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Morphological and molecular diversity of Pieridae butterfly *Eurema hecabe hecabe* (Linnaeus) of Andaman & Nicobar Islands revealed by DNA markers

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Abstract

The present studies aimed to document butterflies belonging to Pieridae family and investigate the extent of genetic diversity in *Eurema* spp in South Andaman, Andaman and Nicobar Islands, India. Molecular characterization of morphologically similar pieridae butterfly; Common grass yellow (*Eurema hecabe hecabe*) of South Andaman was carried out using RAPD and ISSR markers. A total of 250 bands were scored with 14 RAPD primers, of which 28 were polymorphic and the percentage of polymorphism was 11.76%. Similarly, a total of 314 bands were scored with 12 ISSR primers, of which 26 were polymorphic and the percentage of polymorphism was 11.46%. Dendrogram based on average similarity coefficient of 24 primers grouped the population into two distinct clusters. Genetic similarities between these species are discussed on the basis of morphological characters and ISSR data. This shows a bright possibility of the use of ISSR & RAPD-PCR in molecular profiling and identification of Butterfly species of Andaman Islands.

Key words: Polymorphism, Common grass yellow, *Eurema hacabe hecabe,* microsatellites, Lepidoptera, molecular markers, Andaman and Nicobar Islands.

Introduction

Lepidoptera is a very large order that includes some of the most beautiful species and some of the most economically important pests in the class Insecta (Wynter-Blyth 1957). Within the order Lepidoptera, family Pieridae is extensively distributed throughout the world and contains about 1,200 species and 60 genera, out of which 109 species occur in India. The Common grass yellow butterfly (*Eurema hecabe hecabe)* (Lepidoptera: Piridaea),having fairly small butterflies are readily recognised by their bright yellow wings and their habit of gathering in small groups on patches of damp sand or soil. They are distributed almost all over the Oriental, Australian and Afro tropical regions and extend into the temperate zone of the eastern palaearctic region such as Japan and India. Immense role of butterfly in pollination, also contribute to biodiversity maintenance (Woodhull Steve 2005).

The butterfly fauna of the Andaman and Nicobar Islands (Fig.1) has a high degree of endemism showing more than 50% at the subspecies level (Khatri 1993). Andaman and Nicobar Islands show closer affinity to the Indo-Myanmar's-Thai flora, while the Nicobar group of islands is closer to the flora of Malaysia-Indonesia. These islands represent one of the richest repositories of biodiversity in the whole of south and south-east Asia and harbour a wide range of insect fauna which also includes butterflies (Lepidopetra). The archipelago in Indian Ocean is situated around 1100-1300 km away from continental India and also fragmented from each other by sea. The islands have typical tropical maritime climate with prolonged rainy season spanning from May to December and short dry period from January to April. Minimum and maximum temperature in islands ranges from 25 to 32 ^{0}C, relative humidity from 65 to 92 % and wind movement from 15 to 55 km per hour and higher incidence of ultra-violet rays. The climatic factors along with geographical speciation favour rapid evolution which might happen in butterflies too in these Islands (Evans 1932, Ferrar 1951).

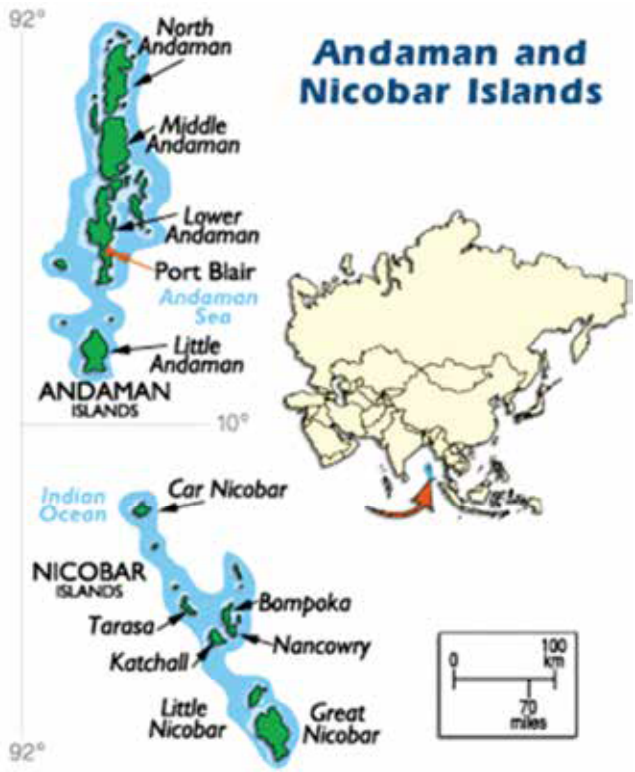

Fig.1. Pictures shows the Geographical location and details of Andaman and Nicobar Islands.

The butterfly fauna from these islands includes 116 genera, 215 species and 230 subspecies (Veenakumari *et al.,* 2008, Khatri and Khazan Singh 2004). The long isolation of these islands from mainland, India and undisturbed ecosystem provides an optimal condition for the preservation and evolution of many local evolved species. Andaman and Nicobar Islands (India) have been included in the list of 19 'hot spots' by the International Union for Conservation of Nature (IUCN) and hot spot refers to an area of ocean which deserves special protection because of wild life and significant wild life habitats(Anonymous 2013). In this regard butterflies abundance also points towards absence of ecological pollution. Though 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 (Haribal 1992, Kehimkar 2008). Morphological identification of butterflies is a difficult task, since basic knowledge on the distribution and dynamics of diversity is mainly lacking. Molecular techniques provide an important tool that facilitates the assessment of genetic diversity and aid genotyping, classification, inventorying and phylogenetic studies (Breyne *et al.,* 1997, Cruickshank 2002, Gupta et *al.,* 1994). RAPD and ISSR markers have been successfully applied to study genetic structure of endangered populations, interspecific study and gene flow between butterflies' populations (Luque *et al.,* 2002, Tiple *et al.,* 2009).

Pieridae, the whites and yellows are a family of butterflies of moderate or small size. The common names refer to the two predominant colours found on the wings of these butterflies along with markings in black. Of the 1051 species of pierids occurring in the world, 81 species in 21 genera are found in India. Andaman and Nicobar Islands have an extremely diverse terrain, climate and vegetation which host the biodiversity hotspots. From these islands only thirteen species were reported by Veenakumari and others from eleven genera from two sub-families, pierinane and coliadinae (Arora & Nandi 1982, Veenakumari *et al.,* 2008, Khatri and Khazan Singh 2004). The work has been reported earlier on this aspect and this is first attempt to analyse intraspecific genetic diversity in common grass yellow butterfly(Fig.2) through DNA based marker in the Bay Islands.

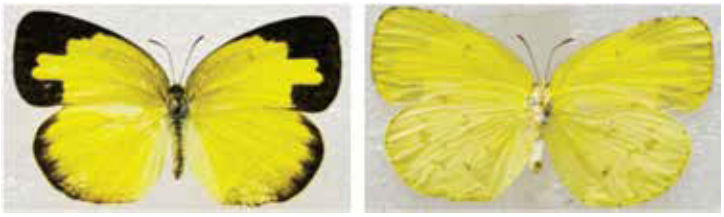

Fig.2. Pictures shows the *Eurema hecabe hecabe* of Andaman and Nicobar Islands.

Table. 1. Morphological character analysis data of the butterfly *Eurema hecabe hecabe* spp.

S.No	Wing span (mm)	Forewing			Hind wing			Length of antenna (mm)	Body length (mm)
		Length (mm)	Width (mm)	Irregular black band	Length (mm)	Width (mm)	Underside reddish brown markings		
1.	36	22	18	Present	20	18	Present	11	18
2.	42	25	19	Present	23	18	Present	12	19
3.	45	26	18	Present	23	18	Present	10	18
4.	38	25	15	Present	23	18	Present	09	16
5.	44	26	19	Present	22	18	Present	10	18
6.	36	25	18	Present	22	18	Present	10	18
7.	46	26	19	Present	23	17	Present	11	17
8.	42	24	18	Present	23	18	Present	10	18
Av.	41.12	24.87	18.00	Present	22.37	17.87	Present	10.37	17.75

Materials and methods

The census procedure used for this study was 'transect recording' or the Pollard Walk (Pollard 1975, Royer *et al.,* 1998). Transects of a known length were walked for identifying local species of adult butterflies. Butterflies resting on plants and those in flight were counted and identified. If an exact determination of the species was not possible, an insect sweep net was used to capture those butterflies in question to facilitate field identification. Only those species that could not be identified in the field were killed and later identified in the laboratory using the available literature and assistance of specialists in the identification of Lepidoptera (Makris 2003, Tolman and Lewington 1997). For the study of genetic relation and fingerprinting analysis eight morphologically similar adult common grass yellow butterflies (*Eurema hecabe hecabe)* (Fig.2) (Table.2) were collected from research farms of ICAR-Central Island Agricultural Research Institute, Port Blair, South Andaman.

Table. 2. RAPD and ISSR primers sequences, number of amplified fragments and olymorphic loci (%) for all the primers.

Sl.No.	Primer (RAPD)	Sequence	Total No. of bands	Average bands	Polymorphic bands	% of polymorphism	PIC
1.	OPAB14	AAGTGCGACC	39	4.875	2	5.12	0.476
2.	OPX01	CTGGGCACGA	08	2.666	2	25.0	0.218
3.	OPX02	TTCCGCCACC	25	3.125	1	4.0	0.476
4.	OPAC04	ACGGGACCTG	14	2.833	3	21.42	0.390
5.	OPM06	CTGGGCAACT	21	2.333	4	19.0	0.440
6.	OPX18	GACTAGGTGG	12	6.000	0	0.0	0.304
7.	OPAM04	GAGGGACCTC	22	2.750	3	13.0	0.451
9.	OPAG8	GGTGGCCAAG	29	4.142	1	3.44	0.495

10.	OPB01	GTTTCGCTCC	11	1.833	3	27.2	0.292
11.	OPX08	CAGGGGTGGA	13	2.600	1	7.69	0.323
12.	OPN03	GGTACTCCCC	21	4.200	1	4.76	0.440
13.	OPA02	TGCCGAGCTG	15	3.750	1	6.66	0.358
14.	OPC20	ACTTCGCCAC	20	2.000	6	30.0	0.430
15.	UBC 807	$(AG)_8T$	13	2.600	2	15.38	0.323
16.	UBC 842	$(GA)_8G$	10	2.500	1	10.0	0.263
17.	UBC 827	$(AC)_8G$	12	2.000	4	33.33	0.304
18.	UBC 812	$(GA)_8A$	16	3.200	1	6.25	0.375
19.	UBC 823	$(TC)_8C$	20	2.500	3	15.0	0.390
20.	UBC 836	$(AG)_8YA$	16	2.288	2	12.5	0.375
21.	UBC 857	$(AC)_8YG$	23	3.833	2	8.69	0.460
22.	ISSR 24	$(GA)_6CC$	36	4.000	12	33.33	0.492
23.	ISSR 11	$(CT)_8RC$	19	1.727	6	31.5	0.417
24.	ISSR 14	$(GACA)_4$	26	2.363	3	11.5	0.482

Total genomic DNA was extracted from the legs/ half abdomen of fresh samples by C-TAB method with slight modifications (Nishiguchi *et al* 2002, Murray & Thomson 1980). The quantitation of DNA in RNA-free sample was done using UV spectrophotometer. Polymerase chain reaction (PCR) was performed in final volume of 20 µl containing 10x assay buffer, 2.5 mM dNTPs, 1.5units of *Taq* DNA polymerase, 10 pmols/reaction ISSR primer / RAPD primer and 100 ng of template DNA. ISSR analysis was done with 12 primers of ISSR series (Clonitec) and RAPD (series obtained from OPERON TECHNOLOGIES Inc. Alameda Calif) analysis was done with 14 primers. The PCR was performed by initial denaturation at 94°C for 5 min. followed by 45 cycles of denaturation at 94°C for one min., annealing at 37°C for one min., extension at 72°C for two min. and final elongation at 72°C for 7 min. For ISSR analysis the annealing temperatures were taken as suggested by manufacturing/ synthesising company. The PCR products were run on 1.2% agarose gel prepared in 1 × TAE buffer containing 0.5 µg/ml of the ethidium bromide at 80 V for 2.0 h. The gel was photographed under UV-transilluminator (Fig.3&4). All the genotypes were scored for presence and absence of the ISSR and RAPD bands. The 0/1 matrix was used to calculate similarity as Jaccard's coefficient using SIMQUAL subroutine in similarity routine. The resultant similarity matrix was employed to construct dendrogram using SAHN based UPGMA to deduce genetic relationship (Rohlf 1999).

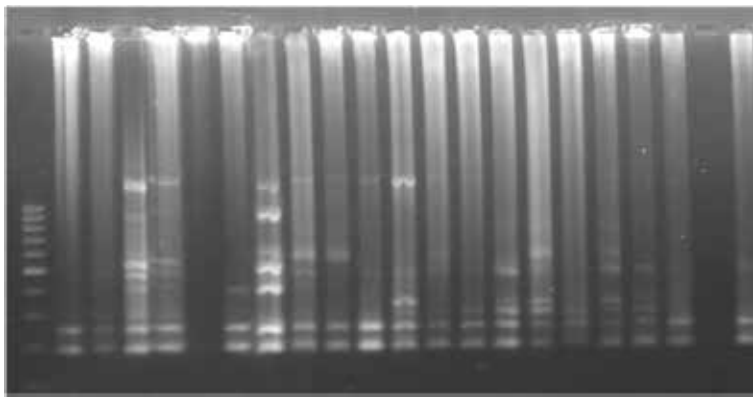

Fig.3. PCR profile of butterfly, Common grass yellow (*Eurema hecabe hecabe*) using ISSR primers.

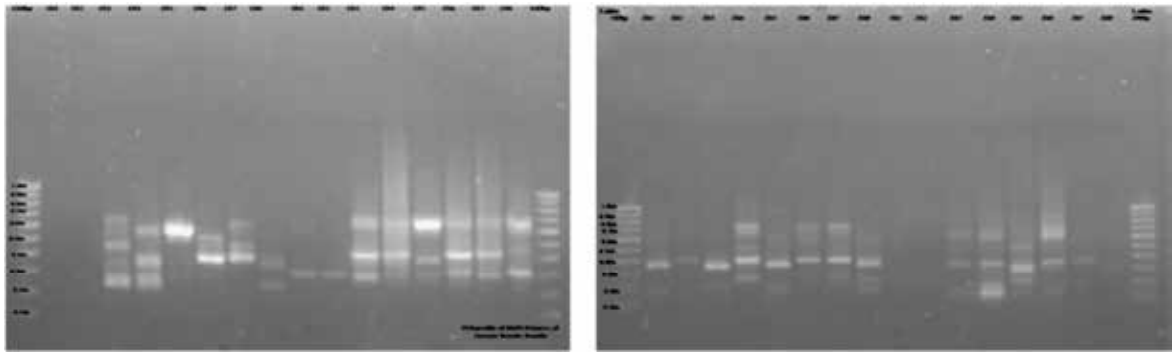

Fig.4. PCR profile of butterfly, Common grass yellow (*Eurema hecabe hecabe*) using RAPD primers.

Results and Discussion

Twenty-four ISSR and Twenty-four RAPD primers were used to study the genetic diversity among *Eurema hecabe hecabe*. Among 24 ISSR primers 10 primers showed amplification and a total of 191 amplicons, of which 36 were found to be polymorphic (11.46%). Primer ISSR24 and ISSR14 produced maximum number of amplicons, while primer UBC827 and UBC842 produced minimum number of amplicons (Table 2). Similar results were obtained with 14 RAPD primers which produced 250 amplicons, of which 28 were polymorphic and the level of polymorphism was 11.2%.

Table. 3. Similarity index among *Eurema hecabe hecabe spp*. of south Andaman based on RAPD and ISSR analysis

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.000																			
2	0.782	1.000																		
3	0.826	0.956	1.000																	
4	0.782	0.826	0.869	1.000																
5	0.782	0.739	0.782	0.739	1.000															
6	0.695	0.826	0.782	0.826	0.652	1.000														
7	0.782	0.826	0.782	0.826	0.739	0.826	1.000													
8	0.652	0.869	0.826	0.782	0.695	0.869	0.782	1.000												
9	0.782	0.869	0.826	0.826	0.739	0.826	0.826	0.695	1.000											
10	0.652	0.782	0.739	0.782	0.608	0.782	0.695	0.739	0.695	1.000										
11	0.739	0.869	0.826	0.869	0.782	0.782	0.782	0.826	0.782	0.826	1.000									
12	0.782	0.739	0.782	0.739	0.913	0.739	0.739	0.695	0.826	0.695	0.695	1.000								
13	0.739	0.782	0.826	0.782	0.695	0.782	0.695	0.652	0.782	0.652	0.739	0.695	1.000							
14	0.695	0.739	0.695	0.739	0.652	0.739	0.739	0.695	0.826	0.782	0.782	0.739	0.608	1.000						
15	0.695	0.826	0.782	0.826	0.739	0.739	0.826	0.695	0.913	0.782	0.869	0.739	0.695	0.913	1.000					
16	0.695	0.826	0.782	0.739	0.739	0.739	0.826	0.695	0.913	0.782	0.782	0.739	0.695	0.826	0.913	1.000				
17	0.565	0.782	0.739	0.608	0.608	0.695	0.695	0.739	0.782	0.652	0.652	0.695	0.565	0.782	0.782	0.782	1.000			
18	0.565	0.608	0.565	0.608	0.608	0.608	0.695	0.565	0.695	0.652	0.652	0.695	0.565	0.782	0.782	0.695	0.826	1.000		
19	0.391	0.608	0.565	0.521	0.434	0.521	0.434	0.565	0.521	0.739	0.565	0.521	0.478	0.608	0.608	0.608	0.652	0.565	1.000	
20	0.478	0.608	0.652	0.521	0.521	0.521	0.521	0.565	0.695	0.565	0.478	0.608	0.478	0.608	0.608	0.695	0.826	0.652	0.739	1.000

The dendrogram generated by UPGMA (Fig. 5) differentiate 4 to 39% similarity and 61 to 96% diversity between the accessions. The dendrogram clearly divided into two major clusters *i.e.* cluster one had genotype of specimen 1.5,12,2,3,4,11,6,8,7,9,15,16,14,10 and 13 showing high similarity of 72%. Cluster two had genotype of specimen 17, 20, 18 and 19 differentiating at 73% similarity coefficient. The accessions 2 and 3 were showing highest similarity 96% and the accession 19 showing 65% similarity with other accessions.

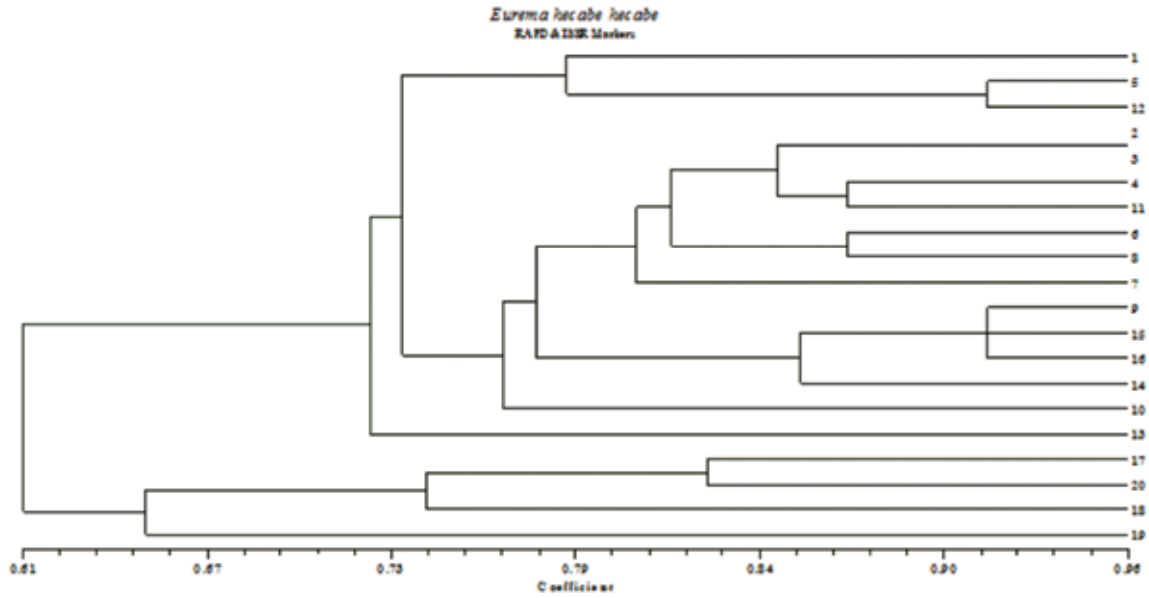

Fig. 5. Dendrogram showing genetic diversity amongst Common grass yellow (*Eurema hecabe hecabe*) genotypes by RAPD and ISSR primers.

The RAPD and ISSR primers used in the present study respectively yielded 28 and 36 polymorphic markers that discriminated twenty genotypes into two main clusters. The number of total polymorphic and discriminate fragments percent was found to be almost similar for ISSR and RAPD marker. The results have demonstrated that both ISSR and RAPD markers are suitable for characterization and assessment of the butterfly species and there is no significant difference in the genetic diversity within the species. This low intra-specific diversity could be due to high degree of gene flow in population through random mating without barrier. The close relationship across the species might be explained by either historical relationship in sharing common ancestral alleles or more likely geographical proximity and large population size which favour genetic interchange (Abbot 2001, Hoole *et al* 1999). The two marker systems, ISSR and RAPD used in the present study have also been used as effective tools to evaluate genetic diversity and phylogenetic relationships in different species of butterflies (Sharma *et al*, Sobti *et al*, Sharma *et al* 2003). Since no such report on genetic diversity using molecular markers was available in these islands for butterfly species, the present study revealed the nature and extent of intra-specific genetic diversity. The results of the present study can be harnessed as a starting point to determine the level of intra and inter-specific genetic diversity and to detect hybrids among these butterfly species in future in the perspective of geographical isolation of Islands.

Conclusions

Molecular and morphological analyses of the pieridae butterfly species *Eurema hecabe hecabe* revealed that morphological indicators were found to be less informative. More differences could be revealed from the molecular study and they were found to be reliable for the differentiation of the species *Eurema hecabe hecabe*. These results will pave the way for in depth studies on the characterization of the species *Eurema hecabe hecabe* which will eventually facilitate documentation for the development of bar-coding of the elite local species in the pristine Andaman and Nicobar Islands

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