Analysis of phylogenetic relationship among species of Danainae butterflies using nuclear and mitochondrial gene markers

Thesis submitted in partial fulfillment
of the requirements for the
degree of Master of Philosophy in Biotechnology

by

Lalhlimpuia Pachuau

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Under the Supervision of

Dr. N. Senthil Kumar
Professor
Department of Biotechnology

&

Joint- Supervision of

Dr. G. Gurusubramanian
Associate Professor
Department of Zoology

Department of Biotechnology
School of Life Sciences
Mizoram University
Aizawl, Mizoram

CERTIFICATE

I certify that the thesis entitled "**Analysis of phylogenetic relationship among species of Danainae butterflies using nuclear and mitochondrial gene markers**'' submitted to the Mizoram University for the award of a degree of Master of Philosophy in Biotechnology by Lalhlimpuia Pachuau is a record of research work carried out by her during the period from 2011 to 2012 under my guidance and supervision and this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles in this University or any other University or institution of higher learning.

Signature of the supervisor

(N.SENTHIL KUMAR)

Signature of the Co-Supervisor

(G.GURUSUBRAMANIAN)

DECLARATION

I declare that the thesis entitled "**Analysis of phylogenetic relationship among species of Danainae butterflies using nuclear and mitochondrial gene markers**" submitted to the Mizoram University for the award of degree of Master of Philosophy in Biotechnology is a bonafide record of work carried out by me during the period from 2011 to 2012 under the guidance of **Prof. N. Senthil Kumar** (Supervisor), Department of Biotechnology and **Dr. G. Gurusubramanian** (Co-Supervisor), Department of Zoology and has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles in this University or other University or institution of higher learning.

LalhlimpuiaPachuau
Candidate

PARTMENT OF BIOTECHNOLOGY

SCHOOL OF LIFE SCIENCES
MIZORAMUNIVERSITY
TANHRIL COMPLEX, AIZAWL – 796 009: MIZORAM, INDIA
Phone: 0389-2330859/2330861 Fax: 0389-2330642/2330644 (O)

This is to certify that ***Lalhlimpuia Pachuau*** son of *Vanlalrinawma Pachuau* has appeared in the M. Phil Course work examination held during February 2012 and has been declared successful. This is in accordance with University Grants Commission (Minimum Standards Procedure for Award of M. Phil/Ph.D degree) Regulations, 2009.

(PROF. N SENTHIL KUMAR)
Head
Department of
Biotechnology,
Mizoram University

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CHAPTER 1

1.0. INTRODUCTION AND REVIEW OF LITERATURE

Butterflies are recognized to comprise somewhere between four and 14 families (Kristensen, 1976); however, the butterflies are generally categorized into five families (Papilionidae, Pieridae, Nymphalidae, Riodinidae, and Lycaenidae). On the other hand, the family Riodinidae has occasionally been included in the Lycaenidae (Ackery, 1984; Ehrlich, 1958).

1.1. Classification of Danainae

Danainae (Milkweed butterflies) are a subfamily in the family Nymphalidae, or brush-footed butterflies (Brower *et al.*, 2010). Historically, this group had been considered a separate family, Danaidae, and the Danaini tribes placed herein were sometimes considered distinct subfamilies in the Nymphalidae.

Historical efforts to circumscribe and arrange the danaines are described in detail in Ackery and Vane-Wright (1984). This comprehensive account of the Danaini included manually performed cladistic analyses of relationships among the tribe's 12 genera (Vane-Wright *et al.*, 2002) and some 157 species based on morphological characters derived mainly from adult specimens. As the authors noted, their results corroborated to a greater or lesser degree prior hypotheses of relationships suggested by

Bates (1862) and Forbes (1939). Kitching (1985) studied immature stages of a subset of Danaini and performed separate cladistic analyses of data matrices scored from egg, larval and pupal stages. His results implied relationships among danaine genera that are largely but not entirely concordant with those of Ackery and Vane-Wright (1984).

1.2. The Danainae Subfamily :

Kingdom : Animalia
Phylum : Arthropoda
Class : Insecta
Order : Lepidoptera
Family : Nymphaelidae
Subfamily : Danainae

Ackery and Vane-Wright (1984) recognized 2 tribes and 4 subtribes within Danainae: Euploeini Comprising Euploeina and Itunina, and Danaini comprising Danaina and Amaurina.

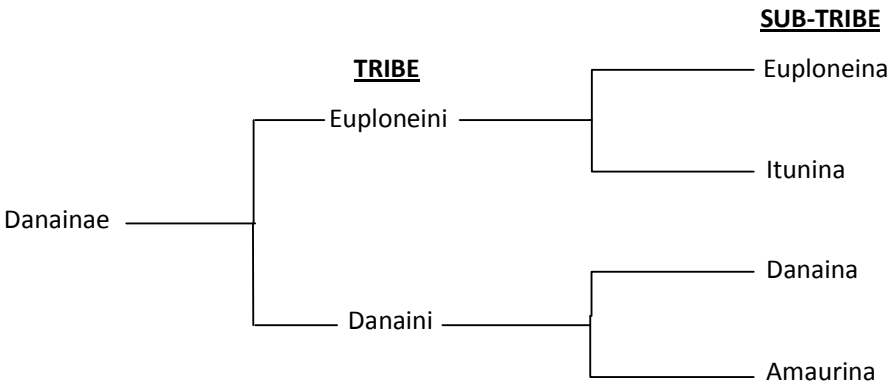

Fig. 1: Classification of Danainae into Tribe and subtribe by Ackery and Vane-Wright (1984).

1.3. Morphhology and unique characters of Danainae

Fig. 2: Plain Tiger (*Danaus chrysippus*) showing extruded Hair-pencil (protruding from the abdomen)

The milkweed butterflies are a charismatic tribe of some 160 large, aposematically coloured species distributed in tropical and temperate habitats throughout the world. Most of the Danaini are found in tropical Asia and Africa. A total of 23 species belonging to six genera are found in India (Kehimkar, 2008). They lay their eggs on various milkweeds on which their larvae feed. Well known members of the group include Plain Tiger (*Danaus chrysippus* L.), the Tree-Nymph or Paper Kite (*Idea agamarschana*) and various Crows (*Euploea* spp.).

The Danaini are united by the presence of paired secondary sexual organs within the male abdomen referred to as hair pencils (Fig 2), and strongly clubbed, spinose, four-segmented fore-tarsi in females (Ackery and Vane-Wright, 1984). They are known for their need of pyrrolizidine alkaloids, essential as precursors of pheromones for the males in their courtship.

Morphological identification of butterflies is usually based on the wing patterns (Evans, 1932; Wynter-Blyth, 1957). Generally it is attributed to the numbers and positions of spots on the wings. It has now been established that classification of closely related lepidopteran species based on morphological features can pose several difficulties on account of attributes that can change as function of environment and prevalence of several biotypes. These factors make morphological criteria not a preferred way for a very accurate differentiation of these species

(Sharma*et al*., 2006). Molecular data may provide a valuable complement to morphological evidence, particularly in situations in which groups are weakly supported or unstable in traditional analyses(Miller *et al*., 1997).

1.4. GENETIC MARKERS

1.4.1. RAPD-PCR

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 (Williams *et al.,* 1990). This technique 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 (Galluser *et al.*, 2004).

On the basis of RAPDs, genetic polymorphisms and genetic diversity in natural populations between species of Nymphalidae have been studied (Galluser *et al*., 2004). Phylogenetic relationships in some genus of Nymphalid butterflies were described using different molecular markers (Pena *et al.,* 2006). Recently, in India RAPD-PCR was successfully applied for molecular characterization of few species of butterflies belonging to family Pieridae (Sharma *et al*., 2006).

1.4.2. Mitochondrial DNA

Mitochondrial DNA molecules are much smaller than the nuclear chromosomes. In animal cells, it is fewer than 20,000 bp and is a circular duplex (Wolstenholme, 1992). Each mitochondrion typically contain 2 to 10 copies of this mtDNA molecules, and the number can rise upto hundreds in certain cells of an embryo undergoing cell differentiation.

mtDNA codes for the mitochondrial tRNAs and rRNAs and for a few mitochondrial proteins. More than 95% of mitochondrial proteins are encoded by nuclear DNA. All of the proteins encoded by the mtDNA are subunits of the mitochondrial respiratory chain (Okimoto *et al*., 1992). The mtDNA encodes 37 genes: 13 for subunits of respiratory complexes I, II, III, IV and V, 22 for mitochondrial tRNA, 2 for rRNA. The 13 protein coding genes in the animal mitochondrial genome are better targets because indels are rare since most lead to a shift in the reading frame (Ghosh S.K, 2012).

1.4.2.1. Mitochondrial Cytochrome Oxidase subunit1 (CO1)

CO1 has been used in Lepidoptera for resolving relatively recent divergence events, particularly at genus and species level (Caterino *et al*., 1999). It is useful in fully resolving topologies within species of Nymphalidae (Wahlberg *et al*., 2003). Its accuracy depends mainly on

the separation between intra-specific (within-species) variation and inter-specific divergence of the selected DNA sequence.

Since Hebert *et al*. (2003) first proposed the use of the CO1 as a barcode to identify animals, in which only 648bp of the mitochondrial CO1 gene near its 5'end is used, DNA barcoding has attracted worldwide attention. The advantage of using CO1 is that it is short enough to be sequenced quickly and cheaply yet long enough to identify variations among species.

1.4.2.2. Mitochondrial D-loop

Displacement loop region (D-loop) is the non-coding control region of the mtDNA, and plays an important role in replication and transcription. Within the D-loop there is a tandem repeated sequence, **flanked by methionine tRNA(Met) on one side and 12s rRNA gene on the other** (Vila and Bjorklund, 2004; Zardoya*et al*., 1995).The Dloop region **remains a largely unused and little known genetic marker in insects**. (Caterino *et al*. 2000; Mardulyn *et al*. 2003).The reason for these might be (i) their difficulties in amplification and/or sequencing, (ii) putative strong selective constraints related to its extreme richness in AT and to the presence of highly conserved structural elements (Zhang and Hewitt, 1997).The D-loop region of mtDNA is particularly interesting due to the high variability level (Brower *et al*., 2010) and the matrilineal transmission and the lack of recombination (Thompson *et al*., 1994).

Phylogenetic analysis is being increasingly used to address research questions in tropical ecology. With the growing wealth of DNA sequence data at hand, molecular phylogenies of extant taxa offer the opportunity to examine the tempo and mode of speciation. This analysis can enhance our understanding of the evolution in groups for which fossil data are lacking (Paradis, 1998). In addition, quantification of DNA substitutions under the assumptions of a molecular clock allows estimation of divergence dates (Karanth *et al*., 2008). Additionally estimation of approximate branching times can also help correlate novel traits or radiation events to geological events (Xiang *et al*., 2000), and allow testing of ecological hypothesis. Despite careful phylogenetic analysis using multiple gene regions and thousands of base pairs, the historical relationships of many groups of organisms remain unresolved (Waits *et al*., 1999).

The evolutionary history of butterflies has been largely a mystery. The lack of robust phylogenetic hypotheses and a temporal framework has inhibited the study of aspects of the evolution of butterflies, such as bio-geographical events and evolution of adaptive traits(Vane-Wright, 2004). It is only recently that studies using molecular methods have provided time estimates for the origin and diversification of butterflies in the Nymphalidae (Wahlberg, 2006; Kodandaramaiah and Wahlberg, 2007; Wahlberg and Freitas, 2007; Peña and Wahlberg, 2008), Papilionidae (Braby *et. al*., 2005; Nazari *et al.*, 2007), and Pieridae (Braby *et. al*., 2006).

Phylogenetic approaches to the study of speciation are becoming increasingly common as molecular phylogenetic analyses of entire species clades become available (Barraclough and Vogler, 2000; Barraclough et al. 1998). These studies rely on the fact that species-level phylogenetic hypotheses permit both the rate and pattern of cladogenesis to be inferred. In combination with ecological and morphological information for the species concerned, phylogenies can be used to test hypotheses regarding which factors promote cladogenesis. The wing patterns of butterflies are traits that play an important role in diversification. A number of groups show great diversity and rapid divergence in color, pattern between both populations and species (Bates,1862; Vane-Wright, 1979). Furthermore, the dual role of wing patterns in signaling to both predators and potential conspecific mates

means that they may commonly cause reproductive isolation between divergent populations. In the case of aposematic warning colors, switches in color pattern between related species can generate both pre- and post-mating reproductive isolation and thus contribute to speciation (Jiggins et al., 2001). Therefore, color patterns are traits likely to play a role in speciation, which are also easily recorded and therefore amenable to comparative analysis.

Mizoram, a state situated in Northeast India, has one of the richest biomes of the country. Many of its rich flora and fauna have been left unexplored, with the inclusion of an in-depth analysis (scientific/morphological/phylogeny). An intensive research on its rich biomes has been left untried. A close study and examination of the Danainae species among itself and its comparison with the already analyzed and sequenced species of the sub-family through phylogenetic analysis could therefore, prove useful in understanding their diversity, evolution and conservation needs.

This study would assess the relationships among Danainae species and to test the monophyly of the various hypothesized Subtribes viz, Forbes (1939); Ackery and Vane-Wright (1984); Brower and Wahlberg (2010); by using two mitochondrial genes (CO1 and D-loop) and nuclear genome analysis through RAPD-PCR.

1.5. OBJECTIVES OF THE STUDY

i. Collection and morphological identification of the Danainae species in Mizoram.

ii. Phylogenetic analysis of Danainae species of butterflies using nuclear (RAPD) and mitochondrial (COI and D-loop) gene markers.

iii. To analyze the nucleotide sequences of the gene markers and to estimate their divergence.

CHAPTER 2

MATERIALS AND METHODS

2.0. Field survey, collection, identification

Butterfly species belonging to Danainae sub-family were collected by hand-nets from different locations of Mizoram during 2011 to 2012. A total of 13 different species belonging to four Genera were collected (Table1) and were preserved in 70% alcohol until analysis. The identification of butterflies was based on the information of Evans (1932), Wynter-Blyth (1957), Wahlberg (2009) and Isaac Kehimkar (2008).

2.1 Preparation of Butterfly DNA

DNA was extracted from legs and tissues of Butterflies using slight modification of Zimmermann *et al.* (2000). Legs or tissue from the thorax stored in 70% alcohol were taken in 1.5 ml eppendorf tube.

Table 1: Specimens collected for the study.

Taxon	Collection locality	Voucher Number	Month and year of collection
Euploea aglea (Long branded blue crow)	Aizawl	C-IV	September 2011
Euploea mulciber (Striped blue crow)	Kolasib	KL-6	May 2012
Euploea midamus (Blue spotted crow)	Aizawl	TH-1	February 2012
Euploea klugii (Blue king crow)	Zawlnuam	ZN-3	March 2012
Euploea core (Common crow)	Zawlnuam	ZN-4	March 2012
Euploea Sylvester (Double branded crow)	Aizawl	LEP-131	April 2010
Parantica aglea (Glassy tiger)	Aizawl	AZ-1	May 2011
Parantica sita (Chestnut tiger)	Lunglei	LL-6	June 2012
Parantica melaneus (Chocolate tiger)	Reiek	RE-1	July 2012
Danaus chrysippus (Plain tiger)	Kolasib	KL-1/ KL-3	June 2012
Danaus genutia (Striped tiger)	Sairang	SR-1	October 2011
Tirumala septentrionis (Dark blue tiger)	Zawlnuam	ZN-1	March 2012
Tirumala limniace (Blue tiger)	Zawlnuam	ZN-2	March 2012

Table 2: List of the four primers and their sequence used in the study.

Sl.No	NAME OF THE RAPD PRIMER	PRIMER SEQUENCE 5’ – 3’
1	OPT-1	GGGCCACTCA
2	OPT-4	GTGTCTCAGG
3	OPT-5	GGGTTTGGCA
4	OPB-12	CCTTGACGCA

Tirumala – strikingly marked with pale blue streaks and spots against a black or dark brown background

Tirumala septentrionis

Danaus – Pale orange, Black wing margins with small white spots along border

Danaus genutia

Parantica – Blueish white transparent marking on dark brown UP.

Parantica aglea

Euploea – Overall glossy black or blue.

Euploea mulciber

Fig. 3: Four species representing each of the four genera of the study

The legs or tissue were then finely macerated with the help of scissors and homogenize in 250 µl of room temperature extraction buffer containing 50mM Tris HCl (pH 8.0), 25 mM NaCl, 25 mM EDTA (pH 8.0) and 0.1% SDS. 2 µl of proteinase K (18mg/ml) was added and gently mixed, and incubated in oven at 60ºC for at least 3 h or at 37ºC overnight. To the sample, equal volume of phenol/chloroform (250 µl) was added and mixed thoroughly until the solution gets homogenized, and spin at 13,000 rpm for 5min and the supernatant was carefully removed using micropipette to a new Eppendorf tube. To the supernatant 15 µl of 5 M NaCl and 450 µl 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 then again spun 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. The pellet was then dried in oven at 60ºC for 15-20 min. 30µl of 1X TE buffer was again added and the pellet was re-suspended by gently flicking the tube and stored at -20℃ for further used.

2.3 PCR Amplification and Analysis

Polymerase chain reaction (PCR) was carried out to amplify the mitochondrial and nuclear genomes viz, 650bp of the CO1 and Displacement loop (variable between species and is approximately 335bp). The Random Amplification of Polymorphic DNA (RAPD) was carried out using 4 decamer primers which were obtained from Bangalore Genei (Tables 2-4)

Initial amplification of the mtCO1 gene was carried out using the forward primer **LCO**-5' TAA TAC GAC TCA CTA TAGGGG GTC AAC AAA TCA TAA AGA TAT TGG- 3' and reverse primer **HCO** - 5' ATT AAC CCT CAC TAA AGT AAA CTT CAG GGT GAC CAA AAA ATC A - 3' combination and thermo-cycling conditions (Tables 3-4) outlined by Zimmermann *et al*. (2000) and Hebert *et al*. (2004).

Amplification of the D-loop sequence was done using the primers designed by Vila and Bjorklund (2004). Primers SeqLepMet and LepAT2B. Primer **SeqLepMet** (5’ TGA GGT ATG ARC CCA AAA GC 3’) lies in the 5’ end of the tRNA-methionine gene, whereas primer **LepAT2B** (ATT AAA TTT TTG TAT AAC CGC AAC3), antisense to SeqLepMet, is located towards the 5’ extreme of the 12s rRNA gene (Tables 3-4).

2.3.1. Data analysis (RAPD)

RAPD-PCR amplifications of Danainae species was performed using condition outlined by (Sharma *et al*., 2006) and the bands on gels were documented using the Gel documentation system (UVI TEC FireReader). Each species was scored for the presence or absence of every amplification product, and the data were entered into a binary data matrix.

Table 3: PCR reagents for RAPD, CO1 and D-loop

PCR parameter	RAPD	CO1	D-loop
Taq Buffer	1x	1x	1x
Magnesium Chloride (Mgcl2)	2 mM	2 mM	2.5 mM
Deoxynucleotide Triphosphate (dNTPs)	2 mM	0.25 mM	0.2 mM
Primer concentration (forward and reverse for both CO1 and D-loop)	10 pm/µl	0.2 pm/µl	0.2 pm/µl
Taq Polymerase	1U	1U	1U
DNA concentration	10-50 ng	10-50 ng	10-50 ng
BSA	10 mg/ml	10 mg/ml	10 mg/ml

Table 4 : PCR conditions for the three markers with a repeat of 30 cycles for RAPD, 35 cycles for CO1 and Dloop.

Markers	Initial denaturation(□C)	Denaturation (□C)	Annealing (□C)	Extension (□C)	Final extension(□C)
RAPD	94 (5 mins)	94 (1 min)	35 (1min)	72 (1 min)	72 (5 mins)
CO1	95 (5 mins)	94 (1 min)	55 (50secs)	72 (90secs)	72 (5 mins)
D-loop	95 (2 mins)	94 (1 min)	51 (90secs)	65 (1min)	65 (7 mins)

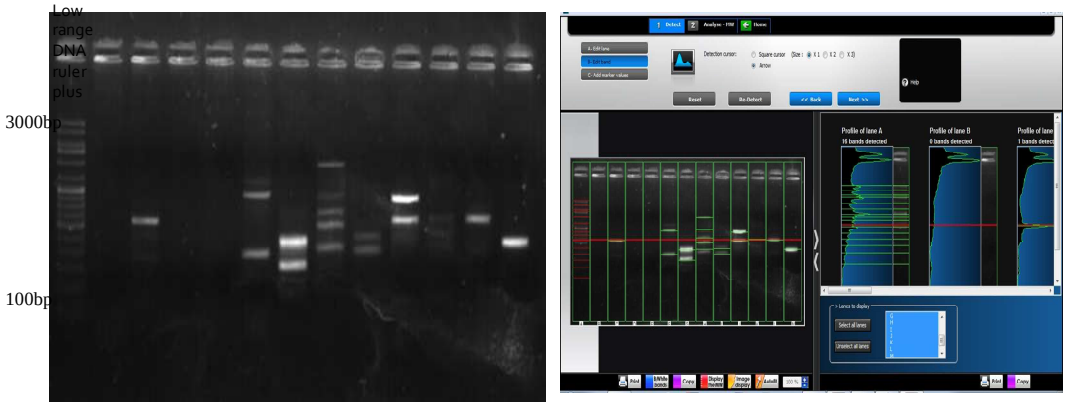

Fig. 4: Gel electrophoresis photo of 12 species of Danainae by OPT-1 primer along with RAPD analysis using Gel documentation system (UVI TEC Fire Reader).

A total of 147 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. The PIC value was determined by applying the formulaPICi = 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 functions that is DI and EMR as described by Prevost and Wilkinson (1999). The DI of the primer is defined as 1- sigma (pi) 2 where pi is the frequency of ith 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**"**. Generating dissimilarity matrices, only polymorphic bands with PIC values higher than 0.08 were used. All the above mentioned statistical analysis was performed using NTSYS-pc software version 2.2020. 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. The Mantel matrix correspondence test was also

used to test the significance of the correlation coefficient of similarity matrix generated with RAPD data (Mantel,1967).

2.3.2. CO1 and D-loop sequences analysis

The PCR amplified products were sequenced (GenBank accession numbers are KC306717 - KC306729 for CO1 and KC306730 - KC306741 for D-loop).Phylogenetic and molecular evolutionary analyses were conducted using MEGA 5.1 version (Tamura*et al.*, 2011).The DNA sequences were aligned by using MUSCLE alignment(MEGA 5.1 version) along with sequences retrieved from the NCBI database.

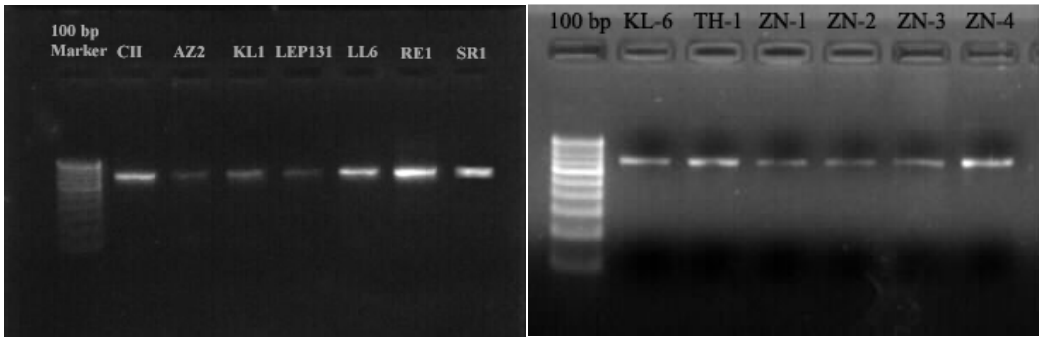

Fig. 5: Gel electrophoresis representation of CO1 gene among 13 species of Danainae under study

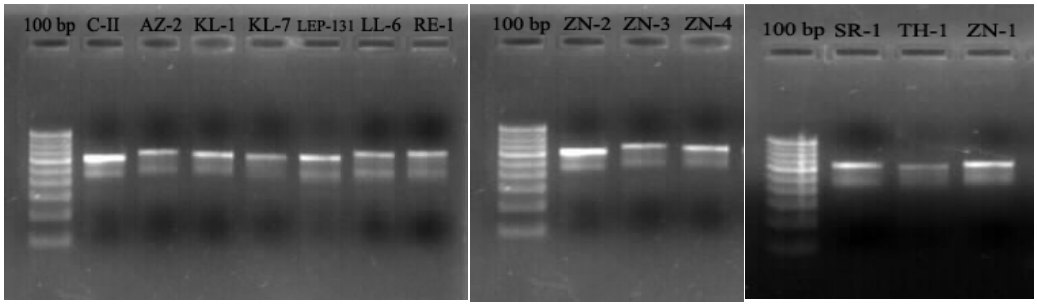

Fig. 6: Gel electrophoresis representation of D-loop marker among 13 species of Danainae under study

The data obtained was used to derive Maximum Likelihood (ML) Phylogenetic tree. The program MODEL TEST (Posada and Crandall, 1998) was used to choose asubstitution model that fit the data the best and to estimate the transition-transversion ratio and gamma shape parameter. For building the parsimony tree, 1000 bootstrap replications was performed, where each bootstrap replication undergoes 10 additional replications with different input order of the taxa. This analysis had been undertaken to determine the robustness of the chosen markers for species identification (DNA barcoding).

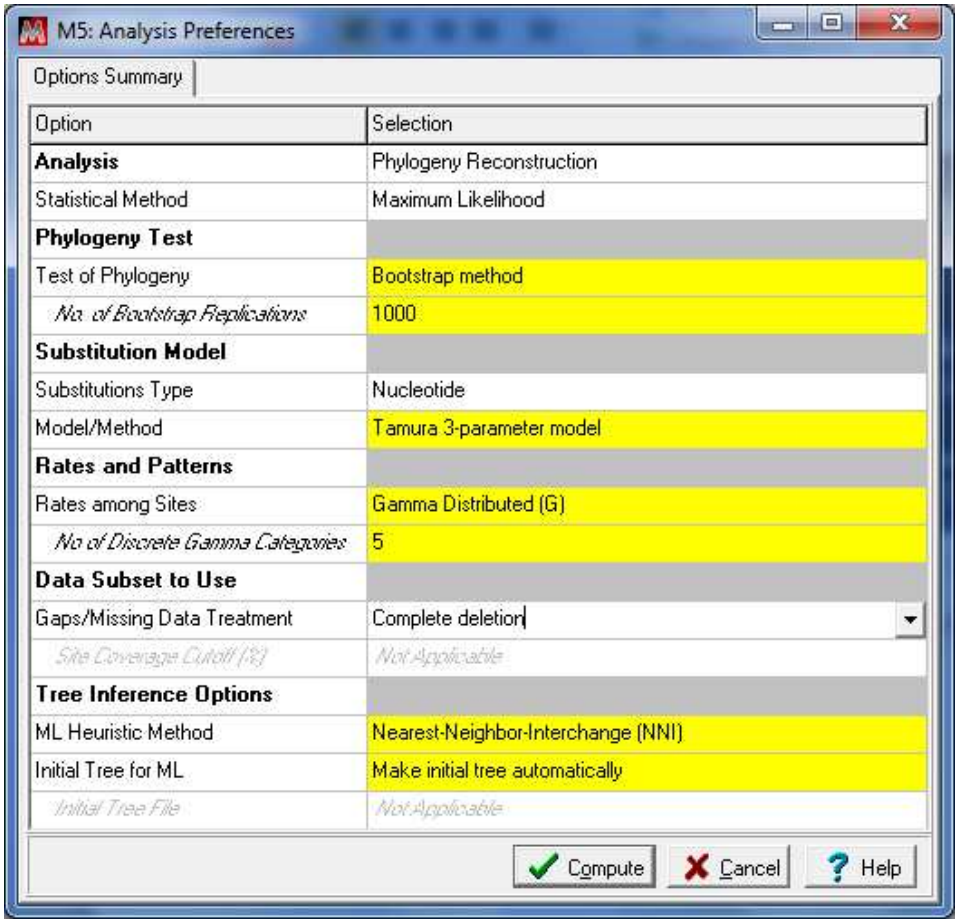

Fig. 7: Parameters used to obtain a phenogram among the butterfly species of Danainae using the two mitochondrial gene markers (CO1 and D-loop). A Maximum Likelihood tree is obtained using Tamura 3-parameter model and a 1000 bootstrap replications, with gaps and missing data along the sequence aligned deleted.

CHAPTER 3

RESULTS

3.0. RAPD

RAPD patterns were visually analyzed and scored from the gel photographs. A series of discrete bands were obtained on amplification of DNA samples of thirteen species of Danainae butterflies with four primers (OPT-1. OPT-4, OPT-5 and OPB-12). Out of the 147 total discrete fragments, Primer OPT-5 gave as many as 62 prominent bands while OPT-1 produces just 19 bands. The amplified ranges of primers was as low as 130 bp as in OPT05 to as high as 1900bp as in OPB-12 and there was a total of 116 (79%) 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.

From the RAPD data obtained, a dendrogram was generated using the UPGMA (Unweighted pair-group method with arithmetical averages). Two Neighbour Joining trees using both Jaccard and NE172 coefficients and one SAHN tree using NE172 coefficients were built. As informative as the bands obtained from the RAPD data maybe in terms of genetic distances or the dissimilarity among the species, the dendrogram tree

generated was not as convincing in terms of the tree clustering among the species in relation to genera-wise; as species were not forming the same clade with other species of the same genera in most cases as in case of the three dendrograms (Figures 7 and 8).

Table 5: RAPD primers, Polymorphic bands, % Polymorphism, PIC, EMR and MI as resolved by the 5 primers among the 13 species of Danainae.

Primer	Total band	Polymorphic band	Polymorphism %	PIC	RP	EMR	MI
OPT-1	19	16	84.2	0.16	2.92	0.84	0.19
OPT-4	31	26	83.9	0.16	4.77	0.84	0.2
OPT-5	62	45	72.58	0.18	9.54	0.73	0.25
OPB-12	35	29	82.86	0.17	5.38	0.83	0.2
TOTAL	147	116	79				

Table 6: Simple matching coefficients of dissimilarity matrix determined by analysis using four RAPD primers from 13 species of Danainae. Lowest dissimilarity value (0.105) found between C-IV and LEP which are the two species belonging to the *Euploean* genus while the highest value (0.478) was between RE-1 and ZN-4, belonging to *Parantica* and *Euploean* genera respectively.

	C-IV	AZ-1	KL-3	KL-6	LEP 131	LL-6	RE-1	SR-1	TH-1	ZN-1	ZN-2	ZN-3	ZN-4
C1V	0.000												
AZ1	0.210	0.000											
KL3	0.260	0.391	0.000										
KL6	0.277	0.295	0.391	0.000									
LEP	0.105	0.210	0.260	0.210	0.000								
LL6	0.243	0.295	0.391	0.295	0.210	0.000							
RE1	0.371	0.434	0.412	0.478	0.332	0.434	0.000						
SR1	0.260	0.391	0.412	0.391	0.260	0.351	0.371	0.000					
TH1	0.226	0.277	0.295	0.243	0.194	0.243	0.371	0.371	0.000				
ZN1	0.163	0.277	0.332	0.277	0.163	0.277	0.456	0.295	0.226	0.000			
ZN2	0.194	0.313	0.332	0.313	0.194	0.313	0.371	0.226	0.260	0.194	0.000		
ZN3	0.178	0.226	0.351	0.260	0.148	0.295	0.391	0.277	0.243	0.178	0.243	0.000	
ZN4	0.210	0.295	0.434	0.295	0.210	0.295	0.478	0.313	0.277	0.243	0.277	0.226	0.000

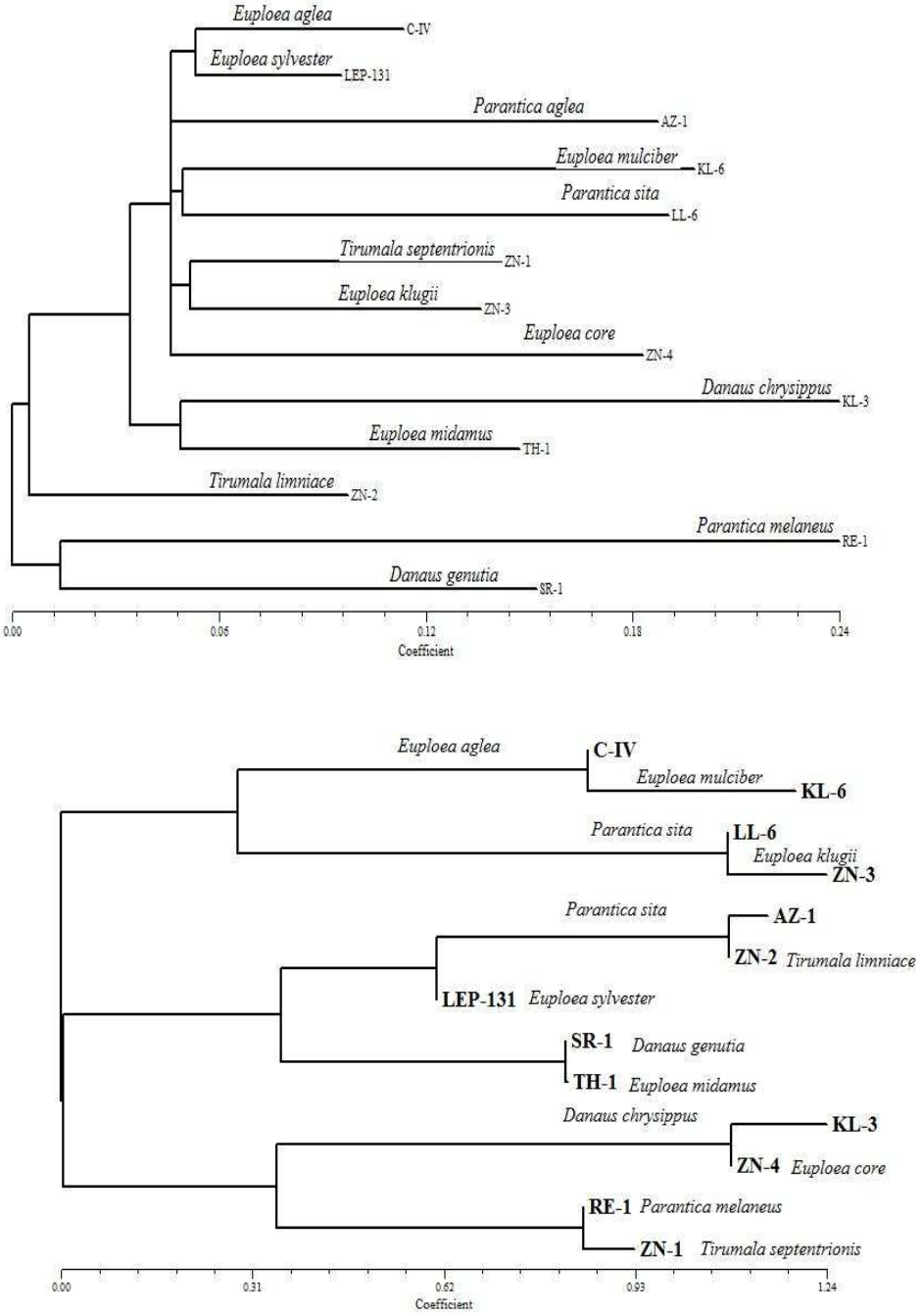

Fig. 8: Dendrogram generated by clustering using UPGMA analysis computed from a pairwisecomparison of RAPDs from 13 species using Neighbour Joining (NJ) method with Jacquard and NE172 Coefficients

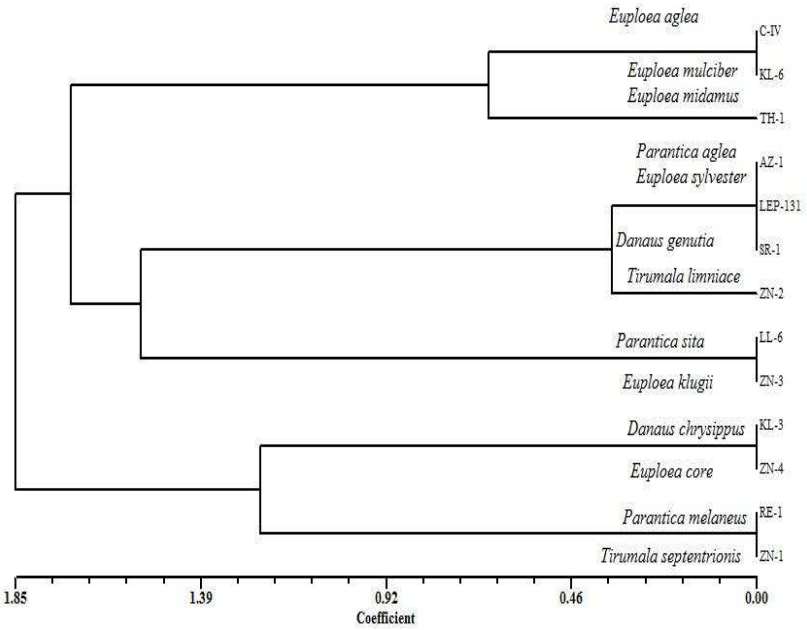

Fig 9: Dendrogram generated by clustering using UPGMA analysis computed from a pairwisecomparison of RAPDs from 13 species using SAHN method with Jaccard Coefficient.

3.1. Mitochondrial markers (CO1andD-loop) sequence analysis

The 13 species of Danainae were sequenced successfully and a phylogram was generated by usingMEGA 5.1 version (Tamura*et al*., 2011) through the parameters as described in materials and method (Fig 6).

The Present study showed similarity with the previous studies i.e Forbes (1939); Ackery and Vane-Wright (1984); Brower and Wahlberg (2010) in its resolution of subtribes and genera relationships. Both the CO1 and D-loop markers are in congruence with the clustering of the Danaina subtribe (Danaus + Tirumala) as done by the previous works. The sister relationship of the Amaurina and Danaina is also moderately supported by the D-loop marker.

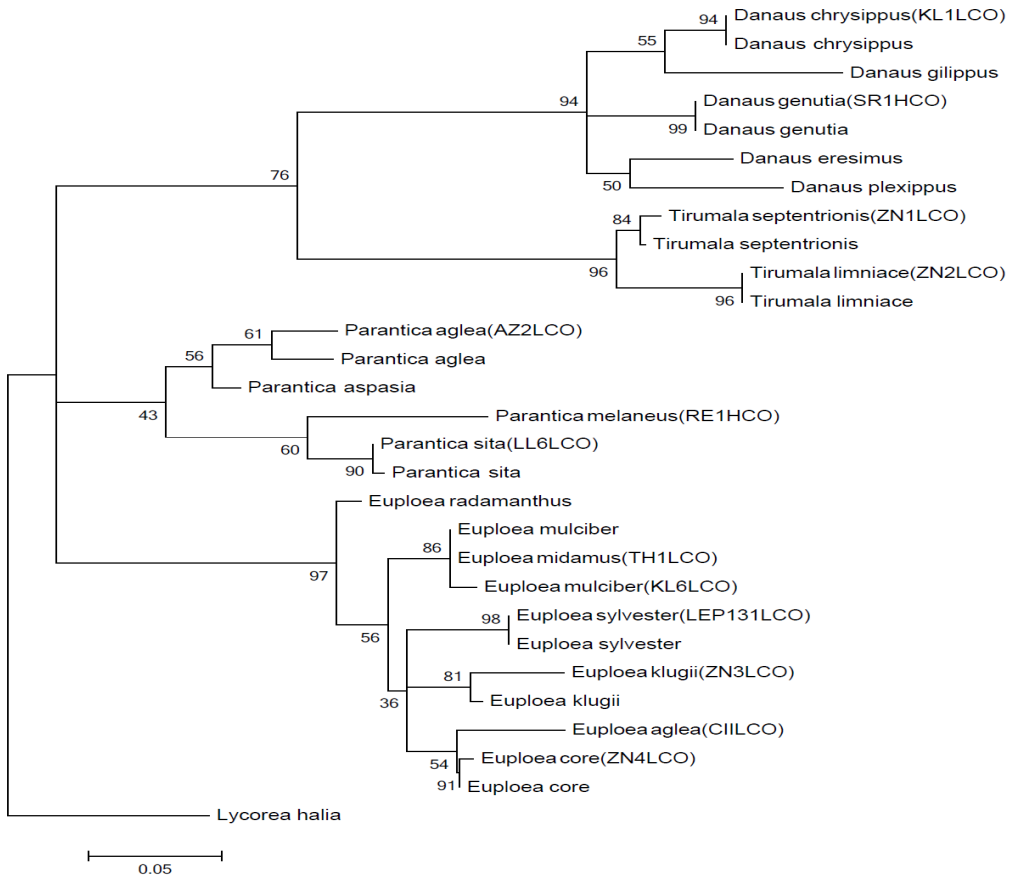

Fig. 10: Maximum Likelihood tree of CO1 gene marker among the 13 species of Danainae (labeled voucher numbers viz, KL1LCO, SR1HCO, ZNILCO, ZN2LCO, AZ2LCO, RE1HCO, LL6LCO, TH1LCO, KL6LCO, LEP131LCO, ZN3LCO, CIILCO and ZN4LCO) under study along with the sequences retrieved from NCBI database and *Lycorea halia* (Danainae belonging to different subtribe) as outgroup. The overall mean distance is 0.130. The Likelihood tree has resolved in a way, giving a separate clade for each genus.

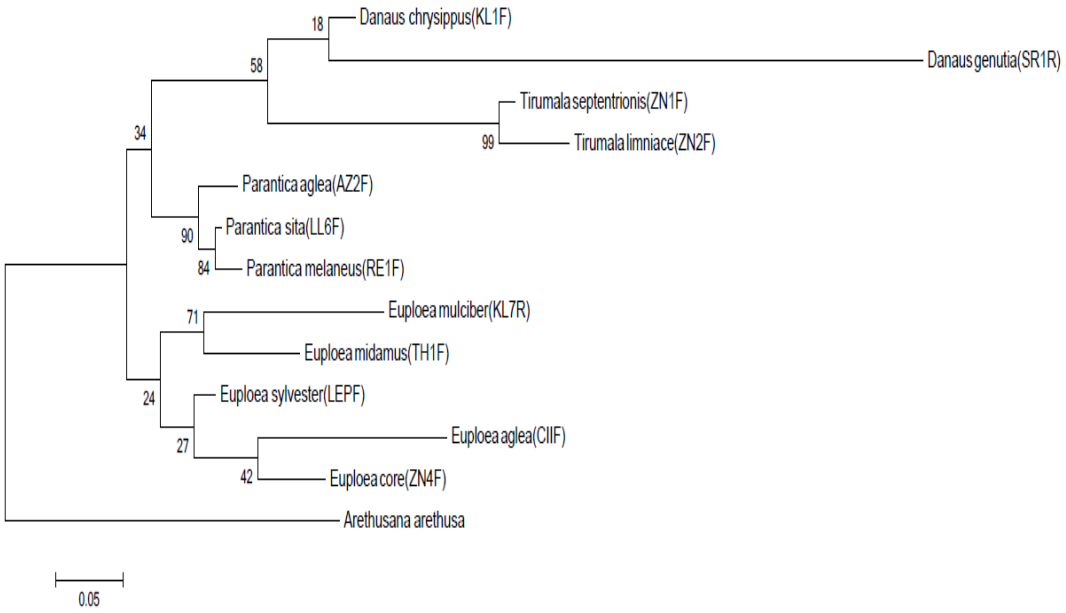

Fig. 11: Maximum Likelihood tree of D-loop gene marker among the 12 species of Danainae with *Arethusana arethusa* as outgroup. Here, the topology of the tree is almost similar to that of CO1 with the clustering of species according to their genus. The overall distance between the species being 0.492.

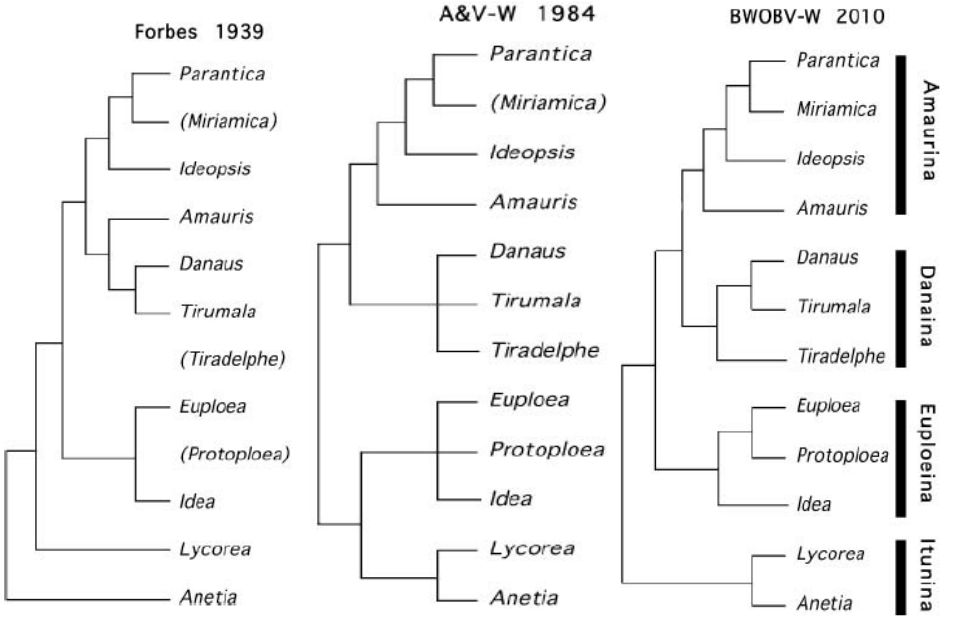

Fig. 12: Comparison of relationships of Danainae genera as implied by Forbes (1939); Ackery and Vane-Wright (1984); Brower and Wahlberg (2010) with the present study.

3.2. Comparison between the CO1 and D-loop gene markers between the 12 Samples of Danainae :

Thirteen species were successfully sequenced for CO1 while 12 out of 13 were sequenced successfully for D-loop. It is from these 12 successfully sequenced species that comparison between the two mitochondrial markers was drawn.

Maximum Likelihood Estimate of Transition/Transversion Bias for CO1 and D-loop is as follows:

CO1- the estimated Transition/Transversion bias (*R*) is 1.02; [+*G*], parameter = 0.4568; the maximum Log likelihood for this computation was -1004.681. There were a total of 257 positions in the final dataset and D-loop- the estimated Transition/Transversion bias (*R*) is 3.13; [+*G*], parameter = 1.0358); The maximum Log likelihood for this computation was -1160.742.There were a total of 174 positions in the final dataset. The transition/transversion bias between the two markers gives a higher value for D-loop (3.13) to that of CO1 (1.02), indicating a higher rate of mutation for D-loop between the two gene sequences.

Table 7: Comparing Tajima's test of neutrality for CO1 and D-loop

Gene	M	S	Ps	Π	D
Co1	12	70	0.272374	0.105589	0.79013
D-loop	12	105	0.603448	0.196273	-0.082926

(NOTE : 'm' = Number of sequences. 'S'= Number of segregating sites. 'Ps'= S/m. π = Nucleotide diversity. 'D'= Tajima test)

Table 8: Comparing Overall mean distance in CO1 and Dloop

Gene	D	S.E
CO1	0.128	0.017
D-loop	0.461	0.093

In comparison between the number of segregating sites (S) per site with the nucleotide diversity (π), the D-loop marker shows higher level of variability as compared to that of CO1, taking into account that a longer sequence of CO1 was used.

The overall mean distance as resolved among the 12 species of Danainae between the two markers indicated a greater distance in D-loop among the species to that of CO1. The overall mean distance, thus resolved indicated a greater range of variability given by D-loop.

Table 9: Comparison of the mitochondrial DNA molecular diversity between 12 species of Danainae in CO1 and Dloop

S.No		CO1	D-loop
1	Conserved sites	484/702	175/515
2	Variable sites	209/702	302/515
3	Parsimony informative sites	126/702	130/515
4	Singleton sites	80/702	142/515

(Note : Parsimony informative site = A site is parsimony informative if it contains two types of nucleotides (or Amino acids), and two of them occur with a minimum frequency of two; Singleton site = It contains atleast two types of nucleotides (or amino acids) with at most,one occuring multiple times.)

The 12 CO1 sequences have an average length of 702bp while that of D-loop have an average length of 515bp. D-loop, even being a shorter sequence, have a higher variable site (302/515) compared to a longer sequence of CO1 (209/702). The greater number of variable sites, parsimony informative sites and Singleton site indicated the greater variability presented by D-loop to that of CO1 for use as a genetic marker.

CHAPTER 4

DISCUSSION

The study of the phylogeny of Danainae butterflies using the Species found in Mizoram (4 genera/13 samples) have **showed congruency in part with the previous studies** of relationships among Danainae by Forbes (1939); Ackery and Vane-Wright (1984); Brower and Wahlberg (2010). The generic relationship implied by our cladogram is in relation with the cladogram from the previously mentioned works. They are remarkably similar, differing from one another only in the position of Itunina and *Amauris*. Relationships among the subtribes and genera are, for the most part, well supported.

RAPD PCR marker has proved to be useful in acquiring the genetic distances of the species but has been found to be of less significance particularly in the identification and classification among species. Although RAPD markers can easily detect differences among populations and species of different organisms, including plants (Ayres *et al.,* 1999), producing discrete bands of various lengths revealing genetic variations as well as similarities (Sharma *et al*., 2006), along with species specific bands, a major drawback of RAPD is the loss of complete genotypic information, due to the fact that most RAPD bands are dominantly inherited (Galluser *et al.*, 2004). Also, the high level of polymorphism generated by the RAPD markers led to difficulty in interpretation of the results, as the fragments generated by the primers

was difficult to assess whether they were specific to a subspecies or simply more frequent in some populations than in others.

Given that CO1 and D-loop are linked on the mtDNA and recombination is believed to be absent, a corelation between the levels of divergence in CO1 and D-loop could be expected (Ruokonen and Kvist, 2002). In comparing pairwise distances from CO1 third codon positions against the D-loop region in certain species, there is an exception where the CO1 third codon position evolved more than three times faster than the D-loop region (Vila and Bjorklund, 2004). However, this took into account only substitutions, and an inclusion of D-loop indels in the calculation of distances is required for a full estimation of the rate of evolution. Overall, the D-loop region showed a level of nucleotide diversity similar to or higher than that of CO1 (Vila and Bjorklund, 2004).

Cladistic relationships among the four genera and 3 subtribes of Danainae were inferred by analysis of the two mtDNA markers (CO1 and D-loop). The number of variable sites, segregating sites, R-value, +G value, Nucleotide diversity and Genetic distance values were observed in higher order in D-loop marker than CO1 with a lower Tajima's Test of neutrality and conserved sites.D-loop was thus, found to be more variable for this study.

Sister relationships were found between the two genera Danaus and Tirumala as well as the subtribes Amaurina and Danaina with the previous works by Forbes (1939); Ackery and Vane-Wright (1984); Brower and Wahlberg (2010). However, the relationship and the tree topology could also be a result of the relatively sparse sampling of species. The increase in sample could affect the overall tree topology.

Thus, from this study, we can conclude that, for phylogenetic studies, more conserved sequences (i.e., mtDNA) gave a more credible result in order to assess the genetic relationships between the Danainae species of butterflies.

SUMMARY

- 13 species of Danainae butterflies were collected from different sites of Mizoram.
- They were categorized into 4 genera and 3 subtribes based on morphological characters according to the information of Evans (1932), Wynter-Blyth (1957), Wahlberg (2009) and Isaac Kehimkar (2008).
- Four RAPD primers were used to determine the polymorphism between the danainae species with less significant characterization of the species according to their genera. However, genetic distances resolved from the RAPD data proved useful. Lowest dissimilarity value (0.105) found between C-IV and LEP which are the two species belonging to the *Euploean* genus while the highest value (0.478) was between RE-1 and ZN-4, belonging to *Parantica* and *Euploean* genera respectively. This is in relation with the previous works by Forbes (1939); Ackery and Vane-Wright (1984); Brower and Wahlberg (2010) and the present work on mitochondrial DNA where *Euploean* genus is a separate cluster from the three other genera (*Parantica, Tirumala* and *Danaus*)

- Two mitochondrial DNA markers (CO1 and D-loop) were used to resolve the phylogenetic relationship among four genera of the Danainae butterflies under study.
- Cladistic relationships among 4 genera and 3 subtribes of Danainae were inferred by analysis of two mtDNA markers (CO1 and Dloop).
- The cladistic relationships given by the resulting work was in congruence with the previous works of Danainae subfamily relationship as done by Forbes (1939); Ackery and Vane-Wright (1984); Brower and Wahlberg (2010).
- A brief comparison between the three markers used, viz, two mtDNA markers (CO1 and D-loop) and RAPD as nuclear markers from the study showed that molecular and phylogenetic characterization of Danainae subfamily using RAPD marker was difficult.
- The two mtDNA markers used had given a better resolution of cladistic relationships where a comparison between the two markers showed that D-loop was showing greater variability in this study between the two mtDNA markers.

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

LALHLIMPUIA PACHUAU, CATHERINE VANLARUATI AND N. SENTHIL KUMAR*

Department of Biotechnology, Mizoram University, Aizawl, Mizoram - 796 004, India

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

N. SENTHIL KUMAR
Department of Biotechnology, Mizoram University, Aizawl, Mizoram - 796 004, India