ladder
- White arrows represents base pare lengths of species specific bands

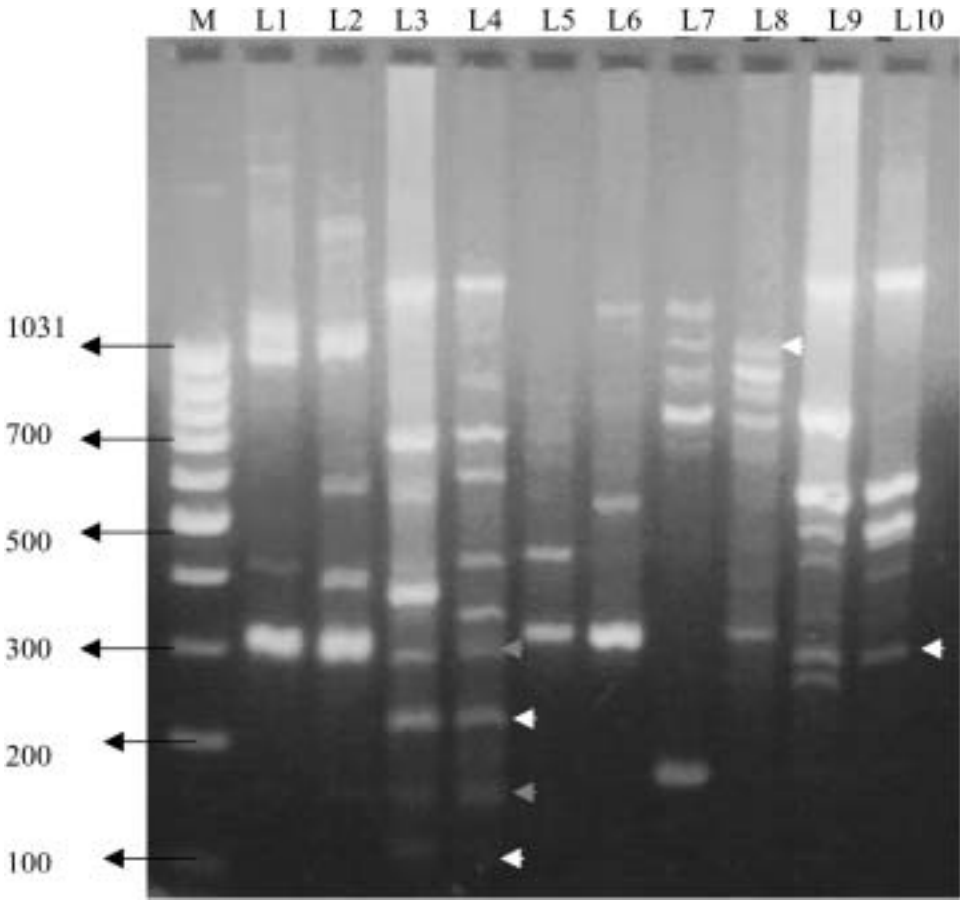

Fig. 3 — Photograph of agarose gel showing bands in amplified products with primer 3.
Lane M Gene Ruler TM 100 bp DNA ladder.
Lane L1 and 2 *Pieris canidia* ♂ and ♀
Lane L3 and L4 *Pieris brassicae* ♂ and ♀
Lane L5 and L6 *Ixias pyrene* ♂ and ♀
Lane 7 and L 8 *Ixias marianne* ♂ and ♀
Lane 9 and L10 *Pontia daplidice* ♂ and ♀
- Black arrows represents base pare lengths of ladder
- White arrows represents base pare lengths of species specific bands
- Grey arrows represents base pare lengths of genus specific band

parison of these patterns, a set of distinct, well separated bands were selected, neglecting the weak and unresolved bands. KAMBHAMPATI *et al.* (1992) found considerable variations among the individuals within closely related species and specific populations of the genus *Aedes*. Genus specific bands have been observed *e.g.,* a band of 570 bp with P1 (Table 4) and two bands of 310 and 160 bp lengths with P3 were amplified in both the species of *Pieris* and these can be considered as marker bands for this genus (Table 6). 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 8).

These conserved regions provided diagnostic profiles for these species. Such diagnostic markers have also been reported in three species of white-fringed weevils (Coleoptera) and two of *Parnassius* (Lepidoptera: Papilionidae) by HARDWICK *et al.* (1997) and ZAKHAROV (2001) respectively. All the primers in the species studied, amplified the male and female specific bands suggesting the genetic variations in the two sexes. There were, however, some bands common to both the sexes (Tables 4-7). These common bands showed intraspecific genetic relatedness. The present results were in accordance with those of SHARMA *et al.* (2003) in which an interspecific genetic relatedness and polymorphisms in the two species of butterflies

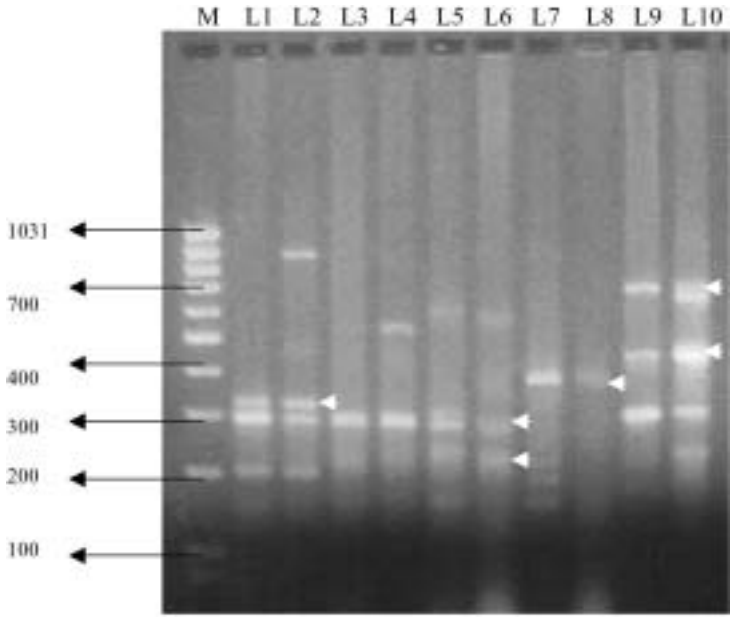

Fig. 4 — Photograph of agarose gel showing bands in amplified products with primer 4.
Lane M Gene Ruler TM 100 bp DNA ladder.
Lane L1 and 2 *Pieris canidia* ♂ and ♀
Lane L3 and L4 *Pieris brassicae* ♂ and ♀
Lane 4 and L5 *Ixias pyrene* ♂ and ♀
Lane 7 and L 8 *Ixias marianne* ♂ and ♀
Lane 9 and L10 *Ponita daplidice* ♂ and ♀
- Black arrows represents base pare lengths of ladder
- White arrows represents base pare lengths of species specific bands

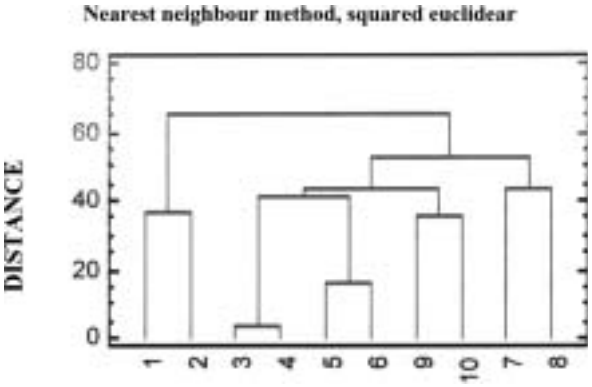

Fig. 5 — Dendogram showing the relationship based on RAPD analysis between different species of family Pieridae with Primer 1.

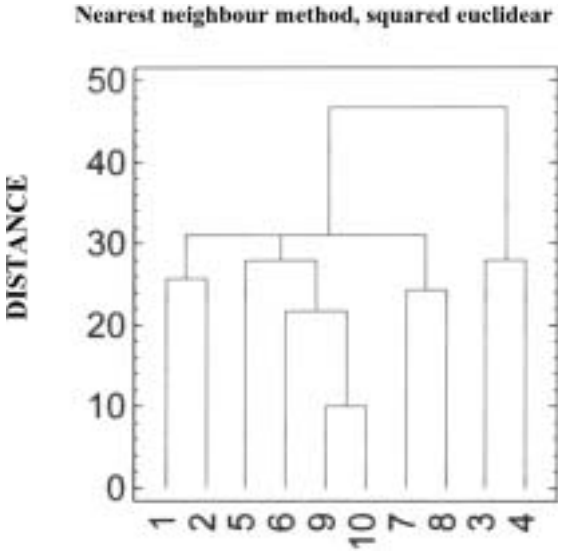

Fig. 6 — Dendogram showing the relationship based on RAPD analysis between different species of family Pieridae with Primer 3.
1. Pieris canidia ♂ *2. Pieris canidia* ♀ *3. Pieris brassicae* ♂ *4. Pieris brassicae* ♀
5. Ixias pyrene ♂ *6. Ixias pyrene* ♀ *7. Ixias mari-annne* ♂ *8. Ixias marianne* ♀
9. Pontia daplidice ♂ *10. Pontia daplidice*

Table 4 — RAPD-PCR products obtained on amplification with primer 1 (5'-AACGGGCAAC-3')

Lane →	L1 *Pieris canidia* (♂)	L2 *Pieris canidia* (♀)	L3 *Pieris brassicae* (♂)	L4 *Pieris brassicae* (♀)	L5 *Ixias pyrene* (♂)	L6 *Ixias pyrene* (♀)	L7 *Ixias marianne* (♂)	L8 *Ixias marianne* (♀)	L9 *Pontia daplidice* (♂)	L10 *Pontia daplidice* (♀)
Band Size						1031				1031
in Base Pairs (bp)	900									900
Approx.										875
	800				800	800	800	800		
	720									
				700			700	700		700
			675	675			675	675		
								655		
							600	600	600	600
	570	570	570	570						
										525
	500	500			500	500	500	500		
									450	450
	435	435								
			410	410				410	410	410
									400	400
	385	385								
							365			
								355		
	340	340								
			320	320			320	320		
					310	310				
	280	280					280	280		
			260	260			260	260		
	250	250								
	235									
							220	220		
	205		205	205	205	205				
									200	200
									190	190
					190					
							170			
	160	160								
		155	155	155						
	135	135								

Table 5 — RAPD-PCR products obtained on amplification with primer 2 (5'-ACCAGGGGCA-3')

Lane →	L1 *Pieris canidia* (♂)	L2 *Pieris canidia* (♀)	L3 *Pieris brassicae* (♂)	L4 *Pieris brassicae* (♀)	L5 *Ixias pyrene* (♂)	L6 *Ixias pyrene* (♀)	L7 *Ixias marianne* (♂)	L8 *Ixias marianne* (♀)	L9 *Pontia daplidice* (♂)	L10 *Pontia daplidice* (♀)
Band Size	940	940			940		940		940	940
in Base Pairs (bp)						900				
Approx.	825	825	825							
									720	720
							700			
									655	655
	625	625					625		625	625
					600	600				
	520		520		520		520			520
				480			480			
			465							
									450	450
		420								
		400			400	400	400			
		365					365	365		365
			335	335						
	325									
					310					
	300					300				
			290	290						
		265								
	255		255	255	255				255	255
						240				
					230					230
		200				200	200		200	200
		140								

Table 6 — RAPD-PCR products obtained on amplification with primer 3 (5'-TCCGAGAGGG-3')

Lanes →	L1 *Pieris canidia* (♂)	L2 *Pieris canidia* (♀)	L3 *Pieris brassicae* (♂)	L4 *Pieris brassicae* (♀)	L5 *Ixias pyrene* (♂)	L6 *Ixias pyrene* (♀)	L7 *Ixias marianne* (♂)	L8 *Ixias marianne* (♀)	L9 *Pontia daplidice* (♂)	L10 *Pontia daplidice* (♀)
Band Size in Base Pairs (bp) Approx.	1031	1031					970	970		
				945			945	945		
								900		
							860	860	860	860
					755					
			725	725						
			700	700			700	700	700	700
					665					
			600	600						
			555			500			500	500
					440				440	
			425	425	425					
	410							410		
		400								400
			385							
				345					345	345
					320	320		320		
	310	310	310	310						
									300	300
									280	
			225	225						
	160	160	160	160						
			115	115						

Table 7 — RAPD-PCR products obtained on amplification with primer 4 (5'-TCTGCCGTGA-3')

Lanes →	L1 *Pieris canidia* (♂)	L2 *Pieris canidia* (♀)	L3 *Pieris brassicae* (♂)	L4 *Pieris brassicae* (♀)	L5 *Ixias pyrene* (♂)	L6 *Ixias pyrene* (♀)	L7 *Ixias marianne* (♂)	L8 *Ixias marianne* (♀)	L9 *Pontia daplidice* (♂)	L10 *Pontia daplidice* (♀)
Band Size in Base Pairs (bp) Approx.		895							650	650
										615
					600					600
						555				
									460	460
							365	365		
	330	330								
					310					
	300	300	300	300					300	300
					275	275				
					235	235				
			215							215
	200	200					200			
							180			
	150				150		150			

Table 8 — Some species specific bands (bp) obtained with different primers in various species

Species	Primer 1	Primer 2	Primer 3	Primer 4
Pieris canidia	435,385,340,250,135	-	1031	330
Pieris brassicae	-	335, 290	725, 115	-
Ixias pyrene	310	600	-	275,235
Ixias marianne	220	-	970	365
Pontia daplidice	450,400,200	720,655,450	300	650,460

i.e., Catopsilia crocale and *Catopsilia pyranthe* of the family Pieridae were found.

The dendrograms drawn on the basis of shared bands were analysed according to nearest neighbour analysis. WILLIAMS *et al*. (1994) constructed dendrograms for *Listronotus bonariensis* populations on the basis of average linkage cluster analysis using Nei and Lei similarity based on shared fragments. It was revealed that data obtained with P1 and P3 resulted in clustering of male and female individuals of all five species, which is in accordance to the study carried out by BALLINGER-CRABTREE *et al*. (1992) in 11 geographical populations of *Aedes aegyptii* using three primers. Thus, these two primers generated sufficient number of bands to put the individuals of the same species in one group. However, P2 and P4 put the individuals of two sexes of *Pieris canidia* and *Pontia daplidice* far apart from each other in their respective dendrograms. This may be due to the use of small number of individuals in the study which shared low number of bands to make proper groups. Besides, the sequences amplified by these primers may be the segments of DNA not common to the individuals of both the sexes that is why there were less common bands between two sexes and hence the males and females were not clustered in the dendrograms. The number of primers is therefore being increased in further studies to sort out the best primers useful in the diagnosis of the species under study.

RAPD therefore, appears to be useful in differentiating species, subspecies and strains in arthropods as also demonstrated by BALLINGER-CRABTREE *et al*. (1992) in *Aedes aegyptii*; CENIS *et al*.(1993) in the identification of aphid species and clones; TOM *et al*. (1995) in gypsy moth; DOWDY and MCGAUGHEY (1996) in Indian meal moth and WENG *et al*. (1996) in silkworm *Bombyx mori.*

CONCLUSION

The present study has revealed that the RAPD-PCR technique is extremely useful for rapid identification of genetic polymorphisms in Lepidopterans because of the reproducibility of the results for each of the species. The bands generated by RAPD are clear genetic markers of four primers used for the amplification of genomic DNA. However, the data will be strengthened by increasing the number of individuals and the primers for the study in future.

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