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MORPHOLOGICAL *VERSUS* MOLECULAR CHARACTERIZATION OF THREE SIMILAR PIERID SPECIES OF BUTTERFLIES

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ABSTRACT

The morphological diversity and genetic similarity of three Pierid species (both male and female) of butterflies- *Pieris canidia*, *Pieris napi* and *Pieris brassicae* has been studied. RAPD-PCR analysis using seven decamer primers produced discrete bands of various size revealing genetic variations as well as similarities among the three species of butterflies. Some species specific bands were obtained using primers: MA04 (750 bp), MA 13 (650 bp), MA 15 (650bp) for *P. napi;* MA04 (1000 bp), MA 05 (1600bp) and MA 13 (1400 bp) for *P. brassicae* and OPT05 (350 bp) for *P. canidia.* The specific bands can be considered as diagnostic bands for these species. The NTSYS-pc result of the morphological characteristics is supported by morphological similarities between the species. Maximum similarity between male and female of the species is shown in *P. canidia* where this species have only mild variation in their wing pattern, including coloration and distribution of spots. Therefore, the male and female of the other two species (*P. napi and P. brassicae*) are more different not only in the prominance of their spots but also in their colorations and spots on their wings. The results were supported by the dendrogram which showed clustering of males and females of each species. The dendrogram generated by using morphological data however, is not in congruence with RAPD data. Similarly, mitochondrial DNA variation among the three species was studied by sequencing 650 bp of the cytochrome c oxidase I gene (CO1). These sequences were then aligned with existing sequences retrieved from NCBI database confirming the genetic similarity of the three species with the same species from other regions and also the genetic dissimilarity between the different species of the same family.

KEYWORDS: Pieridae, RAPD-PCR, genetic variation, morphological diversity, COI barcoding

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INTRODUCTION

Pieridae is one of the most important and commonly seen families of Lepidoptera. 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]. *Pieris*, the whites or garden whites, is a widespread genus of the family Pieridae, with three dominant species found in Mizoram. They are usually white or light yellow, with black patterns on the wing surface[2]. Most of them are small or medium-sized, but some large-sized species are seldom seen especially in tropical countries. 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. Morphological identification of butterflies is usually based on the wing patterns and is attributed to the numbers and positions of spots on the wings which may change according to their environment leading to a new biotype. In order to maintain the existing level of diversity within tropical area, sustainable use of strategies and conservation methods needs to be established. Butterflies are widely recognized as potentially valuable ecological indicators as they are sensitive to and directly affected by any alteration to their habitats, atmosphere and local weather. These factors make morphological criteria not a preferred way for a very accurate differentiation of these species[3].

Recently developed molecular marker techniques provide an important tool that ease the assessment of genetic diversity and facilitate genotyping, classification, inventorying and phylogenetic studies[4]. RAPD marker is well suited for use in the large sample throughout systems required for population genetics and studies of biodiversity. The amplification pattern obtained by RAPD primers were considered to be ideal for identification of species[5].Random Amplified Polymorphic DNA-polymerase chain reaction (RAPD-PCR) randomly amplifies many regions of genomic DNA using random primers and can be used for detecting polymorphisms at many loci between species and populations[6]. RAPD-PCR provides a very versatile and widely applied biotechnology technique in entomology. This method has been widely used in the determination of population structure without prior knowledge of DNA sequences and it gives a good resolution of genetic differences. Using RAPD-PCR, genetic polymorphisms and genetic diversity in natural populations between species of Nymphalidae have been studied[7]. Earlier RAPD was successfully applied for molecular characterization of two species of butterflies belonging to family Pieridae by Sharma *et al*[8]. Present paper describes the feasibility of RAPD in discriminating three species of family Pieridae and the genetic diversity in these species.

The rapid evolution of mitochondrial DNA (mtDNA) sequences has often been used to investigate the relationships of populations within species and the relationships of closely related species. mtDNA are thought to better recover species-level relationships because a smaller effective population size for this essentially non-recombining, maternally inherited genome leads to shorter coalescence times[9]. DNA barcoding proposes the use of DNA sequences to identify and classify an organism. The potential of a 650 bp fragment of the 5'-end of mitochondrial cytochrome c oxidase subunit I (COI)-based species identification system was proposed and partially demonstrated by Hebert *et al*[10]. A major unresolved issue however, is how closely the molecular taxa correspond to what traditional biologists recognize as species, i.e., species defined by DNA barcodes might not always correspond with species recognized by traditional ecological and morphological criteria as discussed by Blaxter[11] and Hebert *et al*[9]. Hence, in this paper, we therefore try to examine whether the morphologically similar species are indeed, closely related phylogenetically by comparing the

morphological and phylogenetic relationship among the three Pierid species, comparing the RAPD with CO1 barcoding, tracing through their evolutionary history.

MATERIALS AND METHODS

Three species of butterflies (both male and female: *Pieris canidia*, *Pieris napi* and *Pieris brassicae)* belonging to the family Pieridae were collected during April – May 2011 from Ramrikawn, Aizawl, Mizoram. The identification of butterflies is based on the information of Evans[12], Wynter-Blyth[13], Wahlberg[14] and Isaac Kehimkar[15] (Table 1; Fig.1). Data obtained will be analyzed by appropriate statistical tools.

Table 1
Morphological features of three species of Pieris (male and female)

Species	Color of the wing	wingspan
P. napi ♂	Black veins, UPF black apex and 1 or no discal spots. The UNH are pale yellow with the veins highlighted by black scales giving a greenish tint	40-60mm
P. napi♀	Veins much darkened as compared to male. Two discal spots on UPF	
P. canidia ♂	UPF with black apex, inwardly dentate outer margins and discal spots. UPH with terminal dots and apical spots	45-60mm
P. canidia♀	Similar to male with additional discal spot on UPF and the spots and dots being more prominent.	
P. brassicae ♂	UP white with black apex FW and apical spot HW. No discal spot FW. UNH pale yellowish.	65-75mm
P. brassicae♀	Similar to male with the addition of two discal spots on UPF.	

(UPF-Upper Forewing, UPH-Upper Hindwing, FW-Forewing, HW-Hindwing, UP-Upper, UNH-Under hindwing.)

Male and Female of the three species of butterflies under study

Figure 1
(a) Pieris napi ♂(UP) Pieris napi♀(UP)

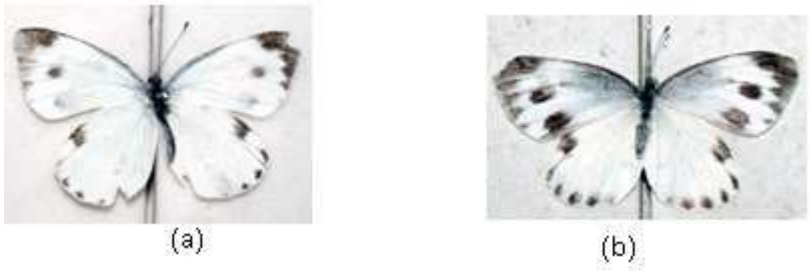

Figure 1
(***b) Pieris canidia ♂(UP) Pieris canidia♀(UP)***

Figure 1
(c) Pieris brassicae ♂(UP) Pieris brassicae♀(UP)

(i) Preparation of butterfly DNA

Genomic DNA was extracted from legs and tissues of Butterflies using slight modification of Zimmermann *et al*[16]. Legs or tissue from the thorax stored in 70% alcohol were taken in 1.5ml eppendorf tube. The legs or tissue were macerate with the help of scissors finely and homogenize in 250µl of room temperature extraction buffer containing 50mM Tris HCl (pH 8.0), 25mM NaCl, 25mM EDTA(pH 8.0) and 0.1% SDS. 2µl of proteinase K (18mg/ml) was added, mixed gently and incubated in oven at 60ºC for at least 3 hr or at 37ºC overnight. To the sample equal volume of phenol/chloroform (250µl) was added and mixed thoroughly until the solution is homogenized, it was then spin at 13,000 rpm for 5min and the supernatant were carefully taken out using micropipette to a new eppendorf tube. To the supernatant 15µl of 5M NaCl and 450µl of ice-cold 100% ethanol were added, mixed gently by inverting the tube several times and then placed in freezer for at least 20 min or overnight. It was again spin at 13,000 rpm for 5 min at cold temperature. Ethanol was poured off without dislodging the pellet, 200µl of room temperature 70% ethanol was added to the pellet and flash spin at 6000 rpm for 1 min and poured off ethanol. The pallet was dried in the oven at 60ºC for 15-20 min. 30µl of 1X TE Buffer was added and the pellet was re-suspended by gently flicking the tube and stored at -20°C for further used.

(ii) DNA Amplification by RAPD-PCR

The DNA was amplified by using seven RAPD random primers. Primers are obtained from Bangalore Genei. The 10µl of reaction mixture contained 1µl of 10X PCR buffer, 1µl MgCl (25mM), 0.2µl dNTPs (2mM), 0.3µl of Taq Polymerase (3U/µl), 0.8µl of BSA (100pmol/µl), 1µl of Template DNA, primer (10pmol/µl) and make up the volume with distilled water. The amplification was carried out in thermal-cycler Gradient (Eppendorf, Germany) using the following condition. Initial Denaturation at 94°C for 5 min followed by 35 cycles of Denaturation, annealing and extension respectively at 94°C, 37°C and 72°C for 1 min each and final extension at 72°C for 5 min. The amplified products were stored at 4°C. The

PCR product was run in 1.5% agarose gel stained with ethidium bromide with Low Range Ruler Plus as Marker and the bands on gels was documented using the Gel documentation system (Figure 2).

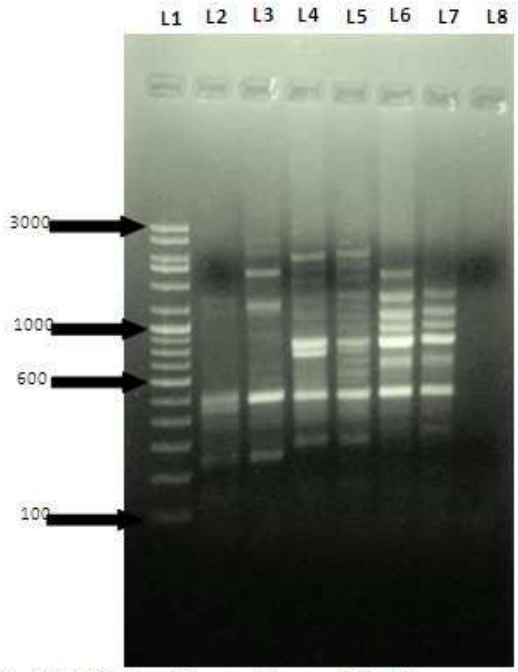

Figure 2 : RAPD banding pattern of the three species (male and female) of butterflies with Primer MA04

Lane 1	Low range ruler plus 3000 bp DNA ladder
Lane 2 and 3	*Pieris canidia* ♂ and ♀
Lane 4 and 5	*Pieris napi* ♂ and ♀
Lane 6 and 7	*Pieris brassicae* ♂ and ♀
Lane 8	Negative control

(iii) PCR condition for CO1

The reaction mixture consists of 2.5µl of 10x PCR buffer, 2µl Mgcl(25mM), 3.13 µl dNTPs (2mM), 0.2µl of Taq Polymerase (5U/µl), 0.8µl of BSA (100pmol/µl), 2µl of Template DNA and 0.5µl of forward and reverse primers (10pm/µl) with 13.37µl of distilled water to make a total volume of 25µl. DNA was denatured at 95 ˚C for 5 minutes, followed by 35 cycles of 30 sec at 94˚C, 30 sec at 55˚C, 1min 30 sec at 72˚C and final extension at 72˚C for 10 minutes by using a DNA thermal cycler Gradient (Eppendorf, Germany).

(iv) Data analysis

Each species was scored for the presence or absence of every amplification product, and the data were entered into a binary data matrix. A total of 254 bands were scored. Only distinct and polymorphic bands were recorded and used in the analysis. Similarity matrix based on Jaccard's similarity coefficient was used to construct unweighted pair group method with arithmetic average (UPGMA) dendrogram. To evaluate the discriminatory power of molecular markers PIC, MI and EMR were calculated (Table 2). The PIC value was determined by applying the formula[17] PICi = 2fi (1- fi), where fi is the percentage of the amplified alleles (bands present) and (1- fi) is the frequency of the null allele (band absent) for i^{th} allele. The MI was calculated as the product of two function that is DI and EMR as described by Prevost and Wilkinson[18]. The DI of the primer

is defined as 1- sigma (pi)2 where pi is the frequency of i^{th} allele, while EMR of a primer is defined as the "product of the fraction of polymorphic bands and the number of polymorphic bands for an individual assay"[19]. Generating similarity matrices (Tables 3 and 5), only polymorphic bands with PIC values higher than 0.08 were used[17]. All the above-mentioned statistical analysis was performed using NTSYS-pc software version 2.20[20]. The cophenetic coefficients between the matrix of genetic similarities and the matrix of cophenetic values were computed using appropriate routines of the NTSYS-pc package. The significance of the cophenetic correlation observed was tested using the Mantel matrix correspondence test [21]. The Mantel matrix correspondence test was also used to test the significance of the correlation coefficient of similarity matrix generated with RAPD data.

(v) Sequence alignment and phylogenetic analysis of the mitochondrial CO1 genome

The analysis of the DNA sequences, phylogenetic analysis and construction of a tree using Maximum likelihood statistical method were performed by using MEGA software version 5.0. The reliability of tree was evaluated by using the Bootstrap method with 500 bootstrap iterations. Bootstrap % refers to the percentage of trees in which the members form clade[22].

RESULTS AND DISCUSSION

RAPD patterns were visually analyzed and scored from the gel photographs. A series of discrete bands were obtained on amplification of DNA samples of three species of butterflies (male and female) with seven primers (MA04, MA05, MA13, MA15, MA23, OPT4, and OPT5). Out of the 254 total discrete fragments, Primer MA05 gave as many as 52 prominent bands while MA23 produces just 28 bands. The amplified ranges of primers was as low as 190 bp as in OPT05 to as high as 2150bp as in MA04 and there was a total of 196 (77.04%) polymorphic bands. 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. For the analysis and comparison of these patterns, a set of distinct, well separated bands were selected, neglecting the weak and unresolved bands.

Table 2

RAPD primers, Polymorphic bands, % Polymorphism, PIC, EMR, MI and Molecular weight range among the male and female butterflies from three Pierid species.

Primers	Seq 5'-3'	Total Bands	Polymorhic Bands	Polymorphism (%)	PIC	EMR	MI	Mol. Weight range (bp)
MA04	TGATCCCTGG	30	28	93.33	0.2521	0.93	0.27	260-2150
MA05	TGCGCCCTTC	52	37	71.15	0.2806	0.71	0.23	280-1950
MA13	CCAGATGCAC	31	19	61.29	0.2944	0.61	0.21	300-1460
MA15	GGCGGTTGTC	45	31	68.88	0.2777	0.68	0.22	290-1230
MA23	AGGCGATAAG	28	24	85.71	0.2619	0.85	0.26	300-1700
OPT04	GTGTCTCAGG	38	30	78.94	0.2709	0.78	0.25	220-1510
OPT05	GGGTTTGGCA	30	24	80	0.2728	0.8	0.25	190-1140
TOTAL		254	193	77.04				

(i) NTSYS Analysis

The amplified fragments were scored manually for their presence (denoted as'1') or absence (denoted as'0') for each primer. For RAPD analysis, data were scored as '1' for the presence and '0' for the absence of band of each species. All calculations were done using computer program NTSYSspc2.2 package. A Dendogram was constructed by using the UPGMA (Unweighted pair-group method with arithmetical averages) with SAHN module of NTSYS software to show a phenetic

representation of genetic relationship as revealed by the similarity coefficient (Figure 3). Cluster-I comprised of 4 species viz. *P. canidia*♂ *and*♀*, P. napi*♂and♀ with *P. brassicae* ♂ and ♀ forming cluster-II. Cluster-I is further subdivided into two sub-clusters, sub-cluster-I consisting of only *P. canidia*♂ and♀ and sub-cluster-II comprised of *P. napi*♂and♀. The dendrogram based on RAPD data showed much similarity with morphological differentiation of these 3 species. *P. canidia* male and female, being a similar species formed one cluster along with both *P. napi* and *P. brassicae*, each forming separate cluster pairwise, in relation to the species of each pairs.

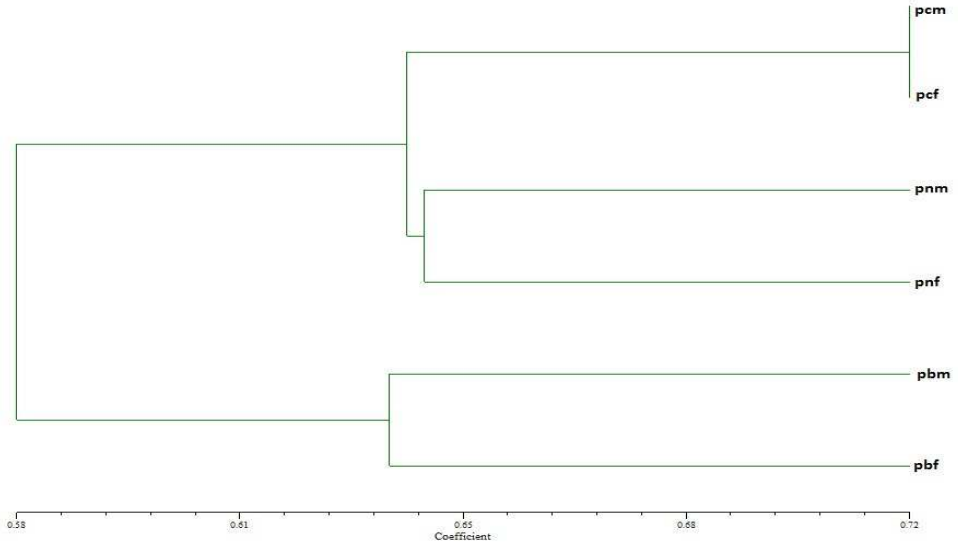

***Figure* 3**
***The dendrogram generated by clustering using UPGMA analysis computed from a pairwise comparison of RAPDs from species of Pieris. PCM - P. canidia*♂ *PCF - P. canidia*♀ *PNM – P. napi*♂ *PNF - P. napi*♀ *PBM -P. brassicae*♂ *PBF - P. brassicae*♀**

Table 3
Simple matching coefficients of similarity determined from analysis using seven RAPD primers using the NTSYS program

	P. canidia♂	*P. canidia*♀	*P. napi*♂	*P. napi*♀	*P. brassicae*♂	*P. brassicae*♀
P.canidia♂	1.000					
P.canidia♀	0.715	1.000				
P.napi♂	0.663	0.617	1.000			
P.napi♀	0.688	0.611	0.642	1.000		
P.brassicae♂	0.617	0.580	0.560	0.565	1.000	
P.brassicae♀	0.617	0.591	0.549	0.575	0.637	1.000

Table 4

Data analysis for three pierid species (male and female) on their morphological characteristics

MORPHOLOGICAL CHARACTERS	*P. canidia*♂	*P. canidia*♀	*P. napi*♂	*P. napi*♀	*P. brassicae*♂	*P. brassicae*♀
Discal spots on UPF	1	1	0	1	0	1
Apical spots on UPH	1	1	1	1	1	1
Toothed inner margin on UPF	1	1	0	0	0	0
White colored UNH	1	1	1	1	0	0
Black apex on UPF	1	1	1	1	1	1
Yellow base of costal region	1	1	1	1	1	1
Marking on UNH	0	0	1	1	0	0
Marking on UNF	0	0	1	1	0	0

The NTSYS result of the morphological characters is supported by morphological similarities between the species. The SM similarity coefficients (Table 5) as calculated using Simqual' sub-program of NTSYS-pc v2.2 software revealed maximum similarity between *P. canidia* male and female (1.000) where the male and female of this species have only mild variation in their UPH (Upper hind wing) with similar spotting, where black spots on the female are only more prominent than male, while the male and female of the other two species (*P. napi and P. brassicae*) are slightly more different not only in the prominance of their spots but also in their colorations and spots on their wings .

Table 5

Similarity coefficient among three Pierid species (male and female) based on Morphological Features

	P. canidia♂	*P. canidia*♀	*P. napi*♂	*P. napi*♀	*P. brassicae*♂	*P. brassicae*♀
P. canidia♂	1.000					
P. canidia♀	1.000	1.000				
P. napi♂	0.500	0.500	1.000			
P. napi♀	0.625	0.625	0.875	1.000		
P. brassicae♂	0.625	0.625	0.625	0.500	1.000	
P. brassicae♀	0.750	0.750	0.500	0.625	0.875	1.000

Clustering of all four Pieridae species was firmly supported by morphological characters (Figure 4).

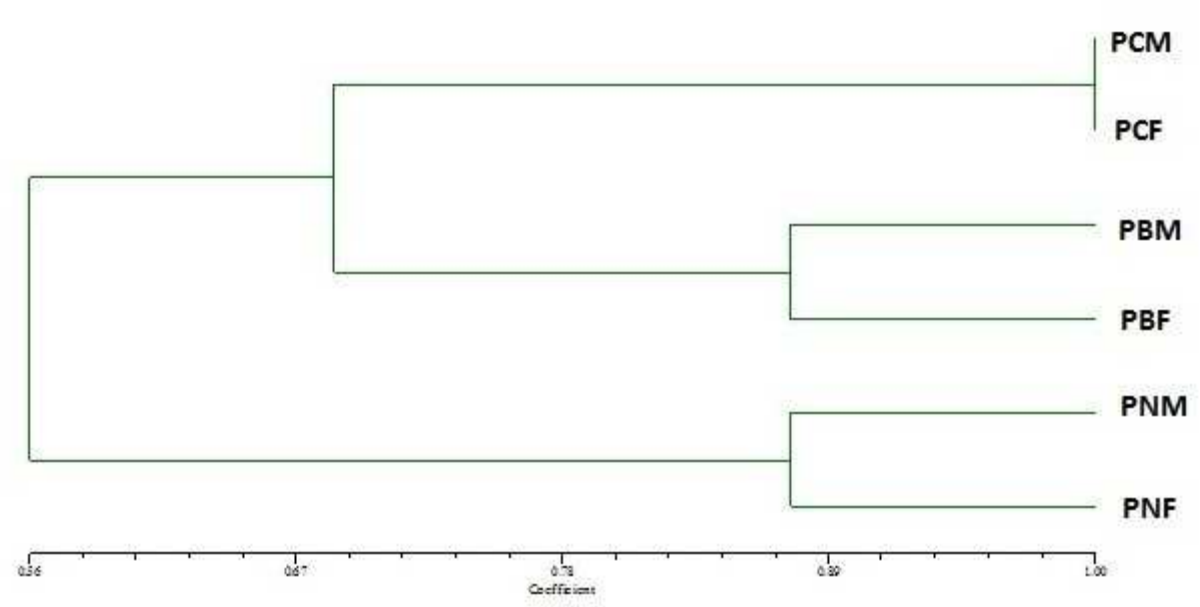

Figure 4
Dendogram of three Pierid species (male and female) based on the morphological data.

(ii) MEGA analysis

The COI gene of the mtDNA was amplified and sequenced from *P. brassicae, P. canidia* and *P. napi* (NCBI GenBank accession numbers - JQ965749, JQ965750 and JQ965751, respectively). Maximum likelihood phylogenetic tree using these sequences and other sequences downloaded from NCBI showed (Fig 5) indicated that the three *Pieris* species under study did not belong to the same clade as compared to a more related species, which in fact is a support for the phylogenetic analysis from the resulting RAPD-PCR technique being observed. Each species form a separate clade from one another, inspite of their similarity morphologically.

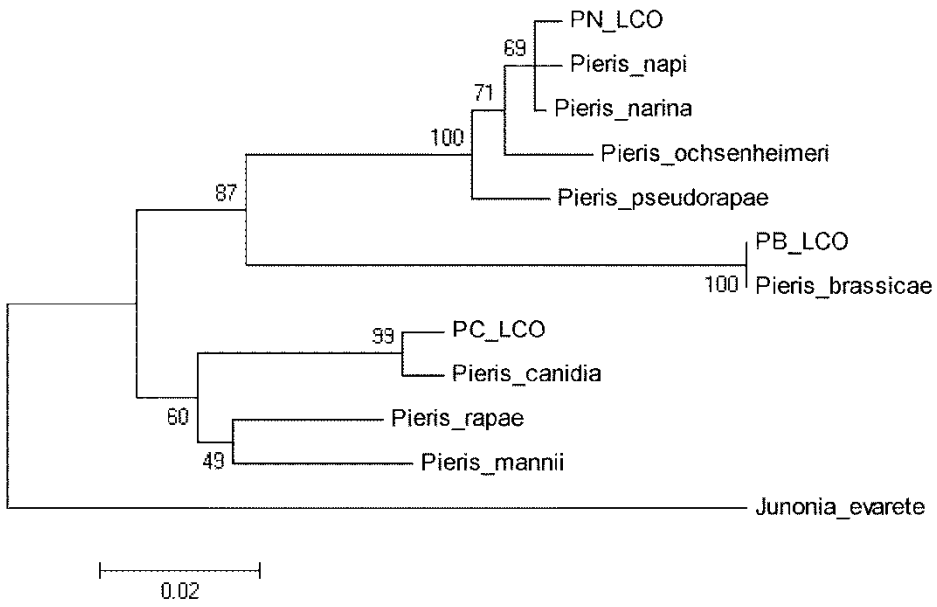

Figure 5

Phylogenetic tree of the Mitochondrial CO1 genome sequences of the three Pierid species (where PN_LCO, PC_LCO and PB_LCO are P. napi, P. canidia and P. brassicae respectively whose samples were obtained from Mizoram) along with 5 different Pieris species and similar species sequences (the three species in study) from the database with Junonia evarete as an outgroup. The tree is based on maximum likelihood method. The number of branches indicates bootstrap values. Bootstrap % refers to the percentage of trees in which the members form a clade.

Because of robustness and simplicity, PCR-based RAPD technique is extensively used in DNA typing of necrophageous insects, gene flow between populations, evaluation of genetic population structure, determination of genetic relationships, phylogenetics in mites and ticks. Sharma *et al*[8] had demonstrated the application of RAPD in discrimination of two Pieridae butterfly species at sex level. Since no DNA sequence information is required to perform RAPDs, it can be widely used for species differentiation**.** According to Wahlberg[13], the utility of mtDNA on its own in assessing the boundaries of traditionally recognized species (eg. Wiens and Penkrot[23]) is suspect. One must combine all possible knowledge, including morphological, ecological and molecular, to understand the species boundaries of groups of very closely related species.

In this study, we showed the genetic and phylogenetic relationship and differentiation of the morphologically similar Pieridae species of butterflies. The three species under study viz, *P. canidia, P. napi and P. brassicae,* through RAPD-PCR technique and COI barcoding are shown to have genetic and phylogenetic relationship as well as specificity for each species, which distinguishes each of them from the species of the same family as shown by the RAPD-PCR banding pattern. This has given them their relatedness as well as differences in evolution. The RAPD technique supported by COI barcoding and morphological analysis have thus indicated that the morphologically similar Pieridae species have discriminating features phylogenetically.

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REFERENCES

1. Ashish, D.T., Sonali, V.P. and Vishal, P.D., Molecular Characterization of Morphologically similar four Pieridae butterflies (Lepidoptera:Insecta) by RAPD-PCR technique. International Journal of Pharma and Bio Sciences 1(2): 163, (2010).
2. Sobti, R. C., Sharma,V. L., Kumari, M., Gill, T. K., Singh, J., Sodhi, M., Mukesh, M., Bansal, M., Arya1, S. and Bisnoi, S., Genetic relatedness of six North-Indian butterfly species (Lepidoptera: Pieridae) based on 16S rRNA sequence analysis. Mole Cellular Bioche 295: 145–151, (2007).
3. Linares, M.C., Soto-Calderón, I.D., Lees, D.C., and Anthony, N.M., High mitochondrial diversity in geographically widespread butterflies of Madagascar: A test of the DNA barcoding approach Molecular Phylogenetics and Evolution 50 : 485–495, (2009).
4. de Silva, D.L., Day, J.J., Elias, M., Willmott, K., Whinnett, A., and Mallet, J., Molecular phylogenetics of the neotropical butterfly subtribe Oleriina (Nymphalidae:Danainae:Ithomiini). Molecular Phylogenetics and Evolution 55 : 1032–1041, (2010).
5. Singh, R., Bajpai, N., and Tewari, R.R., Genetic characterization of Bactrocera (Dacus) flies (Diptera:Tephritidae) Based on RAPD-PCR. International Journal of Pharma and Bio Sciences 2 : 498-503, (2011)
6. Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafaiski, J.A., and Tingey, S.V., DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research 18 : 6531–6535, (1990).
7. Gallusser, S., Guadagnuolo, R., and Rahier, M., Genetic (RAPD) diversity between *Oleria onega agarista* and *Oleria onega* ssp. in north-eastern Peru. Genetica 121 : 65–74, (2004).
8. Sharma, V.L., Bhatia, S., Gill, T.K., Badran, A.A., Kumari, M., Singh, J.J., and Sobti, R.C., Molecular Characterization of Two Species of Butterflies through RAPD-PCR Technique. Cytologia 71(1) : 81–85, (2006).
9. Hebert, P.D.N., Cywinska, A., Ball, S.L., and deWaard, J.R., Biological identifications through DNA barcodes. Proceedings of the Royal Society of London, series B 270 : 313–321, (2003a).
10. Hebert, P.D.N., Ratnasingham, D., and de Waard, J.R., Barcoding animal life: Cytochrome c oxidase subunit I divergences among closely related species. Proceedings of the Royal Society of London, series B (Suppl.) DOI 10.1098/rsbl.2003.0025, (2003b).
11. Blaxter, M., Molecular systematics: Counting angels with DNA. Nature 421 : 122–124, (2003).
12. Evans, W.H., The identification of Indian butterflies. Bombay Natural History Society, (1932).
13. Wynter-Blyth, M.A., Butterflies of the Indian Region. Bombay Natural History Society, (1957).
14. Wahlberg, N., Oliveira, R., and Scott, J.A., Phylogenetic relationships of Phyciodes butterfly species (Lepidoptera: Nymphalidae): complex mtDNA variation

and species delimitations. Systematic Entomology 28 : 257-273, (2003).
15. Kehimkar, I., The Book of Indian Butterflies. Bombay Natural History Society, (2008).
16. Zimmermann, M., Walhberg, N., and Descimon, H., Phylogeny of Euphydryas Checkerspot Butterflies (Lepidoptera: Nymphalidae) Based on Mitochondrial sequence data. Annals of the Entomological Society of America 93 : 347-355, (2000).
17. Roldan-Ruiz, I., Dendauw, J., VanBockstaele, E., Depicker, A., and Loose, M.D., AFLP markers reveal high polymorphic rates in ryegrasses (Lolium spp.). Molecular Breeding 6 : 125–134, (2000).
18. Prevost, A., and Wilkinson, M.J., A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theoretical and Applied Genetics 98 : 107-112, (1999).
19. Milbourne, D., Meyer, R., Bradshaw, J.E., Baird, E., Bonar, N., Provan, J., Powell, W., and Wagh, R., Comparison of PCR-based marker systems for the analysis of genetic relationships in cultivated potato. Molecular Breeding 3 : 127-136, (1997).
20. Rohlf, F.J., NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, Version 2.2, Exeter Software, Setauket, NY, (2000).
21. Mantel, N., The detection of disease clustering and a generalized regression approach. Cancer Research 27 : 209–220, (1967).
22. Tamura, K., Peterson, D., Peterson, N., Stretcher, G., Nei, Masatoshi., and Kumar, S., MEGA5 : Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28(10) : 2731-2739, (2011).
23. Weins, J.J., and Penkrot, T.A., Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). Systematic Biology 51 : 69-91, (2002).