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Morphological and molecular studies of six *Junonia* species of butterflies using RAPD-PCR technique

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ABSTRACT

The genomic DNA of six species of butterflies (*Junonia atlites, J. iphita, J. hierta, J. orithiya J. lemonias* and *J. almanac*), family Nymphalidae, sub-family Nymphalinae, were used for RAPD-PCR analysis using 15 random primers to study the genetic similarity and diversity. A total of 437 bands were scored, of which 357 were polymorphic and the average percent polymorphism was 82.70%. Dendogram constructed using the UPGMA of NTSYS spc2.2 software divided the *Junonia* species into two clades. There is a difference in the branching pattern between the morphological and molecular data, which signifies the need for using molecular tools for taxonomic classification as well as in understanding the evolutionary relationship.

Key words: Butterfly; *Junonia*; RAPD-PCR; morphological characters; UPGMA.

INTRODUCTION

Butterflies are included in biodiversity studies and biodiversity conservation prioritization programmes.[1] India possesses about 1641 species of butterflies representing roughly 9.50% of the total world species. Among butterfly families, Nymphalidae is largest, comprising of 12 sub-families, 40 tribes and 6000 species,[2] of which 296 species are described in India. These are usually medium sized to large butterflies, and are also called brush-footed or four-footed butterflies.

Butterflies are suggested as the key taxa for biodiversity monitoring because they reflect changes in land use patterns, as they are sensitive to and directly affected by any alteration to their habitats, atmosphere, local weather and climate.[3] 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.[3] Recent molecular marker techniques ease the assessment of genetic diversity and facilitate genotyping, classification, inventorying and molecular phylogenetic studies.[4]

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 be-

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tween species and populations.[5] Using RAPD-PCR, genetic polymorphisms and genetic diversity in natural populations between species of Nymphalidae have been studied.[6] Phylogenetic relationships in some genus of nymphalid butterflies were described using different molecular markers.[7] Recently, in India, RAPD-PCR was successfully applied for molecular characterization of butterfly species of the family Pieridae.[8] The main aim of this study is to identify and characterize systematically the status and distribution of *Junonia* species combining both morphological and molecular data and creation of a molecular database for Nymphalidae systematic.[9]

MATERIALS AND METHODS

Collection of butterflies

Nymphalidae butterflies were collected from different parts of Mizoram and were identified by their morphological characters as in Table 1, based of Evans,[10] Wynter-Blyth[11] and Kehimkar.[12]

Preparation of DNA

mtDNA was extracted based on slight modification of Zimmermann *et al.*[13] Legs or tissue from the thorax stored in 70% alcohol were taken in 1.5 ml eppendorf tube. They were macerated and homogenized in 250 µl of extraction buffer containing 50 mM Tris HCl (pH 8.0), 25 mM NaCl, 25 mM EDTA (pH 8.0) and 0.1% SDS. 2 µl of proteinase K (18 mg/ml) was added, mixed gently and incubated at 60°C for at least 3 hr or at 37°C overnight. To the sample equal volume of phenol/chloroform (250 µl) was added and then centrifuged at 13,000 rpm for 5 min and the supernatant transferred to a new tube. 15µl of 5M NaCl and 450 µl of ice-cold ethanol were added, and mixed gently and then placed in freezer for at least 20 min. Then it was again centrifuged at 13,000 rpm for 5 min at cold temperature. Ethanol was poured off without dislodging the pellet, 200 µl of 70% ethanol was added to the pellet and flash spun at 6000 rpm for 1 min and the ethanol was poured off. The pellet was dried at 60°C for 15-20 min. 30 µl of 1X TE buffer was added and the pellet was resuspended by gently flicking the tube and stored at -20°C for further used.

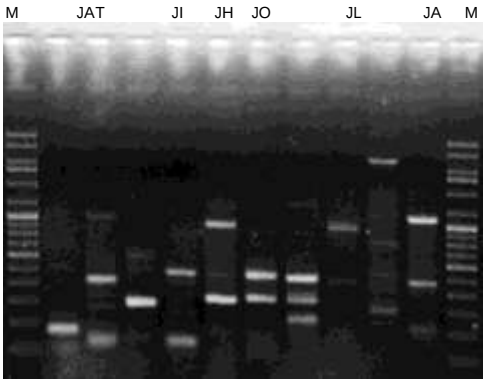
DNA amplification by RAPD-PCR

The DNA was amplified by using 15

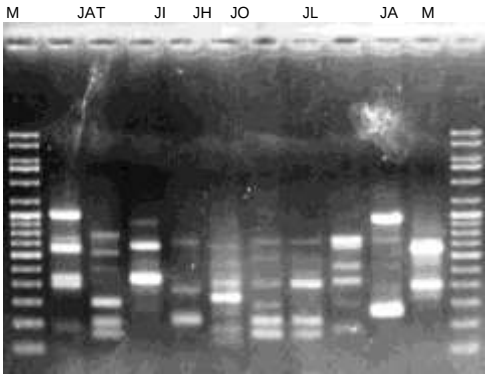
Table 1. Morphological features of six *Junonia* species.

Species	Color of the wing	Eyespots	Wingspan
J. orithya	UPF basal two third black, apex pale brown with white bands, outer discal area below apex and UPH shining blue	Variegated ocelli in both UPF & UPH of S 2 and 5	40-60 mm
J. hierta	Bright yellow, FW apex black bearing yellow markings, dorsum black, costa and termen narrowly black. HW bears prominent oval shining blue patch	Outer discal ocelli in 2 and 5 of both UPF and UPH	45-60 mm
J. iphita	UP pale to dark brown with darker brown bands. FW apex and HW tornus slightly produced. FW apex square-cut and termen concave	UPH with row of small eyespots. UPF with or without small eyespots	55-80 mm
J. atlites	UP with creamy grey with dark brown lines	Complete row of discal eyespots on both wings	55-65 mm
J. almana	UP tawny orange, UPF apex square-cut, not pointed and termen concave. Three narrow black lines along border on UP of both wings	Very large unmistakable on UPH and two smaller eyespots on UPF	60-65 mm
J. lemonias	Brown with black and lemon yellow spots on UP, HW tailed	Ocelli in S 2&5, only those in 2 FW and 5 HW being prominent	45-60 mm

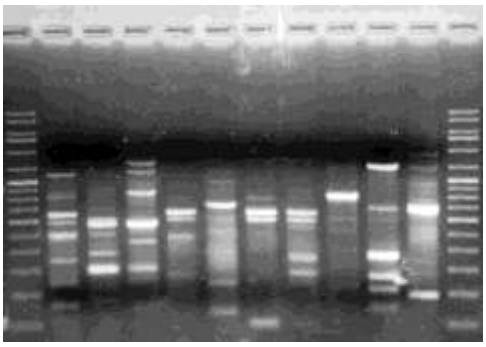
UPF - upper forewing, UPH - upper hindwing, FW - forewing, HW - hindwing, S - space, UP - upper.



Primer opt-4



Primer MA-6



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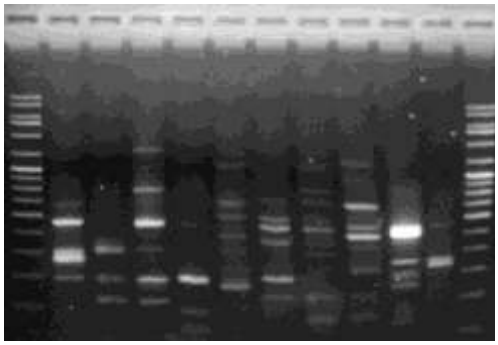
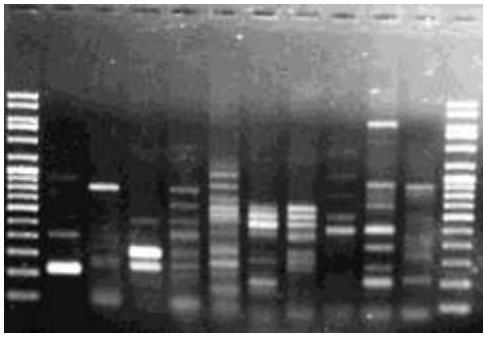
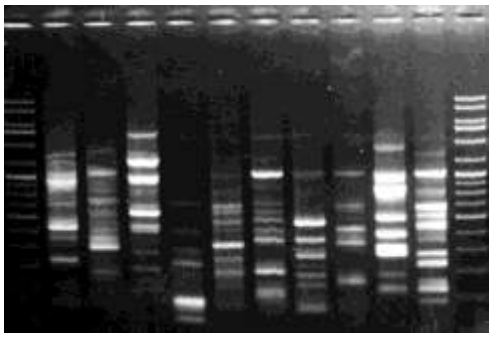


Primer opt – 1



Primer MA –5



Primers MA – 24

Figure 1. RAPD-PCR results using random primers on the DNA extracted from six *Junonia* species. Lane 1: low range ruler plus (M - 100-3000bp). Lane 3: *J. atlites*; lane 5: *J. iphita*; lane 6: *J. hierta*. Lane 7: *J. orithya*; lane 9: *J. lemonias* lane 11: *J. almana*. Lane 2,4,8,10 not to be considered

Table 2. RAPD primer sequences, total bands, polymorphic bands, % polymorphism and polymorphic information content (PIC).

Primer	5′ - **3′ orientation**	Total Bands	Polymorphic Bands	Polymorphism (%)	PIC
MA – 5	TGCGCCCTTC	29	26	89.65	0.31
MA – 6	CTGCTGGGAC	23	20	86.95	0.30
MA – 8	GTCCCGACGA	26	21	80.77	0.30
MA – 10	CACACTCCAG	31	27	87.10	0.31
MA – 13	CCAGATGCAC	34	25	73.52	0.32
MA – 14	TGGGCGTCAA	19	17	84.48	0.28
MA – 15	GGCGGTTGTC	27	25	92.60	0.30
MA – 18	TGGTCAGTGA	35	27	77.14	0.32
MA – 23	AGGCGATAAG	42	33	78.60	0.32
MA – 24	TGACCCGCCT	39	31	79.50	0.30
MA – 26	GACGTGGTGA	22	19	86.40	0.29
OPT – 1	GGGCCACTCA	19	17	89.50	0.35
OPT – 4	GTGTCTCAGG	14	12	85.71	0.31
OPT – 5	GGGTTTGGCA	43	31	72.10	0.31
OPB – 12	CCTTGACGCA	34	26	76.50	0.31
TOTAL		437	357	82.70	0.30

Table 3. Similarity coefficient among six *Junonia* species based on morphological features.

Species	*J. orithya*	*J. hierta*	*J. almana*	*J. lemonias*	*J. iphita*	*J. atlites*
J. orithya	1.000					
J. hierta	0.933	1.000				
J. almana	0.867	0.800	1.000			
J. lemonias	0.467	0.400	0.333	1.000		
J. iphita	0.333	0.267	0.333	0.467	1.000	
J. atlites	0.333	0.267	0.467	0.467	0.867	1.000

Table 4. Similarity coefficient among six *Junonia* species based on RAPD data.

Species	*J. atlites*	*J. iphita*	*J. hierta*	*J. orithya*	*J. lemonias*	*J. almana*
J. atlites	1.000					
J. iphita	0.651	1.000				
J. hierta	0.628	0.631	1.000			
J. orithya	0.637	0.645	0.645	1.000		
J. lemonias	0.620	0.617	0.634	0.654	1.000	
J. almana	0.595	0.620	0.570	0.617	0.584	1.000

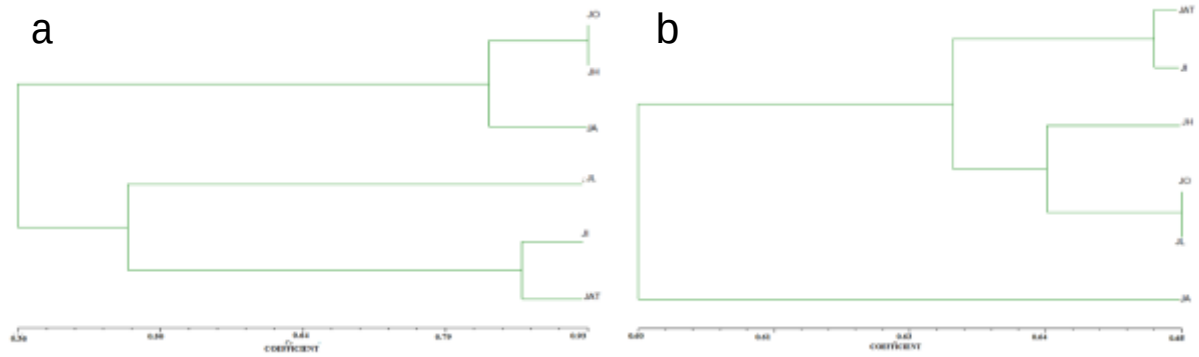


Figure 2. Dendogram of six *Junonia* species based on (a) morphological and (b) RAPD-PCR data. Grey pansy – *J. atlites* (JAT); chocolate pansy – *J. iphita* (JI); yellow pansy – *J. hierta* (JH); blue pansy – *J. orithya* (JO); lemon pansy – *J. lemonias* (JL); peacock pansy – *J. almana* (JA).

RAPD random primers. Primers were obtained from Biosciences, India. 10 µl of reaction mixture contained 1 µl of 10X PCR buffer, 1 µl MgCl (25 mM), 0.2 µl dNTPs (2 mM), 0.3 µl of Taq polymerase (3 U/µl), 0.8 µl of BSA (100 pmol/µl), 1 µl of template DNA, primer (10 pmol/µl) and the volume made up with distilled water. The amplification was carried out in thermal-cycler gradient (Eppendorf, India) using the following condition: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation, annealing and extension respectively at 94, 37 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 were documented using the gel documentation system.

Data analysis

RAPD data was used to construct dendogram following NTSYS-pc software. All calculations were done using computer program NTSYSspc22 package. Pairwise similarity matrices were generated by SM (Simple Matching) coefficient of similarity by using SIMQUAL format of NTSYS software. 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.

RESULTS AND DISCUSSION

The amplified fragments were scored manually for their presence (denoted as '1') or absence ('0') for each primer (Fig. 1). Polymorphism was observed among the six *Junonia* species based on the banding patterns and base pairs. Amplification was obtained in all the 15 random primers tested (Table 2). In all 437 bands were produced of which 357 were polymorphic. On an average 29.13 bands per primer were scored and the average percent polymorphism was 82.70%. Primer OPT-5 produced maximum band of 43 and primer OPT-4 with the minimum band of 14. The polymorphic information content of each RAPD-PCR marker was computed as $PIC_i=2_{fi}$ (1-*fi*),where *f* is the frequency of the amplified allele (band present) and (1-*fi*) is the frequency for null allele.[14] The average PIC value was 0.30.

The SM similarity coefficient revealed maximum similarity (0.654) between *J. orithya* and *J. lemonias* followed by (0.651) between *J. atlites* and *J. iphita* and the minimum genetic

similarity observed among the species was (0.570) between *J. hierta* and *J. almana*. For the morphological characters, the SM similarity coefficient calculated revealed maximum similarity (0.933) between *J. orithiya* and *J. hierta* and the minimum similarity between four species (0.867) *J. orithiya* and *J. almana*, *J. iphita* and *J. atlites* (Tables 3&4).

A dendogram constructed using the morphological characters and 15 RAPD primers aren shown in Figure 2. The dendogram based on morphological characters divided the *Junonia* species into two clades, but the genetic distance among them were different. Cluster-I comprises of 3 species *viz*. *J. orithiya, J. hierta* and *J. almana.* Cluster-II comprises of 3 species *viz*. *J. lemonias*, *J. iphita* and *J. atlites.* Cluster-I is sub-divided into two, sub-cluster-I consisting of *J. orithiya* and *J. hierta* and sub-cluster-II consists of *J. almana.* Cluster-II also further sub-divided into two, sub-cluster-I consisting of *J. lemonias* and sub-cluster-II consists of *J. iphita* and *J. atlites.* The dendogram using RAPD data divided the *Junonia* species into two clades. Cluster-I comprises of 5 species *viz*. *J. atlites, J. iphita, J. hierta, J. orithiya and J. lemonias.* Cluster-II comprises of only *J. almana.* Cluster-I is sub-divided into two, sub-cluster-I consisting of *J. atlites* and *J. iphita* sub-cluster-II consists of *J. hierta*, *J. orithiya* and *J. lemonias*.

The present study reveals that RAPD-PCR is extremely useful for rapid identification of genetic polymorphisms in lepidopteran because of reproducibility of the result for each of the species. Since no DNA sequence information is required, RAPD-PCR can be widely used in identification and differentiation of closely related insect species, although large number of random primers is often required.

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