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Genetic variation within two cryptic species of *Cirrochroa* (Heliconiinae: Lepidoptera) by RAPD-PCR technique

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ABSTRACT

The morphological characters of *Cirrochroa aoris* (Large Yeoman) and *C. tyche* (Common Yeoman) are very similar making identification confusing and difficult. The genomic DNA of the two species was subjected to RAPD-PCR analysis with six decamer oligonucleotides, i.e. MA5, MA6, MA8, OPB12, OPT4 and OPT5. All of them produced discrete bands of various lengths revealing genetic variations as well as similarities between the two species. A total of 50 RAPD bands were generated with 45 polymorphic bands. The percentage polymorphism was 90.58% and all the similarity coefficients between the species were less than 0.2. Results showed a high genetic variation between the two cryptic species. Some species specific bands were obtained with these primers which can be considered as diagnostic bands. All the primers also produced species specific bands.

Key words: *Cirrochroa* spp.; genetic diversity; polymorphism; RAPD-PCR; similarity matrix.

INTRODUCTION

The two species of butterfly genus *Cirrochroa*, namely *C. aoris* Doubleday (Large Yeoman) and *C. tyche* C. & R. Felder (Common Yeoman) belong to the subfamily Heliconiinae of the family Nymphalidae. They are both found in south-east Asia and are very similar in their morphology, wing venation and colouration patterns (Fig. 1), which make them difficult to identify and differentiate.[1] The two species are generally identified on the basis of size, wing shape and colour pattern.[2]

The systematic study of Heliconiinae is confusing and controversial where it is likely that some other genera will eventually be moved to a different tribe as they are studied in detail. Several phylogenic and corresponding taxonomic adjustments have been proposed, though each looking reasonable, every one of them is only weakly supported. Even cladistic analyses of the same type of data often yield contradicting results depending on the exact method of evaluation. Ultimately, the reason is that just a fraction of the evolutionary diversity of Heliconiinae has been sampled.[1]

The use of molecular markers is considered best for analysis of genetic diversity and

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Figure 1. The large Yeoman *Cirrochroa aoris* (left) and common Yeoman *C. tyche* (right) butterflies in the natural habitat.

variety identification because there is no effect of stage of development, environment or management practices. Currently, the commonly used molecular markers are PCR based markers such as random amplified polymorphic DNA (RAPD),[3] amplified fragment length polymorphism (AFLP)[4] and microsatellites/ simple sequence repeat (SSR) markers.[5] Of these, RAPD marker is widely used because of its speed, simplicity and amenability to automation, despite some limitations.[6] RAPD is a non-specific marker and therefore, it is easier to use for minor and underexploited species.[7] The present study was conducted to understand the pattern of genetic variability between the two *Cirrochroa* spp. using RAPD-PCR.

MATERIALS AND METHODS

Collection of specimen

Adult butterflies were collected from different parts of Mizoram (23°09'52.35"N, 92° 56'15.27"E, at an elevation between 40-2165 m) using insect collection net and its legs were preserved in 90% alcohol for further molecular studies.

DNA isolation

Butterflies legs were placed in a centrifuge tube and 250 µl of insect extraction buffer (pH 8; 25 mM NaCl, 50 mM Tris-HCl, 25 mM EDTA and 0.1% SDS) was added and homogenized with micro-pestle and forceps. After adding 2 µl of proteinase K (18 mg/ml) the whole content was incubated at 60°C for at least 3 hours or overnight (under a controlled environment). It was then treated with equal volume phenol and chloroform, centrifuged at 13000 rpm for 5 min at 4°C, and the supernatant was decanted. 5 M NaCl (30 µl) and 425 µl of ice-cold absolute alcohol were added and gently mixed, stored in deep freezer overnight. It was again centrifuged at 13000 rpm for 5 min at 4°C and the ethanol was poured off without dislodging the pellets and 200 µl of 70% alcohol was added. Precipitated DNA was pelleted out by flash spin at 6000 rpm for 1min at 4°C and air dried. Pellet was dissolved in 20 µl of TE buffer (1X - 10 mM Tris-HCl and 1 mM EDTA) and stored at -20°C for further use. The DNA in extracted samples were quantified by bio-photometer (Eppendorf, India) and was diluted for further use.[8]

RAPD-PCR

Denaturation was carried out at 92-96°C. Annealing of primer to each original strand for new strand synthesis was carried out between 36-38°C. Extension was achieved where the polymerase adds dNTPs complementary to the template strand at the 3' end of

Table 1. Primers used for RAPD-PCR.

Primer name	**Primer seq 5′-3′**	mer	MW	Tm	% GC	nM	pmole/µl
MA-5	TGCGCCCTTC	10	2954	32	60	68	680
MA-8	GTCCCGACGA	10	3012	39.3	70	50	500
MA-6	CTGCTGGGAC	10	3043	34	70	51	510
0PB-12	CCTTGACGCA	10	2988	37	60	49	490
OPT-4	GTGTCTCAGG	10	3059	20.4	60	58	580
OPT-5	GGGTTTGGCA	10	3099	40.3	60	56	560

the primers. These 3 steps were repeated for about 30 to 35 times in an automated thermal cycler. This resulted in exponential accumulation of the specific DNA fragments. Six primers were used (Table 1). 30 ng/µl of DNA was used as template for each PCR with 10 µl reaction mixture and PCR reaction was performed in the thermocycler for different six random primers. The reaction set up for amplification is shown in Table 2.

Table 2. PCR reaction set up.

Components	Stock	Final conc	Volume for 10 µl setup
MilliQ water			5.07 µl
BSA	10 mg	10 mg	0.8 µl
dNTP mix	50 mM	2 mM	0.4 µl
MgCl2	25 mM	3 mM	1.2 µl
Taq buffer	10X	1X	1 µl
Primer	560 pmol/µl	10 pmol/µl	0.19 µl
DNA	30 ng/µl	30 ng/ µl	1 µl
Taq DNA pol	3 Unit	1 U	0.34 µl

Agarose gel electrophoresis

Agarose gel (0.8% for genomic and 1.5% for PCR products) was prepared with 1X TAE buffer (40 ml) and stained with 2 µl of ethidium bromide (20 mg/ml). Samples were loaded with loading dye (2 µl) and DNA fragments were electrophorized at 50-100 volts along with DNA ladder (long range ruler) and were visualized in the UV trans-illuminator.

Data analysis

For RAPD analysis, data was scored as '1' for the presence and '0' for the absence of band, which allowed manual binary analysis by comparison of the results obtained for the different species. Only distinct and polymorphic bands were recorded and used in the analysis. The NTSYS-pc software ver. 2.02 was used to estimate genetic similarities with the Jaccard's coefficient.[9] The matrix of generated similarities was analyzed by the unweighted pair group method with arithmetic average (UPGMA), using the SIMQUAL, SIMGEND and SAHN clustering module.[10]

RESULTS AND DISCUSSION

From gel photographs, a series of discrete bands were obtained on amplification of DNA samples of two species of butterflies with six primers, i.e. MA5, MA6, MA8, OPB12, OPT4 and OPT5 (Fig. 2). All the primers produced a large number of bands with different intensities and banding patterns 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. With all the primers, common as well as unique bands were obtained for

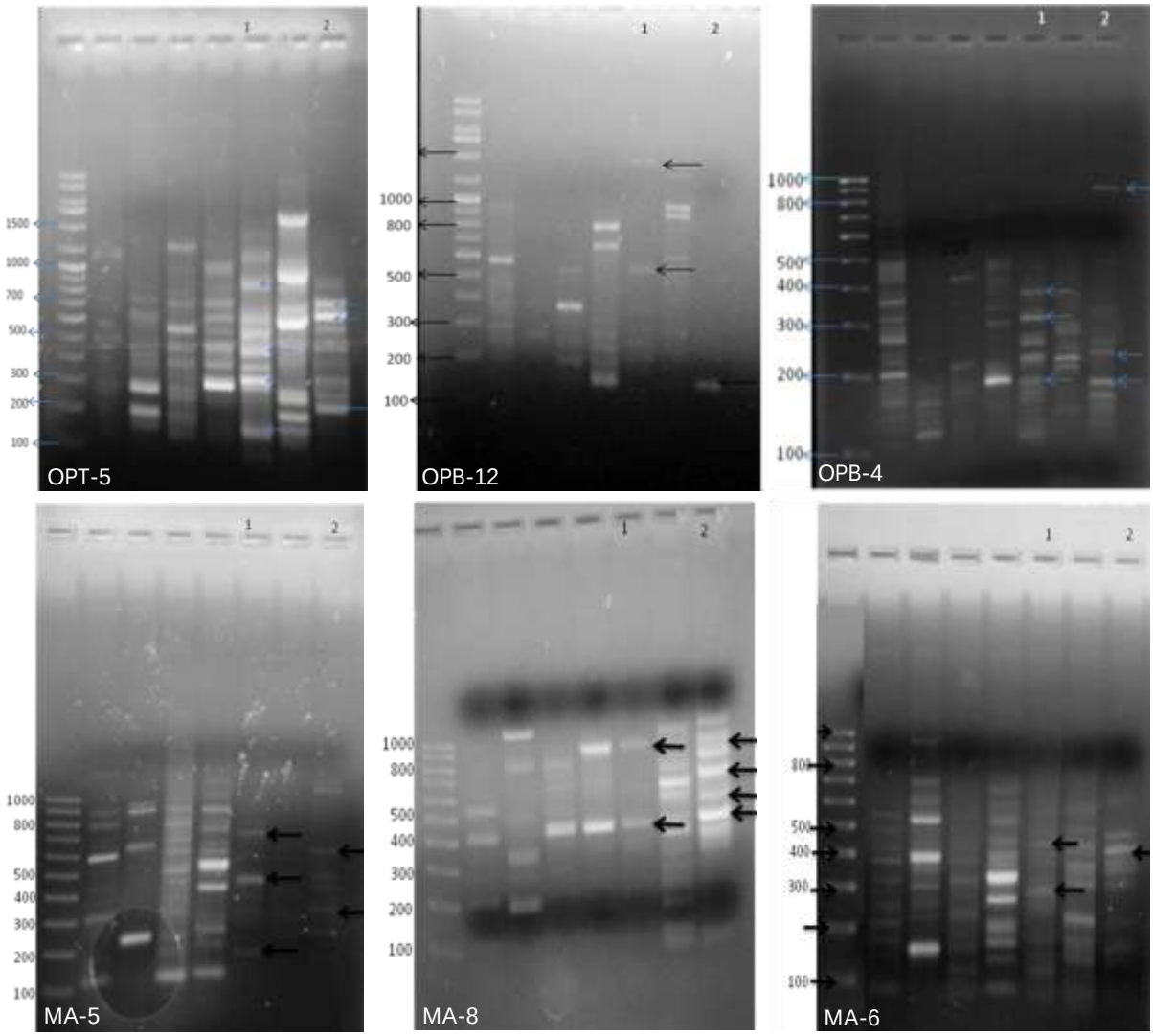

Figure 2. Photograph of agarose gel showing bands in amplified products with six random primers (*C. aoris* lane1 and *C. tyche* lane 2); arrows indicate markers and highlight the distinct bands.

Table 3. Percent polymorphism, polymorphic information content, resolving power and marker index of RAPD primers used in PCR.

Primers	Polymorphic bands	Total no. of bands	% polymorphism	PIC	RP	MI
MA-6	8	8	100	0.5	7	0.5
MA-5	12	12	100	0.5	8	0.5
MA-8	9	11	81	0.5	9	0.5
0PT-5	7	8	87.5	0.5	7	0.5
OPB-12	3	3	100	0.5	3	0.5
OPT-4	6	8	75	0.5	4	0.04
TOTAL	45	50	90.58	0.5	6.34	0.5

Table 4. Similarity coefficient of two *Cirrochroa* species calculated by using NTSYS pc2.2 (SIMQUAL).

Coefficient	similarity
Nei and Li	0.200
Simple matching	0.191
UN1	0.191
UN3	0.237
UN4	0.210
RT	0.106
Jaccard's	0.116
RR	0.106
DICE	0.208
K1	0.132
K2	0.228
O	0.218

both species but the banding patterns were very different in all the primers. Three primers (MA-5, MA-6 and OPB-12) showed 100% polymorphism and primer OPT-4 showed the least polymorphism (75%). The total percent polymorphism, PIC, RP and MI with all the six primers were 90.58%, 0.5, 6.34 and 0.5, respectively (Table 3). SIMQUAL cluster analysis revealed similarity coefficients were not more than 0.2 between the two species and dissimilarity was found out to be 1.52 by using SIMGEND (Table 4).

The presence of species-specific bands these bands suggested the interspecific genetic relatedness between two species (Table 5). These conserved regions provided diagnostic profiles. Similar diagnostic markers have also been reported in five species of butterflies namely, *Pieris canidia, P. brassicae, Ixias pyrene, I. marianne* and *Pontia daplidice*.[11] All the primers in the species studied amplified the species specific bands suggesting the genetic variations in the two species. There were, however, some bands common to both the species (Fig. 4) indicating intraspecific genetic relatedness. The present results were in accordance with those of Sharma *et al*.[11,12]

PCR-based RAPD has been extensively used in DNA typing of necrophageous insects,[13] gene flow between populations and phylogenetic relationship of species.[14] Sharma *et al*.[11,12] had demonstrated the application of RAPD in discrimination of two Pieridae butterfly species at sex level.

In this study, RAPD primers were able to detect high genetic diversity between the two cryptic species of *Cirrochroa* butterflies showing high percentage of polymorphism despite their similar morphological appearances and comparatively less values of similarity coefficients. RAPD, therefore, appears to be useful in differentiating species and subspecies. However, there is need for high level of standardization in RAPD to achieve reliable and comparable results due to poor reproducibility of RAPD markers. Nevertheless, RAPD is still considered a quick and easy assay for estimation of genetic diversity in comparison with other classes of molecular markers.

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Table 5. Some species specific bands (bp) obtained with different primers in two species of *Cirrochroa.*

Species	Primer					
	MA-8	MA-5	MA-6	OPB -12	OPT – 5	OPT-4
C. aoris	900, 400	750, 470	450, 300	1400, 510	450, 300	390, 310
C. tyche	1000, 700, 500	600, 310	400	150	700, 600	900, 250

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