International Journal of Zoology Studies
www.zoologyjournals.com
ISSN: 2455-7269
Received: 26-08-2023, Accepted: 12-09-2023, Published: 27-09-2023
Volume 8, Issue 3, 2023, Page No. 89-93

Evolutionary relationship between butterfly species of family Nymphalidae using RAPD-PCR

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Abstract
The present study was undertaken to evaluate genetic diversity and evolutionary relationships among 16 Nymphalidae species belonging to 4 subfamilies *viz*., Nymphalinae Danainae, Satyrinae and Biblidinae from regions of Amravati. The 9 RAPD markers were screened and the genetic diversity in the form of dendrogram based on binary matrix data set was inferred. Seven of the nine markers generated polymorphic bands, with an average of 22.14 bands per primer scoring 100% polymorphism. The resolution of phylogenetic tree was inferred by Maximum Parsimony and Neighbor-joining methods. The dendrogram exhibits species clustering pattern. Based on binary data matrix the genetic diversity of the species was assessed and it has been observed that numerous taxa exhibit inter-species sister clades that demonstrated shared ancestry. Taxa *V. cardui*, E. core, and *J. lemonias* formed outgroup, showing evidence of distant resemblance to other species.

Keywords: Phylogenetic relationship, Nymphalidae species, Brushfooted butterflies, molecular markers, RAPD

Introduction

The order Lepidoptera is the largest order in the class Insecta, with 174,250 species that serve the terrestrial ecology (Mallet, 2007) [14], 17,000 of which are butterfly species, the most alluring groups of insects in the globe. 1501 species of butterflies have been recorded in India, of which 521 fall under the Nymphalidae family (Kehimkar, 2008) [8]. Numerous studies on butterfly diversity undertaken in Maharashtra have revealed 277 species, 90 of which are members of the Nymphalidae family (Kasambe, 2016) [10]. The butterflies have club shaped filiform antennae, which is covered with olfactory scales for smell (Vincent and Carde, 2009) [19]. They have mandibulate mouthparts for biting and grinding food in caterpillars and siphoning mouthparts for sucking the nectar in adults (Krenn *et al.*, 2006) [11].

Butterflies are the most important pollinators contributing in growth and expansion of flora of tropical regions which represents high abundance and species diversity of insects. (Bonebrake *et al.*, 2010) [2]. They are fragile to changing environment hence, are referred as the key taxa for biodiversity monitoring. They are a crucial link in the food chain for predatory insects, birds, and reptiles. Several studies revealed that habitat specificity is directly linked to the availability of host plants for larvae and adults (Grossmueller *et al.*, 1987; Keil, 1997) [6, 9]. Thus, to keep a check on richness or destruction caused to environment there arises an urgent need to conserve butterflies (Wynter-Blyth, 1957) [21].

Among butterfly systematists the number of families and the relationships of species group within these families have continued to be a source of dispute (Vane-Wright and Ackery, 1984) [17]. This is fuelling epistemic curiosity within researchers to explore different morphological or molecular based character parameters in constructing a phylogenetic tree. Classification of closely related species based on morphological features can mislead correct identification as mimicry is seen in such population. RAPD is one of the technologies that provides a powerful tool to improve species identification and to better understand genetic variability (Sivasankaran *et al*., 2013) [16]. RAPD technique helps in gaining large number of genetic markers using arbitrary oligonucleotide primers (Bardakci, 2001) [1]. Mapping morphological characters onto phylogenetic trees (Zhang *et al.*, 2008) [22] or molecular phylogenetic studies highlighting adaptive phenotypic variation provides valuable insights into the related evolutionary mechanisms. The relationships can either be monophyletic, paraphyletic or polyphyletic (Hennig, 1966) [7]. Monophyletic has all descendants from same ancestor, paraphyletic has last descendants with common ancestor and polyphyletic shows different ancestors (Kitching *et al.*, 1998) [12].

Material and Methods

Present investigation was carried out to create a molecular data for Nymphalidae systematic by considering individuals of more than one genus in a family.

1. Survey, collection and identification

The study areas, which include the Masod National Green Corps Park Garden, the GVISH Apsara Garden, the Late Hribhau Kaloti Garden of Wadali Talav, Sant Gadge Baba Amravati University, and the Chhatri Talav Garden in Amravati, are all located in the Amravati district of Maharashtra situated at $20^0$55'45.95" North to $77^0$45'32.87" East (Maps of India.com). The sites show diverse climatic conditions, favourable for host plant of butterfly species sustaining on them. Initially habitat survey of five distantly located study areas in Amravati city was done by observing abundance of commonly occurring butterfly host plants (Wadatkar and Kasambe 2009) [20].

The first decreased pair of legs' characteristic familial trait served as the basis for the identification of Nymphalidae family. Further, by consulting important publications

written on Butterflies inhabiting India by Isaac Kehimkar (2008) [8], Wynter-Blyth (1957) [21] other species were discovered by the presence of particular other traits like upper and under wing pattern, eye spots and more.

The butterflies namely- *Junonia lemonias, Ariadne Ariadne, Byblia ilithyia, Euploea core, Tirumala limniace, Danaus chrysippus, Danaus genutia, Vanessa cardui, Hypolimnas misippus, Hypolimnas bolina 1*(male), *Hypolimnas bolina 2*(male), *Junonia almanac, Junonia hierta, Melanitis phedima, Melanitis leda, Junonia orithiya,* were captured by sweep netting and hand-picking methods from open grounds, small bushes, damp patches, flowering plants and rotten fruits.

2. Preservation of specimen

The butterfly legs and thorax tissue were preserved in 70% alcohol in labelled tube (Vanlalruati *et al*., 2011) [18].

3. Extraction of DNA

Freshly collected legs tissues were used to elicit genomic DNA using the Genetix DNA sure Tissue Mini Kit.

4. Amplification of genomic DNA through RAPD- PCR method

Amplification of genomic DNA was performed using forward reverse RAPD primers as mention in table 1.

Table 1: Primers used in RAPD-PCR profiling

Primers	Primer sequence (5'to 3')	% of GC content	Mol. Wt. (bp)
OPA 1	CAGGCCCTTC	70%	2964
OPA 2	TGCCGAGCTG	70%	3044
OPA 3	AGTCAGCCAC	60%	2997
OPA 4	AATCGGGCTG	60%	3068
OPA 5	AGGGGTCTTG	60%	3099
OPP 9	GTGGTCCGCA	70%	-
OPN 16	AAGCGACCTG	60%	-
OPN 17	-------	----	-
OPP 18	GGCTTGGCCT	70%	-

PCR conditions were set as per Sharma *et al.,* (2006) [15] with minor modification. Table 2 highlights the PCR program that was used for nymphalidae butterflies.

Table 2: PCR programme

Sr. No.	Phase	Temperature	Duration	
1.	Pre-denaturation	94^0C	5 min	
2.	Denaturation	94^0C	1 min	Total 35 Cycles
3.	Annealing	37.6^0C	1 min	
4.	Extension	72^0C	1 min	
5.	Final extension	72^0C	5 min	
The amplicons were stored at 4°C				

The presence of amplicons was checked by 2% agarose gel electrophoresis for 3-4 hours.

5. Phylogenetic analysis

Phylogenetic analysis of 16 species belonging to 9 genera of 4 subfamilies of Nymphalidae were performed by using Maximum Parsimony (MP) and Neighbor joining (NJ) in MESQUITE software with 1000 bootstrapping support and default parameters.

Result and discussion

DNA was extracted from 16 collected Nymphalidae species belonging to 9 genera of Nymphalinae, Danainae, Satyrinae and Biblidinae subfamilies. RAPD patterns were visualised, analysed and scored from gel images. The data was interpreted in the form of Binary code to view tree in MEGA-5 program and later Molecular phylogenetic tree was created highlighting the interrelatedness among species representing their respective sub families.

1. Molecular data analysis

Distinct banding patterns that appeared after screening with nine primers suggested that there was intraspecies polymorphism. Seven of the nine primers generated polymorphic bands, with an average of 22.14 bands per primer scoring 100% polymorphism. [Table3]

Table 3: RAPD primers showing% of polymorphism

Sr. No.	Primers	5'to 3'orientation	Total Bands	Polymorphic Bands	Monomorphic Bands	Poly- Morphism%
1	OPA1	CAGGCCCTTC	26	26	00	100
2	OPA2	TGCCGAGCTG	34	34	00	100
3	OPA3	AGTCAGCCAC	32	32	00	100
4	OPA4	AATCGGGCTG	13	13	00	100
5	OPA5	AGGGGTCTTG	18	18	00	100
6	OPP9	GTGGTCCGCA	23	23	00	100
7	OPP18	GGCTTGGCCT	09	09	00	100
Pooled			155	155	00	700
Average			22.14	22.14	00	100

2. Phylogenetic analysis

In order to determine the evolutionary status of brush footed butterflies, phylogenetic research was conducted. The present study was based on verification by cladistics analysis of the species and the phylogenetic position of genus and species using molecular decamers. The resolution of phylogenetic tree was improved by adopting Maximum Parsimony, Neighbor-joining methods.

2.1 Genetic distance

Distance matrix methods, introduced by Fitch and Margoliash, in 1967 [4] (Felsenstein, 1985) [3] is a major part of phylogenetic methods. In this method, Branch lengths show projected rates of evolution in various tree branches. When two branches represent sister lineages in a rooted phylogeny, they may reflect the same period of time, but their projected rates of evolution may differ. Both rooted and unrooted trees can be produced using the distance matrix method.

Table 4: MEGA 5- Genetic distance matrix based on RAPD-PCR in 16 Brushfooted butterflies. 1) *Junonia lemonias*, 2) *Ariadne ariadne*, 3) *Byblia ilithyia*, 4) *Euploea core*, 5) *Tirumala limniace*, 6) *Danaus chrysippus*, 7) *Danaus genutia*, 8) *Vanessa cardui*, 9) *Hypolimnas misippus*, 10) *Hypolimnas bolina 1*, 11) *Hypolimnas bolina 2*, 12) *Junonia almanac*, 13) *Junonia hierta*, 14) *Melanitis phedima*, 15) *Melanitis leda*, 16) *Junonia orithiya*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	0															
2	4.076	0														
3	0.323	0.580	0													
4	0.350	4.253	0.580	0												
5	0.580	4.014	0.676	4.014	0											
6	0.276	4.128	0.350	0.381	0.350	0										
7	0.460	4.014	0.255	3.817	3.283	0.512	0									
8	0.676	4.440	0.844	4.174	4.128	0.580	4.014	0								
9	0.580	4.412	4.076	4.288	4.253	3.283	4.174	4.128	0							
10	0.298	4.174	0.580	3.283	0.844	3.817	3.817	4.253	4.128	0						
11	0.255	4.076	0.381	0.350	0.580	0.580	0.580	0.512	4.014	0.220	0					
12	0.844	4.412	3.936	3.817	4.174	0.676	3.936	4.014	4.174	4.288	3.817	0				
13	0.381	4.288	0.512	4.014	4.076	0.676	3.283	0.844	4.174	4.288	0.844	0.512	0			
14	3.817	4.467	4.174	4.215	4.174	3.283	4.174	4.014	4.440	4.288	3.817	3.936	3.283	0		
15	0.237	0.460	0.255	0.323	0.298	0.220	0.298	0.381	3.283	0.460	0.204	0.255	0.255	0.417	0	
16	0.844	0.844	4.076	4.014	3.936	3.936	4.076	4.215	4.492	4.014	0.844	4.174	3.283	3.936	0.220	0

The genetic distance was computed from the pooled data of 07 primers in 16 Brush footed butterflies to construct the phylogenetic tree. The highest genetic distance between *Hypolimnas misippus* and *Junonia orithiya* is 4.492, according to the RAPD-PCR based distance matrix. The species *H. bolina* 2 and *Melanitis leda* were found to have a lower genetic distance of 0.204 as highlighted in table 4.

2.2 Evolutionary relationship of Nymphalidae butterflies

Two methods Maximum Parsimony (MP) and Neighbour joining (NJ) were employed to reconstruct the phylogeny of 16 sampled butterfly taxa.

Maximum Parsimony (MP) tree (Fig 1)

Clade A, B, and C are three distinct primary clades that can be seen in the unrooted MP tree.

Clade A: Exhibit tight clustering of taxa *Byblia ilithyia* and *Danaus genutia*.

Clade B: include five taxa (05) of that *J. hierta* and *M. phedima* constitute the sister clade, while *Vanessa cardui* outgroups this clade. Clade B additionally includes sister taxa *J. almanac* and *M. leda.*

Clade C: include six tax (06), where *D. chrysippus* and *H. misippus* are grouped together as sister taxa. In this clade *H. bolina 1* and *H. bolina 2* forms the sister species, while *Euploea core* and *Junonia lemonias* outgroup the smaller monophyletic clade formed by the *H. bolina 1* and *2* species.

Intriguingly, the *Tirumala limniace taxon* occupies basal position in the phylogenetic tree thereby showing ancestral link to clades A, B, and C. Taxon *Ariadne ariadne and Junonia orithiya* clearly indicate paraphyletic position thus support separate origin out of this three afore mentioned clades.

Neighbor- joining (NJ) tree (Fig 2)

The phylogenetic tree created using the NJ technique shows that 16 taxa are clustered into three large, separate clades, A, B, and C, in accordance with the MP tree.

Clade A: Include six taxa (06). In clade A, the taxon *J. almanac* and *J. hierta* forms sister taxa. To this sister taxa, taxon *M. phedima* forms the outgroup. In this same clade, taxon *A. ariadne and J. orithiya* clusters as sister taxa with taxon *M. leda emerging* as an outgroup. Thus, the monophyletic clade A clearly harbor two bifurcating smaller clades comprising of three (03) species in each.

Clade B: Comprises of four (04) taxa namely *J. lemonias* and *H. misippus* as sister taxa and *E. core* and *D. chrysippus* as another sister taxon clade.

Clade C: includes six (06) which split into three sister taxa comprising of *H. bolina 2, V. cardui* as sister taxa followed by *H. bolina 1, T. limniace* and *E. core* and *D. chrysippus* as corresponding sister taxon.

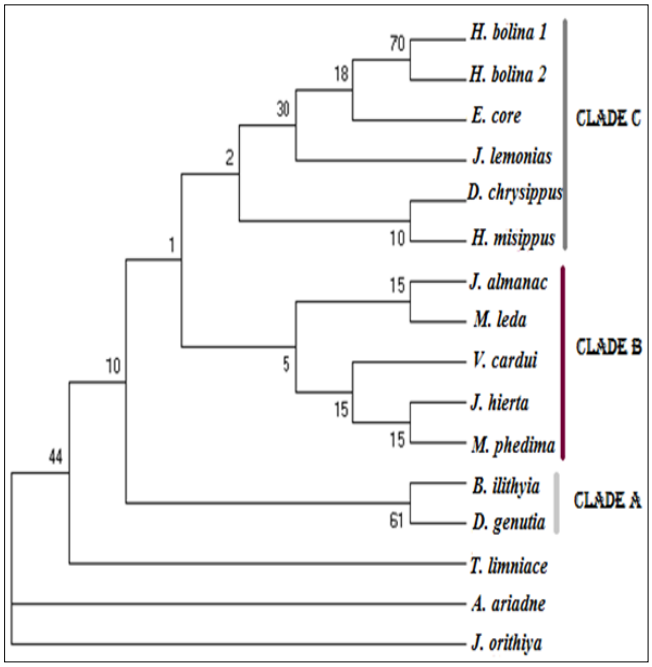

Fig 1: Maximum parsimony tree (Unrooted) of 16 Brushfooted butterflies

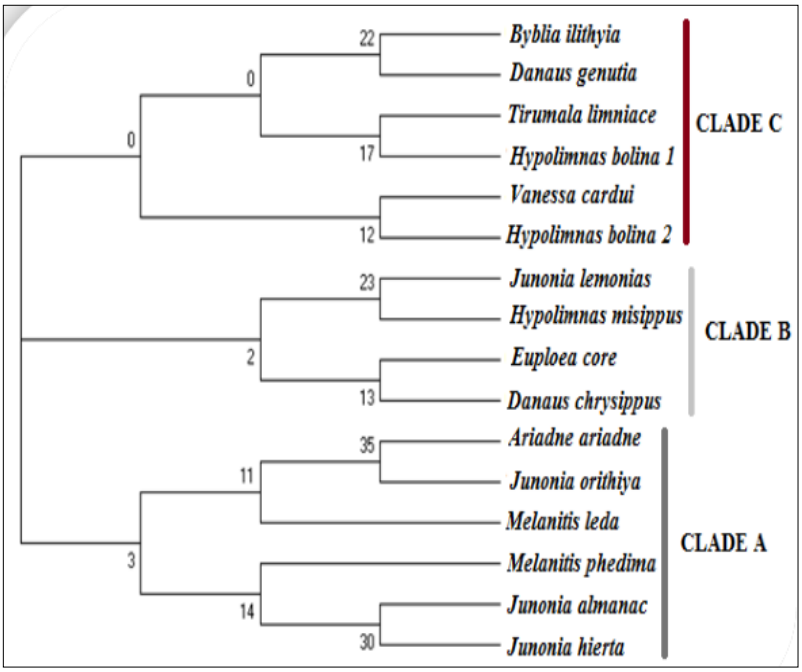

Fig 2: Neighbor- Joining tree (Unrooted) of 16 Brushfooted butterflies

Discussion

The phylogeny given by Freitas and Brown (2004) [5] includes the widest taxon coverage of any morphological study on Nymphalid butterflies to date, and supports the monophyly and relationships of most presently recognized subgroups. A generic-level phylogeny for the butterfly family Nymphalidae was produced by cladistic analysis of 234 characters from all life stages. They described all remaining lineages to be grouped into two main branches: one lineage representing primarily flower-visitors and the other lineage representing primarily fruit-attracted Nymphalid butterflies.

Danainae emerged as an outgroup to other nymphalidae, according to Frietas and Brown (2004) [5]; *Vanessa* and *Junonia* revealed the same site of origin; and Nymphalinae, Biblidinae, and Satyrinae were discovered to be monophyletic. In present work Maximum Parsimony tree shows that taxa *E. core* of Danainae forms an outgroup of two species of nymphalinae *H. bolina1 and H. bolina 2*; taxa of *Vanessa* and *Junonia* showed same point of origin. The Neighbor-joining tree showed that taxa of Nymphalinae, satyrinae and biblidinae formed monophyletic clade.

J. lemonias, *H. misippus* and *J. orithiya* forms out group in tree given by Kodandaramaiah (2009) [13]. Similarly in Maximum Parsimony tree these three forms outgroups with different clade.

It is necessary to conduct comprehensive sampling of the relevant butterfly family, followed by distinctive morphological research, in conjunction with modern DNA techniques, in order to establish a more distinct and vivid evolutionary relationship within the nymphalidae family.

Conclusion

The goal of the current study was to fill the gap between traditional taxonomy and DNA-based taxonomy in order to determine the phylogeny and record the habitat of brushfooted butterflies in the gardens of Amravati**.** It was found that the taxa *A. araidne* and *J. orthiya* supported different origins in the Maximum Parsimony tree. Numerous taxa created inter-species sister clades that demonstrated shared ancestry. *V. cardui*, E. core, and *J. lemonias* were among the taxa that made up the outgroup and showed evidence of distant resemblance to other species The six taxa that make up the monophyletic group in the Neighbor- Joining Tree (*A. ariadne, J. almanac, J. hierta, M. phedima, M. leda, and J. orithiya*) share the same point of origin.

Acknowledgement

The author expresses deep gratitude towards Department of Zoology Government Vidarbha
Institute of Science and Humanities for providing the Evolutionary Biology Laboratory to carry
out molecular study.

Competing interests

Author has declared that no competing interest exist.

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