Molecular Characterization and Phylogeny of Heliconiinae Butterflies (Nymphalidae: Lepidoptera) in Northeast India

Thesis submitted in partial fulfillment of the requirements for the degree ofDoctor of Philosophy in Zoology

by

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CERTIFICATE

I certify that the thesis entitled "**Molecular Characterization and Phylogeny of Heliconiinae Butterflies (Nymphalidae: Lepidoptera) in Northeast India**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology by **ZOTHANSANGI** is a record of research work carried out during the period from 2010-2015 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or institution of higher learning

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Declaration of the Candidate

MIZORAM UNIVERSITY

January, 2015

I, **Zothansangi,** hereby declare that the subject matter of this thesis is the record of work done by me that the contents of this thesis did nir form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University or Institute.

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"God has brought me laughter and everyone who hears about this will laugh with me. Gen 21:6"

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I. INTRODUCTION

Butterflies are one of the most beautiful and colourful creatures on the earth. Along with moths, they are under Order Lepidoptera having two pairs of wings that are partly or wholly covered with tiny overlapping scales and have complete series of transformations (life cycle). Lepidoptera contains more than 180000 species in 128 families and 47 super families. Estimates of species suggest that the order may have more species and is among the four largest, successful orders along with Hymenoptera, Diptera, and Coleoptera (Resh and Carde, 2003). Lepidoptera are found in variety of habitats, but almost always associated with higher plants, especially angiosperms (Gullan and Cranston, 2004).

Butterflies are taxonomically well studied group, which have received a reasonable amount of attention throughout the world (Ghazoul, 2002). Butterflies are classified into two superfamilies; Hesperioidea, consisting of a single family Hesperiidae (Skippers) and Papilionoidea, having four families: Papilionidae (Swallowtails), Pieridae (Whites and Yellows), Nymphalidae (Brush-footed butterflies) and Lycaenidae (Blues) (Kehimkar, 2008). Butterflies are important as pollinators for many species of flowering plants although in general they do not carry as much pollen load as Hymenoptera. They are however, capable of moving pollen over greater distances (Herrera, 1987). Adult butterflies are considered opportunistic foragers that visit a wide variety of available flowers (Courtney, 1986). Butterflies are one of the important food chain components of the birds, reptiles, amphibians, spiders and predatory insects. Butterflies are also good indicator of environmental changes as they are sensitive and directly affected by changes in the habitat (Haribal, 1992). Butterflies are sensitive biota which gets severely affected by the environmental variations and changes in the forest structure and composition as they are closely dependent on plants (Pollard and Yates, 1993). Being good indicators of climatic conditions as well as seasonal and ecological changes, they can serve in formulating strategies for conservation (Kunte, 1997). Many butterfly species are strictly seasonal and prefer only a particular set of habitat (Kunte, 1997)

and they are good indicators in terms of anthropogenic disturbance and habitat quality (Kocher and Williams, 2000). The diversity of butterfly species is high in natural habitats than the modified ones (Nayak *et al.*, 2004). Variation in size, shape, colour and behavior within species of butterflies is quite common. Environmental factors such as geography, climate and season can also have their impact by influencing the movement, distribution and life cycle of butterflies, thus resulting in different forms. The four major types of variation found among butterflies are individual variability, sexual dimorphism, seasonal and geographical variability (Ackery and Vane-Wright, 1984; Kehimkar, 2008).

The **subfamily Heliconiinae** is an assorted group and includes the Costers, Lacewings, Fritillaries, Leopards, Vagrants, Yeomans and Rustic. The eggs of these groups are pale blue or yellow and are laid singly or in clusters. The larvae in general appearance are dark or conspicuous banded with numerous spines and may have a pair of dark horns on the head. Adults fly slowly to display bright warning colours (Beltran *et al.*, 2008).They are commonly called heliconians or longwings as the fore wings are always elongated tipwards. Eyes are large, antenna long and typically brightly pigmented and upper wings usually tawny in colour with transverse lines of black spots. All members of the subfamily possess yellowish abdominal scent glands from which the butterflies evert and emit acrid odour when disturbed (Ross, 1976). They are usually unpalatable to predators as larvae feed on distasteful plants characteristically Passifloraceae vines and Urticaceae.

Heliconiinae is one subfamily of Nymphalidae, 21 species have been described so far in India (Kehimkar, 2008). These butterflies have played a key role in understanding evolutionary biology and ecology and it would be difficult to point a group of Neotropical butterflies that have contributed more to our knowledge of the biological processes in the tropics. The derived members of the tribe have undergone rapid speciation and divergence, while also exhibiting impressive mimetic convergence in wing patterns. The subfamily

Heliconiinae has really only been delimited as it is now since 1991, when Harvey (1991) placed the argynnines and acraeines with the heliconiines. A recent phylogenetic study of the subfamily by Penz and Peggie (2003) suggests that the subfamily should be divided into 4 tribes. The phylogenetic relationships of various groups in Heliconiinae have been extensively studied, especially in the tribe Heliconiini by NSG (2009).

The vast amount of information gathered on this group spans a variety of topics in ecology, evolutionary biology and conservation biology (Muller and Beheregaray, 2010). They tend to show highly localized endemism in the region and high species diversity. However, the higher phylogenetic relationships of major groups of butterflies remain poorly hypothesised. This lack of knowledge is critical, since several disciplines in comparative biology (evolution of host plant preferences, mimicry and behavior) depend on robust phylogenetic hypotheses to provide a framework for interpreting the evolution of putatively adaptive character systems. Despite several recent important efforts to elucidate the higher level relationships of butterflies (Silva *et al.*, 2010), there is still only fragmentary knowledge about patterns of relationships among lineages within the subfamily, one of the most diverse groups of butterflies and has been the focus of several recent phylogenetic studies (Kodandaramaiah *et al.*, 2010; Muller and Beheregaray, 2010). Although many studies on morphological, ecological and molecular attributes of several species of Heliconiinae are available, very little is known about the species of the Oriental Zoogeographic region especially from the north-east India.

The **Northeast India** lies between 22°N and 29°5'N latitude and 88°00'E and 97°30'E longitudes, covering nearly 262379 sq. km. area and shares international border with Bhutan, China, Myanmar and Bangladesh (Chatterjee *et al.*, 2006). Average rainfall in this region often exceeds 2000 mm, hosting more than 50% of the total Indian butterfly species (Kehimkar, 2008). The region has been divided into two biogeographic zones - Eastern

Himalaya and Northeast India (Rodgers and Panwar, 1988). The Eastern Himalaya comprising of Arunachal Pradesh and Sikkim is more mesic due to high degree of precipitation resulting from direct confrontation of monsoon laid wind blowing from Bay of Bengal by abruptly raising hills. The Northeast India biogeographic zone (Assam, Nagaland, Manipur, Meghalaya, Mizoram and Tripura) is most significant one and represents the transition zone between the Indian, Indo-Malayan, Indo-Chinese biogeographic regions as well as a meeting place of Himalayan mountains with that of Peninsular India (Rao, 1994). In the tropical zone, besides several tree species like the towering Hollong and Cinnamon, several evergreen shrubs and woody climbers, tree ferns, screw pine, wild banana, giant bamboos and ferns are conspicuous in the hill forests. Pitcher plants, several orchids and rhododendrons are endemic to the regions (Kehimkar, 2008). The region is geographical 'gateway' for much of India's flora and fauna and as a result the region is one of the richest in biological values with vegetation types ranging from tropical rain-forest in the foothills to alpine meadows and cold deserts. The Northeast India contains more than one-third of the country's total biodiversity. The region represents important part of Indo-Myanmar bio-diversity hotspot, one of the global biodiversity hotspots (www.biodiversityhotspots.org).

This region needs imperative awareness for conservation because of high degree of host plant and butterfly endemism and the grave threats it faces. Global climate is changing rapidly with unpredicted consequences for the reason that elements of biodiversity responds closely to climate (IPCC, 2001). In this area, the shifting cultivation known as "Jhum" is the major threat to biodiversity of insects especially to butterflies. To salvage the issue of extinction crisis, we need to prioritize and target conservation strategies in relation to systematics (conventional and molecular) and biodiversity of butterflies. This approach is a realistic platform to build a much more flexible system, with the potential for the rapid and accurate identification of butterflies using modern tools.

A **phylogeny** is an evolutionary tree that shows how different species are related to each other (Baldauf, 2003; Harrison and Langdale, 2006). One of the fundamental applications of phylogeny is in classification. Phylogeny help to systematically classify organisms in an evolutionary framework and less prone to errors and individual biases (Sidow and Thomas *et al.,* 1994, Zhang *et al.,* 2012). Morphology based classification can be compared and contrasted with molecular systematic with phylogeny (Lewis 2001). Therefore, phylogenies can be built using molecular data or morphological data, but in recent times molecular data are increasingly being used.

RAPD-PCR (Random Amplified Polymorphic DNA-polymerase chain reaction) uses short synthetic oligonucleotides (10-12 bases long) of random sequences as primers to amplify nanogram amount of total genomic DNA under low annealing temperature by PCR where a single oligonucleotide of random sequence is employed and no prior knowledge of the subjected genome is required and can be used for detecting polymorphisms at many loci between species and populations (Williams *et al.,* 1990). This technique amplifies anonymous fragments of DNA from any genome. The profile of amplified DNA primarily depends on nucleotide sequence homology between the template and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites. The size distribution of amplified fragments varies among species. Closely related taxa have similar fragment distributions, while distantly related ones are more divergent (Bardakci, 1999). Thus, RAPD-PCR and has been widely used in the determination of population structure without prior knowledge of DNA sequences while giving a good resolution of genetic differences, genetic polymorphisms and genetic diversity in natural populations between species.

DNA Barcoding is a relatively new concept that has been developed for providing a rapid and accurate species identification using standardized DNA sequences as tags. In fact, it started with the seminal work of Hebert *et al.* (2003). In DNA barcoding, the unique nucleotide sequence patterns of small DNA fragments (400-800 bp) are used as specific reference collections to identify specimens and to discover overlooked species. Any barcoding system should aim to acquire data for at least a nuclear and an organellar gene from single specimen for study of evolutionary pattern and taxonomic and systematic studies (Blaxter, 2004). In barcoding technology, the major point of focus is the DNA macromolecule and every sample which has to be characterized based on DNA barcodes access to its mitochondrial DNA (Ghosh, 2012). Barcode sequence data provide a shared genomic corner stone for the variable repertoire of genes that are used to build the phylogenetic tree. It can be used as a link between the deeper branches of the tree to its shallow, species-level branches (Hajibabaei *et al.,* 2007)

The mitochondrial DNA **(**mtDNA) is a good choice for DNA barcoding because of its fast mutational rate which gives a significant variation between species, lack of introns, limited exposure to recombination and its haploid mode of inheritance (Lin and Danforth, 2004). These genes are generally easier to amplify than nuclear genes and conserved mitochondrial markers are widely available (Simon *et al.,* 1994). Mitochondrial genes have been for many years the most commonly used source of data for studies of insect molecular phylogeny, biogeography and phylogeography (Morgan *et al.,* 2009: Simon *et al.,* 1994). Hebert *et al.,* (2003) established that the mitochondrial gene Cytochrome *c* Oxidase 1 (CO1) can serve as the core of a global bio-identification system for all animal phyla. Rach *et al.*, (2008) have shown that mitochondrial gene NADH Dehydrogenase Oxidase Subunit 1 (ND1) is well suited as an alternative or complement to CO1, since ND1 sequences have been shown to be highly informative at different taxonomic levels in dragonflies.

Although many studies on morphological, ecological and molecular attributes of several species of butterflies from world over are available, very little is known about the Indian species especially in northeast India. This region also needs imperative awareness for conservation because of high degree of host plant and butterfly endemism and the grave threats it faces. The present study, targeting the systematic and biodiversity of the subfamily may contribute towards the knowledge about Heliconiinae butterfly diversity, distribution and its evolutionary relationships in the region.

II. REVIEW OF LITERATURE

2.1 Heliconiinae

The subfamily Heliconiinae is composed of a diverse group of species distributed widely in the New and Old World tropics, and it also includes the largely Holarctic fritillaries (Argynnini). This subfamily represent one of the best-known groups; for example, pioneering studies in mimicry, were based upon studies of the tribe Heliconiini (Turner, 1976; Smart, 1991; Thompson, 1994) making the group of special interest to radiation (Mallet, 1989). According to the most recent version of the classification of Nymphalidae, the subfamily comprises 504 species placed into 40 genera and 4 tribes. A recent phylogenetic study of the subfamily by Penz and Peggie (2003) suggests that the subfamily should be divided into 4 tribes.

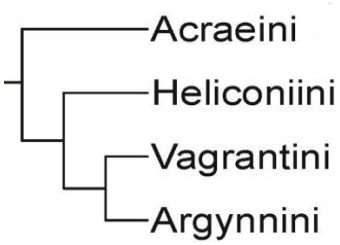


Figure 1. Four tribes of Heliconiinae (Penz and Peggie, 2003).

Acraeini - is a diverse group of unpalatable tropical butterflies. They are usually yellowish in colour with few dark markings and sparsely scaled wings. FW long and HW rounded. The center of diversity is in Africa, where there are hundreds of species involved in mimicry complexes that make their taxonomy a great challenge. It is not clear based upon the most recent data that any of the genera as currently circumscribed are monophyletic (Silva-Brandão *et al.*, 2008).

Heliconiini – Passion-vine butterflies have played a key role in understanding evolutionary biology (mimicry) and ecology (mutualism between insects and plants) (Brown

1981). The larvae of all heliconiines are spiny, have two spines on the head capsule, and can be either solitary or gregarious. Many species have irritant spines that probably discourage predators. The placement of eggs by ovipositing females is correlated with the systematic subdivisions within the Heliconiinae (Walhberg and Brower, 2008).

Vagrantini –This group of genera is distributed from east Africa through India, Sri Lanka, China, Taiwan, the Malay Archipelago and Australia (Fruhstorfer, 1911; Corbet and Pendlebury, 1992). Fruhstorfer separated this group from other fritillaries on the basis of the primitive genital organs, which are in strong contrast to the richly ornamented ones of *Argynnis* (Fruhstorfer, 1911). It includes Vagrant, Leopards, Rustic and Yeomans mainly Southeast Asian and Australasian genera (Walhberg and Brower, 2008).

Argynnini- commonly known as Fritillaries is a group of fairly uniform, small to medium sized butterflies. Almost all species have an orange-brown ground colour with a pattern of black spots and streaks on the upper side, and some pearly white to silvery white spots on the underside, particularly on the hindwing. Simonsen (2006) and Simonsen *et al.* (2006) reduce the number of traditional argynnine genera to six. Most Argynnini species are distributed in the temperate, alpine and arctic regions of the Holarctic (Walhberg and Brower, 2008).

2.2 Heliconiinae in India

In India, 21 species of Heliconiinae from 14 genera are recorded by Kehimkar (2008) while Kunte and Kodandaramaiah (www.ifoundbutterlies.org/#!/tx/8- Heliconiinae) recorded 21 species from 10 genera. Most of the species were found to inhabit the Himalaya, Northeast and South regions of India. Out of the total Indian Heliconiinae, 14 species are found recorded in the Northeast region and majority of the species found in Northeast region are

high elevation fliers usually above 500 meters upto 2500 meters above sea level (Kehimkar, 2008)

In Mizoram, Gupta (2007) reported 5 species from four genera under the subfamily Heliconiinae which were *Cethosia biblis, Cethosia cyane, Argynnis hyperbius, Phalanta phalantha, Vindula erota,* all under tribe Heliconnini. One species *Acraea issoria* under the subfamily Acraeinae was also reported. Zothansangi *et al.* (2011 and 2013) reported another three species *Cirrochroa aoris, Cirrochroa tyche and Vagrans egista* from the state of Mizoram.

2.3 Butterfly Distribution

The population status of butterfly species in any area would help us to understand the status of ecosystems as they are good indicator species (Karaman, 1992). The life history of Lepidoptera reveals that butterflies are exposed to a wide range of environmental influences and are highly sensitive to the changes in temperature, humidity and light levels (Erhardt 1985; Warren *et al.,* 2001). Climatic changes impact the diversity of species and are expected to exacerbate the ecosystems. The elevational distribution of various groups of insects and their diversity has been investigated in different mountainous regions of the world during the past century. The altitudinal distribution of butterflies and moths belonging to the order Lepidoptera has also been studied in various mountains at different latitudes. However, mostly the research on distribution of Lepidoptera has been carried out in various parts of South America (Boggs and Murphy, 1997; Fleishman *et. al.,* 1998). The elevational distribution and diversity of Asian Lepidoptera was studied by Adams (1985), Wolda (1978), Pyrcz and Wojtusiak (2002). In India, most research on distribution of Lepidoptera has been carried in the Southern and Central parts of India (Ramesh *et al.,* 2010; Shamsudeen, 2010; Tiple, 2011).

Despite the large amount of information accumulated on climatic and weather factors that affect a single or several species, detailed analyses are lacking concerning the effect of climatic conditions and their relative importance on butterfly community composition and dynamics. In tropical regions with distinct wet and dry seasons, many insect species attain maximum adult abundance during the wet season, probably in response to changes in plant physiology and growth (Didham and Springate, 2003; Wolda, 1989), in particular the abundance of new foliage (Fensham, 1994; Novotny and Basset, 1998; Shapiro 1975). In regions with no distinct dry season, adult abundance and activity are generally less variable (Hebert, 1980; Wolda and Galindo, 1981), although some species still show clear temporal fluctuations in relation to more subtle variations in rainfall and host-plant dynamics (Hamer *et al*., 2005). In southern Thailand, Boonvanno *et al*., (2000) found out that there was no significant correlation between butterfly abundance, species richness and relative humidity, temperature, and precipitation. In the temperate climate of England, there was a relation between temperature/precipitation and population size fluctuations in 28 butterfly species (Roy *et al*., 2001). In a Mediterranean climate in Spain, Stefanescu *et al.* (2004) found butterfly species richness to be negatively correlated to temperature and positively correlated only to precipitation, except in conditions of extreme cold and rain. Climate variables had strong effects on habitat generalists, whereas host-plant richness and habitat diversity contributed relatively more for habitat specialists. Considering total effects (direct and indirect together), climate variables had the strongest link to butterfly species richness for all groups of species (Menéndez et al., 2007).

In the humid tropics due to deforestation of primary forests, secondary forests and plantations are becoming increasingly widespread land-use systems in human dominated areas (Barlow *et al.,* 2007). Several studies (Bowman *et al*., 1990; Lawton *et al*., 1998; Ramos, 2000) have discussed the potential of butterfly diversity in secondary forests but

diversity and species richness of butterflies across different secondary vegetation gradients were poorly understood. Majumdar *et al.* (2012) revealed that mature secondary forests are more important for butterfly communities while exotic grasslands have a negative impact on species composition.

Regional or local checklists come in many forms. The only major zoogeographical regions for which complete, modern Lepidoptera checklists are available are North America (Hodges *et al.,* 1973) and Australia (Nielsen and Kristensen, 1996). A combination of features has conspired to render butterfly one of the most studied groups of organisms. Despite the unquestionable species richness, butterflies are far more homogeneous, structurally and ecologically, than the other larger insect groups. Paradoxically, the popular appeal of Lepidoptera may have adversely affected professional research on the order. The colourful vestiture of scales and hairs on the wings, body and appendages of Lepidoptera certainly often offers excellent diagnostic features at the lower taxonomic levels, but at the same time this vestiture conceals most of those exoskeletal traits found to be so useful in supraspecific classification, and which are more easily and routinely studied in insect taxa where the exoskeleton is exposed. The combination of a large number of species and little structural diversity can impede progress in the overall taxonomic treatment of the group (Kristensen *et al.*, 2007). The study of evolution frequently requires understanding the history of the population, species or clade under study. In analyses of processes of adaptation or molecular evolution, resolution of the relationships between species is required (Hurst and Jiggins, 2005).

2.4 Host Plant of Heliconiinae

The diets of many butterflies and moths change dramatically with development from herbivory in the larvae to nectarivory in the adults. Nutrition is a key determinant of reproduction therefore; reproductive strategies need to be considered in light of nutrient availability (Wheeler 1996). The host plants of Heliconiinae butterflies are usually distasteful. *Acraea violae, Vindula erota* and *Cethosia* feed on Passifloraceae, species of *Argynnis, Acraea issoria* and *Phalantha alcippe* usually feed on Violaceae while *Cirrochroa, Cupha, Rustic, Phalantha phalanta* feed on Flacourtiaceae. Rustic and Leopards lay eggs singly while Costers and Lacewings are gregarious. The caterpillars of heliconiinae are usually dark or conspicuous banded with numerous spines and may have a pair of dark horns on the head (Kehimkar, 2008). Host plants vary greatly in their food value for different butterflies, the evolution of host plant specificity must ultimately be based on the nutritional requirements. Host choice is extremely selective, species specific and quality is the key determinant of fecundity of butterflies and it can also influence feeding, growth and reproduction (Awmack and Leather, 2002; Shobana *et al.*, 2010). Butterfly diversity may serve as a surrogate for plant diversity because butterflies directly depend on plants, often in highly co-evolved situations (Singh, 2010). Ackery (1988) reviewed the host plant associations of nymphalid butterflies and concluded that if used in conjunction with morphological characters, host plant associations should be contribute toward a resolution of the inter-relationships of the many widely recognized groupings within the family.

The availability of plant nutrient and species of host plant can strongly influence the physiology of butterflies. Moreover, the changes of phytochemicals in the host plants may play an important role in affecting the performance (growth and food utilization) and efficiency of butterflies (Huang *et al.*, 2008). Physiological and biochemical changes in a

plant due to environmental factors may alter its nutritional value for herbivores. In some cases, these host plants' nutritional and allelochemical changes might improve the quality of the host plant and therefore, can be considered beneficial to herbivores (Mattson and Haack, 1987). However, most studies have indicated various responses of herbivores against host plants which are induced with phytochemical changes (Waring and Cobb, 1992). Larvae that fed on less nutritious foliage consumed more food (higher consumption rate and longer feeding period) but the overall performance was poor than larvae that fed on more nutritious foliage (higher growth rate and food processing efficiencies). From a plant chemistry perspective, highly-fertilized plants have higher nitrogen and water contents, but have similar amount of defensive compounds (Huang *et al.,* 2008). Quantitative estimation of phytochemical parameters and identification of the well suited variety of host plant in some Lepidoptera become primary importance since the growth and development of larvae are mainly influenced by yield and nutritional quality of leaf used as feed (Murthy *et al.,* 2013). Insects that feed on mature grasses ingest far lower allellochemicals concentrations as well as a far lower diversity of these chemicals, compoared to insects feeding on dicots (Berneys and Barbehenn, 1987). Little is known about the antioxidant enzymes of Lepidoptera, previous work in insects were done primarily on caterpillars (Ahmad *et al.,* 1987, Pritsos *et al.,* 1988; Barbehenn *et al.,* 2001). A variety of antioxidant enzymes protect tissues and extracellular fluids from oxidative damage. The detoxifying enzymes SGOT and SGPT deactivate the toxicity and pesticidal activity of *Chrystella parasitica* in some Lepidoptera. (Balasubramanian et al., 2008)

2.5 Molecular markers

The incorporation of molecular markers (sequence data) has revolutionized phylogenetics over the past two decades (Hajibabaei *et al.,* 2007) and the application of molecular methods in insects and particularly in Lepidoptera has increased exponentially within the last decade (Zhang *et al.,* 2013; Fordyce, 2010; Hebert *et al.,* 2003; Brower and DeSalle, 1998; Weller *et al.,* 1996; Brower, 1994; Sperling and Harrison, 1994). Many of these studies are based on sequences from single gene, usually in mitochondrial DNA. Different genes are constrained to change in different ways (Simon *et al.* 1994) and can thus be informative at different levels of systematic hierarchy. It has recently been acknowledged that combining data sets can give more reliable results (Kluge, 1989; Brower and Egan, 1997). The ideal molecular systematic approach would include both nDNA and organellar DNA such mtDNA markers and that discrepancies between partitions be used to enrich the interpretation of the evolutionary history of the taxa under consideration (Rubinoff and Holland, 2005).

2.5.1 RAPD-PCR

Random Amplified Polymorphic DNA-polymerase chain reaction provides a very versatile and widely applied biotechnology technique in entomology. Molecular techniques based on DNA sequence polymorphism are now used in population genetics studies, systematic and molecular taxonomy to get an answer to systematic related problems (Nagaraja and Nagaraju, 1995; Tom *et al,.* 1995; Weng *et al.,* 1996; Zhou *et al*., 2000; Zakharov, 2001). The use of a single primer (usually 8-10 bp long) that attaches to both strands of DNA and low annealing temperatures increase the likelihood of amplifying multiple regions representing a particular (multi) locus (Arif and Khan, 2009).

Nuclear coding sequences are commonly used to draw phylogenetic relationships among related species of lepidopterans and the sequence data inferred relationships among nymphaliid butterfly taxa which were remarkably similar to the trees based on combined morphological and ecological data (Alaine *et al.,* 2005). On the basis of RAPDs, Wilkerson *et al.* (1993) distinguished two African populations of cryptic mosquito species and phylogenetic relationships among the species were studied among the groups of *Drosophila* by Robe *et al.* (2005). RAPD-PCR was successfully used to study for characterization, genetic variations and population structure of different groups of moth (Lepidoptera) by Tom *et al.* (1995); Weng *et al.* (1996) and Zhou *et al.* (2000) respectively and revealed that the technique is useful for various entomological applications (Heckel *et al.,* 1995; Dowdy and McGaughey, 1996). Genetic polymorphisms in natural populations (Haag *et al.,* 1993) have been studied and genetic diversity between species of Nymphalidae was described by Galluser *et al.* (2004). Zakharov (2001) studied natural hybridization between two species of Papilionid butterflies using RAPD-PCR. 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; Tiple *et al.,* 2010). Vanlalruati *et al.* (2011) used fifteen random primers for comparing molecular data with morphological data of six *Junonia* species and showed the differences in the branching pattern between the morphological and molecular data, signifying the need for using molecular tools for taxonomic classification as well as in understanding the evolutionary relationship. RAPD appears to be useful in differentiating species, subspecies and strains in insects (Ballinger- Crabtree *et al.,* 1992; Canis *et al.,* 1993 and Dowdy and Mcgaughey, 1996). However, there are several disadvantages that must be taken into account when using the technique. RAPD-PCR cannot distinguish whether a DNA segment is amplified from a

locus that is heterozygous or homozygous as RAPD primers are dominant (Bardakci, 1999). Although RAPD is a simple and inexpensive technique, its major limitation is the inability to differentiate between homozygote and heterozygote, this marker is therefore regarded as a dominant type (Arif and Khan, 2009). The drawback reduces the information provided by each locus because each primer can amplify several loci and there are many commercially primers, the loss of information per locus can be easily balanced by using a high number of loci (Jain *et al.,* 2010).

2.5.2 Mitochondrial DNA

Mitochondrial DNA is a histone free, double stranded circular molecule, much smaller than the nuclear chromosomes. In animal cells, it is less than 20000 bp and is a circular duplex (Wolstenholme and Jeon, 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, and 2 for rRNA (Figure 2). The 13 polypeptides involved in the oxidative phosphorylation process are ND1-4, -4L, -5, and -6 of complex 1(NADH Dehydrogenase); cytochrome b oxidase (cytB) of complex III (bcl complex); COX1-3 of complex IV (cytochrome c oxidase); and ATP6 and -8 of complex V (ATP synthase). These 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. There is a non coding region approximately 1000 base pairs long, the control region or d-loop (Ghosh, 2012).

2.5.2.1 Cytochrome Oxidase Subunit 1

The Consortium for the Barcode of Life (CBOL) has so far accepted mitochondrial encoded cytochrome oxidase 1 (**CO1**) from complex IV as the default DNA barcode region for vertebrates and insects and promotes its use as many other clades as possible (Hebert *et al.,* 2003). Its accuracy depends mainly on the separation between intra-specific (within-species) variation and interspecific divergence of the selected DNA sequence. CO1 seems to possess a greater range of phylogenetic signal than any other mitochondrial gene. In common with other protein coding gene, its third position nucleotides showed a high incidence of base substitutions, leading to a high rate of molecular evolution (DeSalle *et al.,* 1987). Due to its rapid rate of evolution and short coalescence time, the phylogenies based on mtDNA sequences are often well resolved even between recently separated populations and species complexes (Avise, 1994). The mtDNA degrades slower than nuclear DNA hence can be used in degraded or old samples (Arif and Khan, 2009).

Hebert *et al.* (2003) created CO1 profile for 200 lepidopteran species; it was 100% successful in correctly identifying the specimens. Comprehensive analysis of DNA barcodes from adult and caterpillars of neotropical skipper *Astraptes fulgerator* exposed a formerly described single species comprised in fact a total of 10 species, each with distinct ecological characteristics (Hebert *et al.,* 2004). In Lepidoptera, COI has been used for resolving relatively recent divergence events, particularly at the genus and species level (Caterino *et al*., 2000; Zimmermann *et al*., 2000). It is useful in fully resolving topologies within species of the Nymphalidae (Wahlberg *et al*., 2005) as well as within groups of the genus *Heliconius* (Brower and Egan, 1997). Janzen, Hallwachs and colleagues employed barcoding to create a reference sequence library for more than 25000 specimens representing >2000 species of moths and butterflies and their parasitoids (Janzen, 1973; 2003; 2004; Janzen *et al.,* 2005). Even the introduction of species or subspecies to some new areas can be determined

efficiently by using these molecular markers. Rod *et al.* (2006) determined the single introduction of the potential citrus pest *Papilio domeleus* in Domonican Republic through mitochondrial Cytochrome oxidase-I gene sequences and confirmed that the butterflies had originated in Southeast Asia.

2.5.2.1 NADH Dehydrogenase 1

The NADH dehydrogenase complex (or mitochondrial respiratory complex I) catalyzes the oxidation of NADH by ubiquinone. This reaction is linked to proton transfer across mitochondrial membranes. Complex I is composed of a large number of subunits, several of which are encoded by mitochondrial DNA (mtDNA). These genes (for short, ND genes) have been identified in a variety of organisms including insects (Clary *et al.*, 1988; Garesse, 1988). Nosek and Fukuhara (1994) found six new open reading frames for NADH dehydrogenase subunit proteins of different organisms, i.e., ND1, ND2, ND3, ND4L, ND5, and ND6 in yeasts, in which the phylogenetic trees generated from ND1 and cytochrome oxidase subunit 3 sequences of yeast revealed that mitochondrial ND gene sequences tend to diverge much faster than other mitochondrial genes

Weller and Pashley (1995) used sequenced data from ND1 gene of the mitochondrial DNA to determine the sister taxon to butterflies and the relationships among the macrolepidopteran superfamilies. While current barcoding studies have primarily focused on a single marker gene (CO1) gene as a source for identifying diagnostic barcodes (Hebert *et al.*, 2003; Armstrong and Ball, 2005; Blaxter *et al.*, 2005; Janzen *et al.*, 2005), other markers have been suggested as equally well suited (Markmann and Tautz 2005; Monaghan *et al.*, 2005; Savolainen *et al.*, 2005). Hadrys *et al.* (2006) used mitochondrial gene NADH Dehydrogenase Oxidase Subunit 1 (ND1) sequence analysis in dragonfly studies that

revealed strong inter and intraspecific differences in the population structures of all species. The phylogenetic status of Afrotropical galerucines (beetles) was investigated with molecular and morphological analysis by Stapel and friends and from their study, the tree based on ND1data set showed much lower resolution in the basal branching pattrern than that of ITS data set but it resolved phylogenetic relationships between closely related species (Stapel *et al.*, 2008). ND1 gene alongwith other mitochondrial markers was successfully used to present the phylogeny of butterflies belonging to the genus *Euphydryas* (Zimmerman *et al.*, 2000). Wahlberg and Freitas (2007) used ND1 gene to infer the phylogenetic relationships of genera and tribes in the ecologically and evolutionarily well-studied subfamily Nymphalinae. Comparing ND1 and 16S nucloetide sequences, the phylogenetic relationships among species of genus *Parnassius* and its related taxa were analysed and the results were in agreement with other previous phylogeny based on other mitochondrial genes (Katoh *et al.*, 2005).

2.5.3 Distal-less genes (Colour pattern)

Butterflies have uniquely vivid and varied wing-colour patterns. Throughout their evolutionary history, organisms have evolved numerous complex adaptations to increase their chances of survival and reproduction. Butterflies have decorated their wings with bright coloured scales to scare off predators. Recent studies showed that these traits appeared to be built by number of genes (Monteiro and Podlaha, 2009) such as *wingless* genes, *Ultrabithorax, aristaless, distal-less and doublesex*. 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). Gene sequence data provide an excellent source of characters that are likely to be independent of the morphological and ecological characters under study. In addition, the mode of

transmission and genetic basis of sequence data can be determined explicitly and they facilitate estimation of evolutionary rates (Avise, 1994).

The first molecular clues have been provided about the developmental mechanisms and evolution of eyespot pattern with respect to *dll* (Nijhout, 1991; Brakefield *et al*., 1996; Reed and Serfas, 2004). Exuberant diversity has evolved in colour pattern, between species and often through genetic polymorphism and phenotypic plasticity within a species. From *dll* expression study, the divergence of species having eyespot and ones lacking eyespot was observed (Brakefield *et al*., 1996).

Computational packages and online servers are the current tools used in the protein sequence analysis and characterization (Sivakumar *et al.,* 2007). The physicochemical and the structural properties of the proteins are well understood with the use of computational tools. Today, number of computational tools has been developed for making predictions regarding the identification and structure prediction of proteins. The statistics about a protein sequence such as number of amino acid, sequence length, and the physico-chemical properties of a proteins such as molecular weight, atomic composition, extinction coefficient, GRAVY, aliphatic index, instability index, etc. can be computed by computational tools for the prediction and characterization of protein structure. The amino acid sequence provides most of the information required for determining and characterizing the molecule's function, physical and chemical properties. Sequence analysis and physicochemical characterization of proteins using biocomputation tools have been done by many researches and reported (Yuri, 2003; Sivakumar, 2006 and 2007; Ling, 2007; Kumar, 2005). The objective here is to retrieve and characterize the *distal less* genes of Heliconiinae butterflies from data base and study their physico-chemical properties and to test the gene as marker for inferring phylogeny of butterflies.

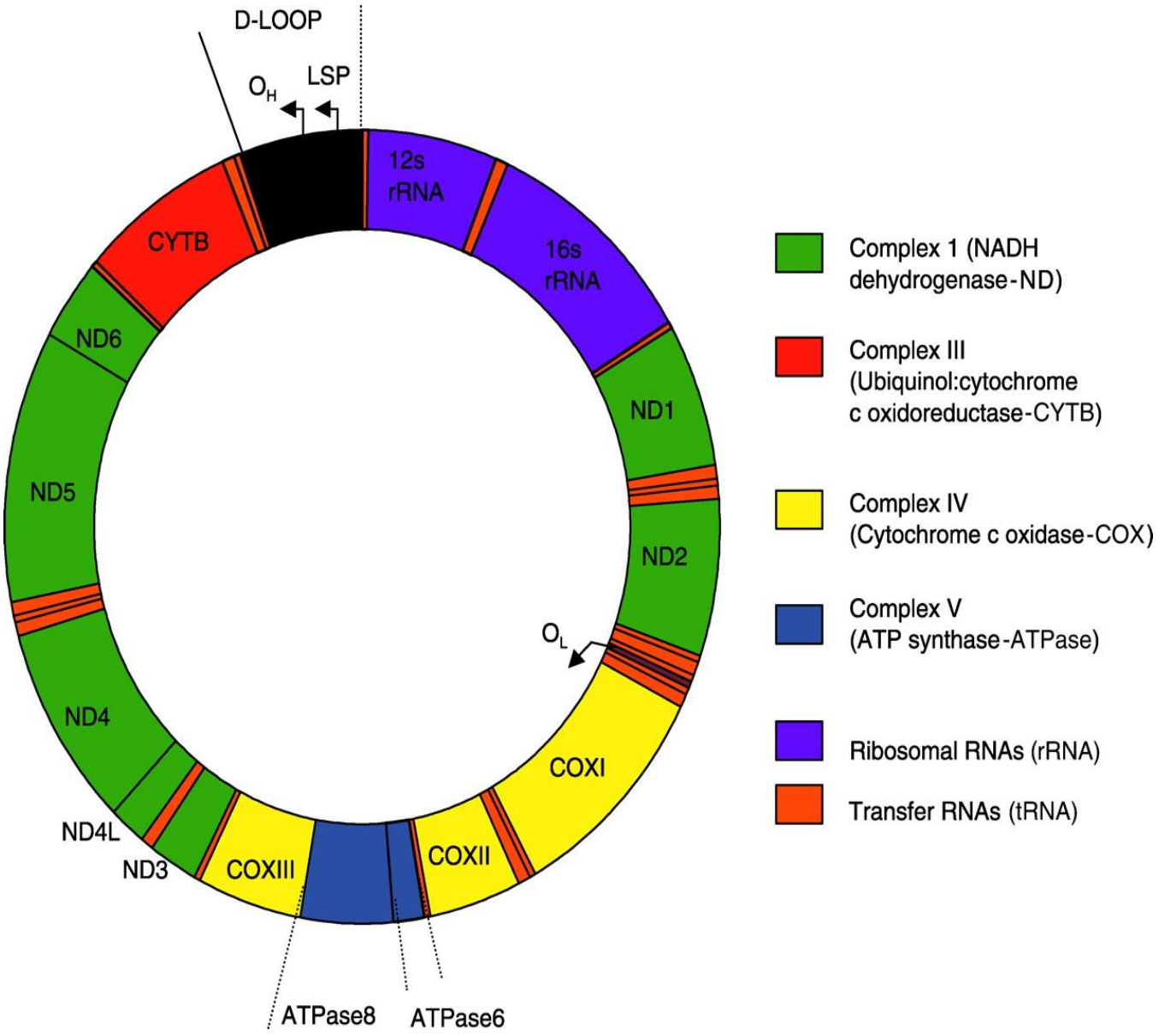


Figure 2. Mitochondrial gene markers showing ND1 and CO1.

2.6 Phylogeny of Heliconiinae

The incorporation of sequence data has revolutionized phylogenetics over the past two decades (Pagel, 1999) and an increasing number of large scale projects are underway to resolve different branches of the Tree of Life (http://tolweb.org). Barcode of Life projects create a perfect taxonomic sampling environment for conducting phylogenetic studies on different branches of the Tree of Life and this sampling has become a key factor in obtaining a robust phylogeny (Zwickl and Hillis, 2002; Pollock *et al.*, 2002).

The classification of the nymphalid subfamily Heliconiinae seems to be in a perpetual state of refinement. Although this may be troublesome to those who seek stability, over the course of 250 years it has linked generations of systematists by a thread of evolutionary thought. The Heliconiinae originated as section Heliconii of the genus *Papilio* (Linnaeus, 1758). A century later, Doubleday (1848) placed Neotropical butterflies with elongated wings and a closed hindwing cell in the family Heliconiidae. The naturalist Bates (1862) then made a major step towards understanding the relationships among Neotropical Heliconiidae and Old World Danaidae and Acraeidae. He divided Doubleday's Heliconiidae into two groups: acraeoid Heliconiidae, including Heliconius and Eueides; and danaoid Heliconiidae, including genera classified currently in the Danainae and Ithomiinae. The subfamily, as it is now, has been delimited really only since 1991. Kirby (1871), Muller (1886), Fruhstorfer (1911), Michener (1942), Ehrlich (1958), Ackery (1984, 1988), Harvey (1991), Freitas (1999) and Brower (2000) contributed to this group of butterfly classification that span 129 years. These eleven studies differ in their intent; examined species, sources of data and methods of analysis, yet all agreed that *Acraea, Heliconius, Cethosia* and *Argynnis* are closely related. In 2003, a comprehensive phylogenetic analysis of this group of butterflies based on early-stage and adult morphology was done by Penz and Peggie (2003). In their

study, parsimony analyses of forty-nine species in twenty-nine genera indicated that Heliconiinae can be divided into four main groups: (1) Pardopsis, Acraea and Actinote; (2) Cethosia plus Neotropical genera; (3) Oriental genera and (4) fritillaries. In their findings, argynnines were the most derived monophyletic group within Heliconiinae implying that species diversification within this group occurred more recently than the emergence of ancestral acraeines and Neotropical heliconiines. The classification of the Heliconiinae was then revised based on their results. However, recent morphology and DNA-based analyses (Freitas, 1999; Brower, 2000) by contrast, placed Neotropical taxa as more derived than acraeines, fritillaries and other heliconiines as the Neotropical taxa possess characters not present in the latter taxa.

The genetics and phylogeny of Heliconiinae have been subjected to various analyses and proposed models (Emsley, 1964; Brown, 1979) and the biogeography of the group is well-known (Penz and Peggie, 2003; Brown, 1987). Systematic analysis of the evolution of mimicry in *Heliconius*, since Eltrigham (1912) has been considered as a model that provides an opportunity to develop an integrative theory of biodiversity (Gilbert, 1984). Heliconians are well-suited to outdoor insectaries and glasshouses and thus are fit candidates for genetic and physiological experimentation. This has permitted the accumulation of enough genetic information to give a considered understanding of the evolution of Mullerian mimicry (Macdougal and Stamp, 1998; Papageorgis**,** 1975; Brown, 1981). The Heliconiinae, has recently been expanded following recent higher-level taxonomic changes, although new taxa continued to be described, even in recent years (Huertas, 2004) It has recently been proposed that the subfamily Heliconiinae be enlarged to include various other groups traditionally treated as separate families or subfamilies, such as the Acraeidae and Argynnidae (Harvey, 1991). Following this approach, Heliconiinae has been organized in four tribes: Heliconiini, Acraeini, Vagrantini and Argynnini (Penz and Peggie, 2003; Harvey, 1991).

Recovering higher-level phylogeny naturally is complicated because long divergence times can add noise to all character systems, and limited taxon and character sampling can cause further analytical problems (Sanderson, 1996; Graybeal, 1998; Zwickl and Hillis, 2002). Obtaining sequence data is needed to understand the basis for any incongruence between DNA and morphology (Brower, 2000; Freitas, 1999), an issue of pressing importance in recent phylogenetic analyses of butterflies and also a detailed analysis of Oriental genera and a detailed analysis of fritillaries should be considered for future analyses of Heliconiinae (Penz and Peggie, 2003).

Despite the long-standing interest in these butterflies, the phylogenetic relationship among the various tribes and genera has remained remarkably obscure especially in the Oriental butterflies. Improving our understanding of the phylogenetic resolution of such scientifically popular taxa should be a high priority, so that this abundance of knowledge can be placed in an evolutionary frame work (Wahlberg *et al.*, 2005). Several phylogenies of Heliconiinae have been proposed, but though looking reasonable each and 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. Hoping that the present work on the genes mentioned will contribute and advance evolutionary relationship and systematics of Heliconiinae and may establish a set of suitable molecular markers which can either confirm or decisively contradict phylogenetic results based on morphological characters. The present project work here may serve as a guide to future systematic work on Heliconiinae and Nymphalidae as well by providing a preliminary diversity and phylogenetic framework in the region.

2.7 OBJECTIVES

The main aim of the present investigation is to study the status and evolutionary relationships of Heliconiinae butterflies by the following objectives:

- Diversity of Heliconninae butterflies in Northeast India (Distribution pattern and Effect of leaf quality on the life cycle of *Acraea issoria*)
- Molecular characterization and phylogeny among the Heliconiinae using molecular markers (RAPD-PCR and DNA Barcoding techniques)
- Physico-chemical characterization structural elucidation of *distal-less* genes (Colour pattern) using computational tools and servers.

III. MATERIALS AND METHODS

3.1. Survey, sample collection and identification

Butterfly species belonging to Heliconiinae subfamily from Northeast region were surveyed. Direct searching and observation with opportunistic sample collection method was used for collecting butterflies from different habitats. In this method, target species or group of species (Heliconiinae) was observed visually and it was a very effective method for day flying butterflies, dragonflies, damselflies and moths (Sutherland, 1996). Adult butterflies were caught with butterfly net and were killed by pinching its thorax with thumb and fore finger. The killed butterflies were immediately kept in butter paper envelopes. All the specimens collected were identified, labeled and kept in refrigerator and the legs were stored in labeled centrifuged tubes and preserved in 95% alcohol at 4ºC till further analysis (Rondon and Korp, 2010). Adult specimens were identified with the help of identification keys provided by Evans (1932), Wynter-Blyth (1957), Ehrlich (1958), Ackery (1984), Haribal (1992) and Kehimkar (2008). {Permission for sample collection was taken from the Chief Wildlife Warden/concerned forest officers for the respective states}

3.1a. Sites of Collection

Heliconiinae butterflies were collected from several habitats throughout the year for the period of four years (2009-2013) in Assam, Meghalaya, Sikkim, Tripura, Arunanchal Pradesh and Mizoram (Fig. 3). The local sites from each state with its coordinates were given in Table 1. The collection sites vary in elevation, climates and vegetation but were usually along streams, roadside gardens, secondary forests and parks.

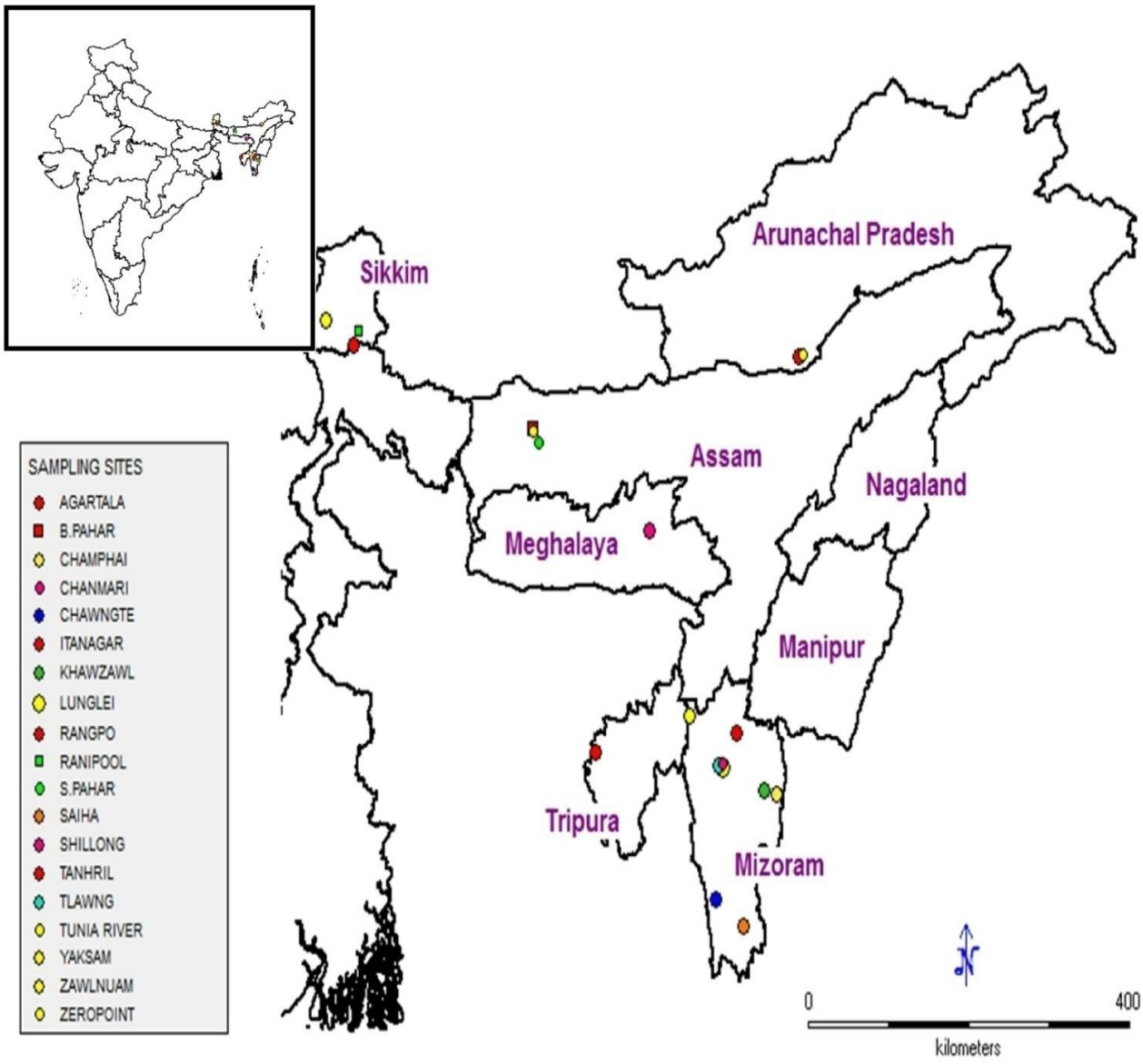


Figure 3. Map showing collection sites sites in Northeast India.

Table 1. Sampling sites with coordinates.

Different States	Collection Sites	Coordinates
1.Mizoram	Tanhril	N 23°43.940' E092°39.826'
	Chanmari	N23°44'19" E92°43'02.1"
	Tlawng	N 23°42'43" E 92°39'51"
	Saiha	N 22°24.021' E092°57.554'
	Lunglei	N 23°42.192' E 092°42.863'
	Zolnuam	N 24°07.953' E092°19.994'
	Chawngte	N22°37'17" E92°38'24"
	Champhai	N 23°28.701' E093°19.585'
	Khawzawl	N 23°30.896' E093°11.193'
2.Meghalaya	Barapani	N 25°.39'9" E 91°52'44"
	Nehu Campus	N 25° 36'44" E 91° 53' 51"
3.Tripura	Agartala	N 23°50' E 91°16'
4.Sikkim	Ranipool	N27°16.841' E088°35.729'
	Rangpo	N27°10.532' E088°31.992'
	Yuksam	N27°21.915' E088°13.281'
5.Assam	Bageswari pahar	N 26°28.344' E090°34.102'
	Tunia river	N 26°28.201' E090°33.791'
	Sunyasi pahar	N 26°22.308' E090°37.874'
6.Arunachal Pradesh	Chimpu	N 27°04.584' E093°35.162'
	Zero point	N 27°05.755' E093°37.370'

3.2 Distribution pattern

Study of butterfly diversity was done in family Nymphalidae with respect to forest types and Subfamily Heliconiinae of Mizoram with respect to vertical distributions.

3.2.1 Diversity of Nymphalidae butterflies in different forest types of Mizoram

The simple classification of forests in Mizoram falls under three broad types (Fig. 4):

1. Tropical Wet Evergreen Forest type
2. Tropical Semi-Evergreen Forest type
3. Mountain Sub-Tropical Forest

Tropical wet-evergreen forest (Forest type 1): This type of forest is found in western part of Mizoram adjoining Bangladesh and Tripura as well as Assam and usually has high precipitation. The vegetation comprised of *Gmelia arborea, Michelia champaca, Dysoxylum binectariferum, Cordic wallichii*. Different bamboo species like *Melocana bambusoides, Bambusa tulda, Dendrocalumus* spp., *Teinostachyum dullooa* were also found. *Bischofia javanica, Eugenia jambolona, Fiscus benghalensis, Podecarpus neirifolia, Sapium insigne, Artocarpus lokoocha* were species of ecological importance found in this forest type. *Phrynium capilatum, Caryotaurens* spp. *Punanga gracilis and Thysonoloena agrestis* thrived under the thick jungles (Pachuau, 1994). Eight sites from different parts were observed and studied from this forest. Buhchang (Kolasib), Sairang, Zawlnuam (Mamit) Chawngte (Chawngte) Dampa (Mamit), Lawngtlai (Lawngtlai). January was the coldest month with average temperature of 19°C while August with average temperature 30°C was the highest during the survey period. July had the highest humidity which was 97% and 562.7 mm of rainfall in September was the highest during the survey period.

Tropical semi-evergreen forest (Forest type 2): This type of forest covers 50% of the total area in Mizoram and occupies the central part of the region. The dominant species were *Schima wallichii, Cedrella tuna, Tarminalia mycriocarpa, Tremeles flora* and *Duabanga sonneratiodes.* The most common species among the variety of this forest are *Lyonia ovalifolia, Quercus helferiana, Saurauja punduana, Erythrina stricta, Helica excela, Albizzia stipulate, Bauhinia variegate, Bombox malabricum* and *Sapindus barak* (Pachuau, 1994). From this area of forest type 2, ten sites were covered for the present studies-Sairang, Sihhmui, Tlawng, Reiek roadsides, Mizoram University Campus, Thenzawl, Tlangnuam, Bawngkawn, Sailam, Hmuifang, Sumsuih and Durtlang. During the survey period, January got the lowest average temperature (18°C) while August (24.8°C) got the highest average

temperature. Highest Humidity (97%) was observed in August whereas highest rainfall (1168.6 mm) was recorded in September.

Mountain sub-tropical forest (Forest type 3): This type of forest is found on the higher elevation, mostly in the eastern part. The vegetation of this type is dominated by *Rhododendron arboretum, Quercas dealdata, Prunus ceracoides, Myricanagi, Quercas icana, Emblica officinalis, Rhus javanica, Pinus kesiya, Clerodendron colebrookianum, Rubus ellipticus, Rubus sirmanicus, Didymochlaena truncatula, Quercas serrata, Quercas xylocarpa* (Pachuau, 1994). Khawzawl, Mualkawi, Tiau, Champhai, Khawbung, Keifangtlang were the sites observed from this forest area. January was the coldest month having average temperature 14.5°C whereas May showed the highest temperature of 23.75°C in a year. July had the highest humidity (90.8%) and September received the heaviest rainfall of 801.46 mm during the survey period.

3.2.2 Sampling methods

Sampling was done by using direct searching and observation with opportunistic sample collection method (Sutherland, 1996) with slight modification of transect method described by Pollard (1977) in the three different forest types of Mizoram from January, 2010 to December 2013. Line transects of about 1 Kilometer in length and 5 meter in width was laid in each of the sites selected. Each transect was observed and sampling was done usually under appropriate weather conditions (www.ukbms.org)

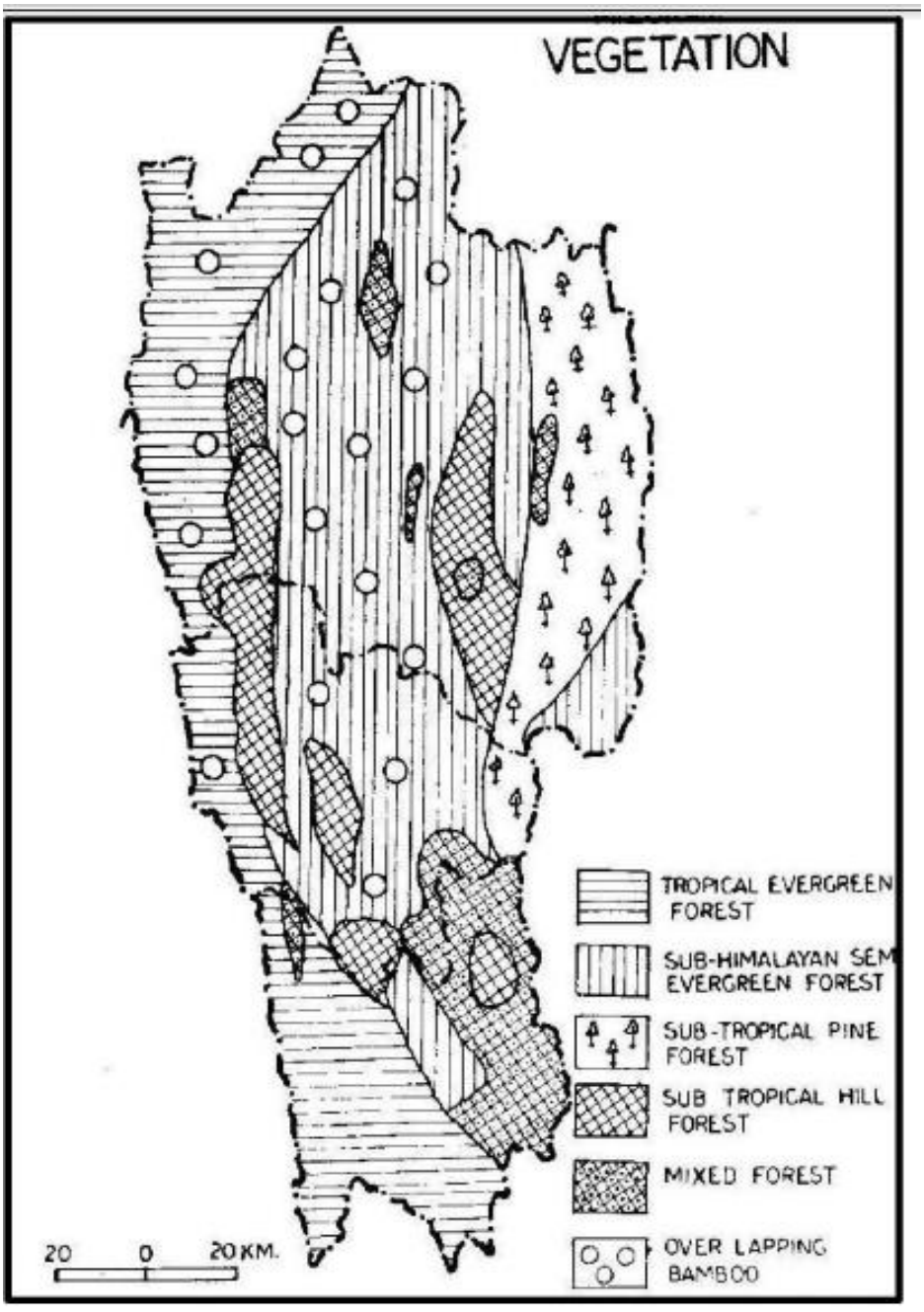


Figure 4. Types of forests in Mizoram (Permission from Pachuau, 1994).

3.2.3 Vertical distributions of Heliconiinae in Mizoram

For the diversity study with respect to Vertical distribution, the altitudinal range in Mizoram was divided into 7 categories as <200 m, 201 m – 400 m, 401 m – 600 m, 601 m – 800 m, 801 m – 1000 m, 1001 m – 1200 m and 1201 m – 1400 m covering 7 districts out of the total 8 districts of the state (Table 3). Field study was done by using the method of sampling along the elevational transect following Pyrcz and Wojtusiak (2002) which consisted of 22 collection sites (Table 3) with lowest collection site at Zawlnuam which is 45m ASL and highest collection site at Champhai with 1400 m ASL. Field survey along transects, in different types of vegetation and habitats on sunny days at different seasons of the year.

The distribution of each species in all the seven altitudinal ranges have been generated from the data collected from all sampling sites. Shannon and Simpson diversity indices were calculated as a measure of diversity at each altitudinal range. Berger-Parker dominance has also been evaluated for each altitudinal range.

3.2.4 Diversity calculation

The total number of individuals collected under each identified species in different habitats was recorded and diversity indices namely Berger Parker (dominance) index, Shannon's diversity indices (H), evenness index (e^H/S) and Simpson index were calculated using Biodiversity Pro software (McAleece *et al.*,1997) and Sigma Plot 12.0. The diversity was statistically correlated with meteorological data from each of the sites (temperature, rainfall and humidity) collected from Directorate of Economics and Statistics and Directorate of Science and Technology, Aizawl, Mizoram (Table 2).

Table 2. Monthly average temperature, humidity and rainfall in the three forest types (2009-2013).

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Forest type 1												
Temperature (°C)	19	19.15	23.7	24.4	24.2	24.02	25	30.38	27.1	26.3	23.2	17.1
Humidity (%)	58.3	51.1	52.3	77.1	85.5	95.7	97	92.4	90.1	85.7	79.9	73
Rainfall (mm)	13.2	11.22	92.5	296.77	398.33	479.17	288.33	454.64	562.7	315.27	15.87	20
Forest type 2												
Temperature (°C)	18	20.16	22.92	24.13	23.6	23.75	24.56	24.8	24.76	23.99	21.25	18.52
Humidity (%)	61.53	54.52	54.33	73.24	82.29	89.09	89.37	90.05	89.03	81.7	75.03	64.73
Rainfall (mm)	11.53	6.23	94.66	253	379.54	493.33	372.37	477.83	1168.56	688.3	71.266	19.33
Forest type 3												
Temperature (°C)	14.5	16.95	21.7	23.25	23.75	22	22.75	23	22	21	18.75	18
Humidity (%)	40.4	49.1	48.1	69.1	81.4	90.6	90.8	90.1	86.8	80.7	67.2	47.1
Rainfall (mm)	10.63	11.3	75	198.32	228.67	286.57	339.5	330.58	801.67	389	39.63	17.47

Table 3. Sampling sites of Nymphalid and Heliconiinae butterflies. The district of the sampling site are shown in parantheses.

Altitude range (meter asl)	**Surveyed sites**
<200	Buhchang (Kolasib), Sairang, Zawlnuam (Mamit) Chawngte (Chawngte)
201-400	Lengpui (Mamit) Sihhmui, Tlawng (Aizawl)
401-600	Dampa (Mamit), Reiek roadsides (Aizawl)
601-800	MZU campus (Aizawl), Lawngtlai (Lawngtlai) Thenzawl (Serchhip)
801-1000	Tlangnuam, Bawngkawn, Sailam (Aizawl)
1001-1200	Lunglei (Lunglei), Khawzawl (Champhai), Durtlang (Aizawl)
1201-1400	Champhai, Mualkawi (Champhai), Hmuifang, Sumsuih (Aizawl)

The diversity indices used are as under

Shannon index (Magguran, 2004)

$$H' = -\sum p_i \ln p_i$$

Where, H' = Shannon index of diversity,

*p*i = the proportion of important value of the ith species (*p*i = *n*i / N, *n*i is the important value index of ith species and N is the important value index of total species).

Shannon's equitability (J) can be calculated by dividing H by Hmax

$$H_{max} = \ln S.$$

Simpson index (Magguran, 2004)

$$D = \sum n(n-1)/N(N-1)$$

Where n_i is the number of entities belonging to the *i*th type and *N* is the total number of entities in the dataset

As D increases, diversity decreases. Simpson's index is therefore usually expressed as 1-D or 1/D.

Berger-Parker index (Magguran, 2004)

$$d = N_{max}/N$$

Where Nmax= the number of individuals in the most abundant species

The Berger-Parker index, d, is a simple dominance measure. It expresses the proportional abundance of the most abundant species.

Species abundance in each altitudinal range has also been plotted using rank abundance curve. In this, species are plotted in sequence form most to least abundant along the horizontal (or X axis) and their abundance are displayed log10 format (Y axis) (Magguran, 2004)

3.3 Effect of leaf quality of host plant on the butterfly life cycle

The preliminary survey on host plants of the Heliconiinae butterflies showed that most of the species were polyphagus and feeding on distasteful leaves of the family Passifloraceae, Violaceae and Flacourtiaceae. Only the larvae of Lacewings and Costers were gregarious, while others remained solitary. *Acreae issoria* (Yellow Coster) one species of Heliconiinae was selected to study the effect of the host plant quality on butterfly life cycle. This butterfly has a monophagous larvae feeding on *Debregeasia salicifolia*, found mainly in the subtropics but also found at higher elevations in the tropics, mainly found along water courses, at elevations ranging from 1500 - 2400 m, shady and moist places by streams (Uniyal and Kumar, 1997).

Larval observations were made weekly at their natural sites (Durtlang, Aizawl- 1384 m ASL). Few larvae were collected for daily observations in the laboratory where the larvae were divided in groups, fed with young and matured leaves of different host plant. The suitability of the host plants from its nutritional point of view was assessed by various biochemical analyses such as total protein, total lipid, total carbohydrate, total amino acid, and water content (Shobana *et al.*, 2010; Murthy *et al.*, 2013)

3.3.1 Estimation of total protein

Total protein was measured using Lowry's method (Lowry *et al.*, 1951)**.** The leaves were homogenised in 70% ethanol using mortar and pestle and homogenate was centrifuged at 5000 rpm for 10 minutes. Supernatant was discarded and pellet was suspended in 10 ml of trichloro acetic acid (TCA) for 30 min to precipitate proteins, centrifuged at 5000 rpm for 10 min and supernatant was discarded. The protein pellet was dissolved with 0.1N NaOH. The protein content was estimated using BSA as standard and optical density was recorded at 660 nm.

3.3.2 Estimation of total carbohydrate

Total sugars were estimated using Anthrone reagent (Plummer, 1971). The carbohydrate was extracted by acid extraction method (Smith *et al.,* 1964). The leaves were incubated with 0.5 N Sulphuric acid in boiling water bath for 1 hr. The hot solution was filtered through a Whitman filter paper. The total carbohydrate content was estimated using Glucose standard (1 mg/ml) optical density was recorded at 620 nm.

3.3.3 Estimation of total amino acids

Amino acids were measured by Ninhydrin reagent (Moore, 1948)**.** The leaves were crushed with mortar and pestle with water: chloroform: methanol extraction buffer in the ratio of 3:5:12 (Hacham *et al.,* 2002). The solution was Centrifuged (6000 rpm) thrice and pellet was collected. The extracted amino acids was mixed with 2% Ninhydrin reagent and total amino acid was estimated using phenyl alanine as standard and optical density was taken at 570 nm.

3.3.4 Estimation of total lipid

Lipid content was measured by gravimetric method **(**Bligh and Dyer, 1959).The leaves were centrifuged at 3000 rpm (5 min) with Chloroform: ethanol (1: 2) in a weighed beaker and kept overnight in oven for drying. The weight of the beaker containing the lipid was taken again. The difference of the weight was recorded as weight of lipid.

3.3.5 Estimation of total water content

The leaf water content was determined by gravimetric method (Vijayan*et al.,* 1997)**.** Weight of fresh leaves were taken and dried in a hot air oven overnight. The weight of dry leaves was again recorded. The water content of the leaves was calculated by taking the difference of the weight of the leaves.

Host-plant *Debregeasia salicifolia*

Larva of *Acraea issoria*

Pre-pupa

Pupa

Freshly-emerged adult

Adult butterfly basking in the sun

Figure 5. Photographs showing host plant and different stages in the life cycle of *Acraea issoria* (Yellow coster) butterfly.

3.3.6 Pattern of gut-based enzymes in Acraea issoria

The pattern of the gut enzymes - SGOT (Serum Glutamic Oxaloacetic Transaminase), SGPT (Serum Glutamic Pyruvic Transaminase) (Reitman and Frankel, 1957) and ALP (Alkaline Phosphatase) were analysed from different regions of the gut (foregut, midgut and hindgut) and were measured using commercial and experimental kits (Kind and King's Method, 1954). Following the protocol of Barbehenn (2002) and according to the Reitman and Frankel method (1957) the pattern of gut enzymes were assayed in the enzyme preparation. The insects were chilled and dissected, the midgut contents included portion of food bolus that extended into the anterior hindgut. 0.5 g enzyme preparation was homogenized with 10 cm 3 0.05 M buffer, Tris-HCl pH 8.5, and the obtained suspension was filtered through two layers of cheese cloth and centrifuged at 2,800 rpm for 20 min. All steps of the extraction were conducted at 4°C. The incubation mixture contained the substrate incubated at 30°C for 10 min. Enzyme extract was added and the mixture was incubated for 60 min, and then mixed with solution of 2, 4-dinitrophenylhydrasine in 1 M HCl. After 20 min, the reaction was stopped by the addition of 0.4 M NaOH, and absorbance was measured on a Hewlett Packard UV-Vis Spectrophotometer at 505 nm. Samples were assayed within 1 h from the time of preparation with reagents using kits obtained from M/s.Crest Biosystems, Goa, India. The patterns of the three enzymes were compared from the densitometric values in each of the foregut, midgut and hindgut among different larval instars (Tietz, 1970)

3.4 Morphological characterization and its analysis

The thirteen species of Heliconiinae butterflies (*Cirrochroa aoris, Argynnis laodice, Vindula erota, Phalantha phalanta, Acraea issoria, Vagrans egista, Cethosia biblis, Cethosia cyane, Argynnis hyperbius, Argynnis children, Cirrochroa tyche, Cupha erymanthis, and Phalantha alcippe*) collected from NE India were identified and their morphological characters were analysed. As most of the identification keys of butterflies are based on their wing pattern, the observations were focussed mainly on the wings, colour, shapes, spots and markings. Twenty three characters were selected and their presence (1) and absence (0) of each character was marked accordingly and a binary matrix was developed (Table 4).

The binary matrix was used to calculate Jaccard's genetic distance coefficient using the SIMQUAL module. These distance coefficients were used to construct dendrogram using UPGMA (Unweighted Pair Grouped Method Arithmetic Average) employing SAHN (Sequential Agglomerative Hierachical and Nested Algorithm) with 1000 bootstrap value. All the parameters were computed using NTSys-pc software version 2.2.

Table 4. Morphological characters of 13 species of Heliconiinae.

	C	V	R	LY	CY	CL	SL	YC	RL	LL	IF	LS	ES
R1	1	1	1	1	1	1	1	0	0	0	1	1	1
R2	0	1	1	1	1	1	1	0	1	1	1	1	1
R3	1	1	1	1	1	1	1	1	1	1	1	1	1
R4	1	0	0	0	0	0	0	0	0	0	0	0	0
R5	0	0	0	0	0	0	0	0	1	1	0	0	0
R6	0	0	1	1	1	1	1	1	0	0	1	1	1
R7	0	0	0	0	0	0	0	1	1	1	0	0	0
R8	0	0	0	0	0	1	1	1	0	0	0	0	0
R9	0	0	0	0	0	0	0	0	1	1	0	0	0
R10	0	0	0	0	0	0	0	0	0	1	0	0	0
R11	0	1	1	1	1	1	1	0	1	1	1	1	1
R12	1	1	0	1	0	0	0	0	0	0	0	0	0
R13	1	1	0	0	0	0	0	0	0	0	0	0	0
R14	1	0	0	1	1	0	0	0	0	0	0	0	0
R15	1	1	1	1	1	1	1	1	1	1	1	1	1
R16	0	1	1	1	1	0	1	1	1	1	0	0	0
R17	0	0	0	0	0	1	0	0	0	0	0	0	0
R18	0	0	0	0	0	0	1	0	0	0	0	0	0
R19	0	0	0	0	0	0	0	0	0	1	0	0	0
R20	0	0	0	0	0	0	0	0	0	0	1	1	1
R21	0	0	0	0	0	0	0	0	0	0	0	1	0
R22	0	0	0	0	0	0	0	0	0	0	1	0	0
R23	0	0	0	0	0	0	0	0	0	0	0	0	1

C- *Vindula erota*, V-*Vagrans egista* R-*Cupha erymanthis* LY-*Cirrochroa aoris* CY-*C.tyche* CL-*P.phalantha* SL-*P.alcippe* YC- *A.issoria* RL-*Cethosia biblis* LL *C cyane*, IF-*Argynnis.hyperbius* LS-*A.childreni* ES *A.laodice*. R1 Tawny Colour, R2 Black spots on UP, R3 Black Line margin, R4 Eye spot (ocelli,) R5 Scalloped HW order, R6 Rounded HW, R7 V-shaped marginal markings, R8 Black bar at end cel l(UPF,) R9 V-shaped marking at outer discal FW R10 White band across apical UPF R11 Discal spots on UPH R12 Produced FW, R13 HW toothed at Vein 4, R14 doubled black wavy marginal lines. R15 UPF cell with dark lines, R16 UPF apex black, R17 UPF pinkish tinge, R18 UP Purple iridescence, R19 Uvariegated R20 UNH Siver markings, R21 Discal silverstripe straight, R22 UNH irregular and broken Silverstripes, R23 UNH midcell band reddish brown.

3.5 Genomic DNA Extraction

DNA was extracted from the legs of butterflies which were stored in 70% alcohol at 4°C prior to extraction. Protocol of Zimmermann *et al.* (2000) was followed for extraction of DNA with some modifications. The leg was washed with distilled water, dried and macerated with the help of scissor in 1.5 ml Eppendorf tube, the leg tissue was homogenized with pestle and 250 µl of extraction buffer (50 mM Tris HCl, 25 mM NaCl, 25 mM EDTA, 0.1% SDS) was added and mixed gently. 2 µl of proteinase *k* (20mg/ml) was then added and incubated in an oven at 56°C for 1½ h. 250µl of equal volume of phenol/chloroform was added to the tube, mixed thoroughly and centrifuged at 13000 rpm for 5 min. Supernatant was carefully transferred in a new eppendorf tube. 420 µl of ice cold absolute ethanol was added, mixed gently by inverting the tube several times and then kept in -20°C for 30 min. The tube was then centrifuged at 13000 rpm for 5 min at 4°C. Ethanol was poured off without dislodging the pellet, and 200 µl of 70% ethanol was added, recentrifuged at 6000 rpm for 2 min. The ethanol was poured off and the pellet was dried in an oven for 15 min. 30 µl of distilled was added to the tube, the pellet was resuspended by gently flicking the tube and was stored at -20°C for further use.

3.6 Estimation of genomic DNA by agarose gel electrophoresis

0.8 g agarose was dissolved in 30 ml of 1X TAE buffer (2 ml of 50X TAE and 98 ml of distilled water) from the stock of 50X TAE buffer. 1 µl of 10 mg/ml ethidium bromide was added. It was allowed to solidify and DNA sample was run at 60-100 V. DNA concentration was also determined by spectrophotometeric method (Bio-photometer plus; Eppendorf, Germany) according to the manufacturer's instruction. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. A 50 ng/µl DNAstock was prepared from the isolated DNA for further experiments.

3.7 DNA amplificationby RAPD PCR

PCR was performed with six random RAPD primers obtained from Biosciences India (Table 5) following Gallusser *et al.*(2004). 20 µl of reaction mixture contained 1 µl of 10X PCR buffer, 1.2 µl MgCl (3 mM), 0.4 µl dNTPs (0.2 mM), and 0.3 µl of Taq polymerase (5 U/µl), 0.8 µl of BSA (100 pmol/µl), 2 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 conditions: initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation, annealing and extension respectively at 94°C, 37°C and 72°C for 1 min each and final extension at 72°C for 5 mins. The amplified products were stored at 4°C.

3.8 Data analysis (RAPD)

RAPD-PCR amplification was performed using condition outlined by Sharma *et al.*, (2006) and the bands on gels were documented using the Gel documentation system (UVI TEC Fire Reader). Each species was scored for the presence or absence of every amplification product and the data were entered into a binary data matrix. Only distinct and polymorphic bands were recorded and used in the analysis.

To evaluate the discriminatory power of molecular markers PIC, MI and EMR were calculated from genotypes generated from RAPD. The PIC value was determined by applying the formula PICi = 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 ith allele. The MI was calculated as the product of two functions that is DI and EMR as described by Prevost and Wilkinson (1999). The EMR defines number of loci (bands) simultaneously analyzed per experiment. A single parameter, MI is a universal matrix that represents amount of information obtained per experiment in a given marker system. Another aspect in which

marker systems differ is their ability to determine relationship between accessions based on genetic similarity estimation. Only polymorphic bands with PIC values higher than 0.08 were used for generating dissimilarity matrices.

Table 5. List of primers used for RAPD-PCR.

Primer Name	5'-3' Orientation	Mer	OD	µg	Mw	Tm	%GC	Nano Mole	Picomoles/ µl
OPT-01	GGGCCACTCA	10	3.3	106	3013	39.3	70	48	480
OPT-04	GTGTCTCAGG	10	5.5	178	3059	20.4	60	49	490
OPT-05	GGGTTTGGCA	10	5.4	174	3099	40.3	60	53	530
OPB-11	GTAGACCCGT	10	4.7	147	3028	26.7	60	35	350
OPB-12	CCTTGACGCA	10	4.5	148	2988	37	60	58	580
OPB-15	GGAGGGTGTT	10	5.4	168	3139	31.2	60	56	560

OD=Optical density, Mw=Molecularweight, Tm=MeltingTemp, %GC=% of GC content

Similarity matrix based on Jaccard's similarity coefficient was used to construct unweighted pair group method with arithmetic average (UPGMA) dendrogram.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. All the above mentioned statistical analysis was performed using NTSYS-pc software version 2.2.

3.9 DNA amplification by CO1 gene and ND1 gene

Amplification of CO1 gene was carried out with universal primers described in Hebert *et al.* (2003) forward primer LCO 5' TAA TAC GAC TCA CTA TAG GGG GTC AAC AAATCA TAA AGA TAT TGG 3' and reverse primer HCO 5' ATT AAC CCT CACTAA AGT AAA CTT CAG GGT GAC CAA AAA ATC A 3'. 25 µl reaction mixture contained: 1X Taq buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTP, 0.1 pM each primer, 10 mg/ml

BSA and 5U Taq DNA polymerase. The PCR thermal regime for amplification was: 5 min at 95°C for initial denaturation, followed by 30 cycles of 30s at 95°C for denaturation, 40s for annealing at 48°-57°C, elongation for 30s at72°C and a final elongation for 6 min at 72°C.

ND1 gene was amplified by PCR performed with specific primers, P850 (fwd) 5'-TTC AAA CCGGTG TAA GCC AGG -3' and P851 (rev) 5'-TAG AAT TAG AAG ATC AAC CAG-3' described in Rach *et al.* (2008). The 25 µl reaction mixes contained: 1X amplification buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.1 pM each primer, 10 mg/ml BSA and 5U Taq DNA polymerase. The PCR thermal regime for amplification was: 5 min at 95°C for initial denaturation, followed by 30 cycles of 30s at 95°C for denaturation, 40s for annealing at 46°-58°C, elongation for 30s at 72°C and a final elongation for 6mins at 72°C

3.10 Sequencing and alignment

The PCR products of CO1 and ND1 genes were quantified using Bio-photometer and were diluted, sequenced using Sanger's di-deoxy method and sequencing reactions were carried out in one direction on a sequencer (GCC Biotech, Kolkata). The raw sequences obtained were first checked for its accuracy and edited by comparing with the associated chromatogram using Bioedit software ver. 7.5.1.0 (Hall, 1999). All the sequences were checked for contaminations using BLAST. The reverse strand sequence was reverse complemented and was overlapped with the forward strand sequence to get the maximum length. Sequences were aligned and checked using Pairwise Sequence Alignment (EMBOSS-water, EBI) and Finch TV version 1.4 (Patterson *et al.*, 2004) followed by manual adjustments. All the protein coding sequences were translated into amino acids and their ORFs checked [Sequence Manipulation Suite (Bioinformatics.org) and ORF Finder (NCBI)]. The sequences for ND1 and CO1 were deposited in the GenBank. The CO1 and ND1 sequences available for Heliconiinae were downloaded from GenBank along with the locality

and the accession numbers. 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 of other Heliconiinae retrieved from the NCBI database.

3.11 MEGA analysis of CO1 and ND1

The PCR amplified products were sequenced and MEGA 5.1 version (Tamura *et al.,* 2011) was used for conducting Phylogenetic and molecular evolutionary analyses. The DNA sequences were aligned by using MUSCLE alignment (MEGA 5.1 version) along with sequences retrieved from the NCBI database. The data obtained was used to derive Maximum Likelihood (ML) Phylogenetic tree with nucleotides distances (p-distance), transition/transversion rate ratios, nucleotide diversity and Tajima's test of neutrality (D). The p-distance is the proportion (p) of nucleotide sites at which two sequences being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared considering the proportion of nucleotide sites that are different (Nei and Kumar, 2000). The overall ts/tv bias (R) was calculated by the formula: R = [A x G k1 + T x C x k2] / [(A + G)x (T + C)], where k1 = purine and k2 = pyrimidine. The Tajima's test of neutrality (Tajima, 1989) compares the number of segregating sites per site with the nucleotide diversity. (A site is considered segregating if, in a comparison of *m* sequences, there are two or more nucleotides at that site; nucleotide diversity is defined as the average number of nucleotide differences per site between two sequences). If all the alleles are selectively neutral, then the product *4Nv* (where *N* isthe effective population size and *v* is the mutation rate per site) can be estimated in two ways, and the difference in the estimate obtained provides an indication of non neutral evolution. For Tajima's test of neutrality (D), *m* = number of sequences, *S* =Number of segregating sites, *p*s = *S*/*m*, *Θ*= *p*s/a1, *π*= nucleotide diversity (Nei and Kumar, 2000). The program MODEL TEST (Posada and Crandall, 1998)

was used to choose a substitution 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.

3.12 Phylogenetic analysis

The phylogenetic analyses were performed with TNT (Goloboff *et al.,* 2003), using Maximum Parsimony (MP); RaxML (Silvestro and Michalac, 2012) for Maximum Likelihood (ML). Bayesian analysis was carried out with MrBayes v. 3.1 (Huelsenbeck and Ronquist, 2001). The purpose of performing Bayesian analysis was to investigate the effect of more restrictive assumptions on the results.

Maximum Parsimony analyses (MP) were performed on the entire data set using the New Technology Search implemented in TNT by traditional search using bootstrapping, with all characters equally weighted. A strict consensus tree was computed whenever multiple equally parsimonious trees were obtained. The stability of each branch was determined using the non-parametric bootstrap test (Felsenstein, 1985) with 1000 replicates and 100 random taxon additions. Clade robustness was evaluated by using the bootstrap (Felsenstein, 1985) in TNT. A bootstrap support (PBS) value was to obtain a consensus tree that includes all and only those clades found in all the trees. All characters were equally weighted, other relationships (those in which the trees disagree) are shown as unresolved polytomies. Multistate taxa were interpreted as uncertainty, topological constraint was not enforced and the generated 50% consensus trees were saved as .tre file.

ML analysis was performed with RaxML (raxmlGUI, Silvestro and Michalac, 2012). Phylip file was generated for RaxML analysis using ALTER Alignment Transformation

Environment (Pena *et al.*, 2010). For ML analysis, the bootstrap was set at 1000 and the model was set at GTR+GAMMAI.

Bayesian analyses (Huelsenbeck and Ronquist, 2001; Huelsenbeck *et al.*, 2001) were carried out with MrBayes v3.1.2set under the model GTR + G. Bayesian posterior probabilities for the clades were obtained using Metropolis-coupled Markov chain Monte Carlo (MCMC) analysis as implemented in MrBayes. The analysis was run twice for 1 million generations, with every 100th tree sampled until the final average standard deviation of the split frequencies fell below 0.01and the first 20% sampled generations discarded as burn-in (based on a visual inspectionof when log likelihood values reached stationarity). The purpose of this analysis was to investigate the effects on the results under a different tree-building method. Such sensitivity analyses may help identify potential instances of long branch attraction (Giribet, 2003) and can provide a valuable heuristic tool to guide subsequent sampling strategies for refinement of the current hypothesis. Convergence and ESS values were assessed with Tracer v1.5 (Rambautand Drummond, 2007). Clades that are recovered under parsimony and Bayesian analyses were referred as stable.

3.13 *In-silico* characterization *distal-less (dll)* genes

The sequences of colour pattern genes (*distal-less*) of different butterflies species were retrieved from Swiss-Prot database in FASTA format for analysis. The physico-chemical characterization of the above proteins were computed using Expasy's Protparam tools and server for the following parameters viz., amino acid composition, theoretical isoelectric point (pI), molecular weight, negative and positive residues, extinction coefficient. CYS_REC predicted the presence of disulphide bridges (SS bonds) in distalless gene sequences. CYS_ REC also identified the positions and total number of cysteines present and predicted the most probable SS bond pattern of pairs (based on the matrix of pair scores) in

the submitted FASTA format protein sequence. (http://sun1.softberry.com/berry.phtml?topic=cys_rec&group=help&subgroup=propt.) The tool Rasmol (http://openrasmol.org/) was then used to visualize the modelled 3D structures and to identify the SS bonds (3D co-ordinates data).

With the eleven distal-less protein sequences retrieved from database, a phylogenetic tree was constructed using maximum likelihood method with 100 bootstrap support using MEGA 5.

IV. RESULTS

4.1 Survey and collection of Heliconiinae butterflies from Northeast India

During the period of the present study (Jan 2010-Dec 2013), thirteen species belonging to eight genera of Heliconiinae subfamily were collected and identified from different sites of Northeast India (6 states) for their molecular analysis. The species collected were *Acraea issoria* (Hubner, 1818), *Cethosia biblis* (Drury, 1770), *Cethosia cyane* (Drury 1770), *Cirrochroa tyche* (Felder and Felder, 1861), *Cirrochroa aoris* (Doubleday, 1847) *Phalantha phalanta* (Drury, 1773), *Phalanta alcippe* (Stoll, 1872) *Argynnis hyperbius* (Linnaeus, 1763) *Argynnis Childreni* (Gray, 1831), *Argynnis laodice* (Pallas, 1771), *Vagrans egista* (Cramer, 1780), *Vindula erota* (Fabricius, 1793) and *Cupha erymanthis* (Drury, 1773). These thirteen species make 62% of Indian species of Heliconinae (Figs. 6, 7, 8, and 9)

Out of these thirteen species, eleven species were found to be present in Mizoram in which *V. egista* (Zothansangi *et al.,* 2013), *C. erymanthis* and *A. children* were recorded for the first time from the state of Mizoram. From Meghalaya seven species of Heliconiinae were collected mostly from Shillong area with one species *P. alcippe* (Small Leopard) from Balpakram National Park, Garo Hills. Three species namely *P. phalanta, V. egista* and *C. biblis* from Tripura, another three species from Assam and two species from Arunachal Pradesh were collected. In Sikkim, *A. childreni, A. laodice, C. cyane, C. biblis* and *P. phalanta* were collected (Table 6).

Acraea issoria (Yellow Coster)

Acraea issoria

Cethosia biblis (Red Lacewing)

Cethosia biblis (Under wing)

Cethosia cyane (Leopard Lacewing)

Male and Female of *C.cyane* mating

Figure 6. Photograph of species of Heliconiinae of Northeast India.

Vagrans egista (Vagrant)

Vagrans egista

Vindula erota (male)

Vindula erota (Female)

Phalanta phalantha (Common Leopard)

Cupha erymanthis (Rustic)

Figure 7. Photographs of species of Heliconiinae of Northeast India.

Cirrochroa aoris (Large Yeoman)

Cirrochroa aoris

Cirrochroa tyche (Small Yeoman)

Phalanta alcippe (Small Leopard)

Figure 8. Photographs of species of Heliconiinae of Northeast India.

Argynnis hyperbius (Indian Fritillary)

Male and Female of *A. hyperbius* mating

Argynnis laodice (Eastern Silverstripe)

Argynnis children (Large Silverstripe)

Figure 9. Photographs of species of Heliconiinae of Northeast India.

Table 6. Species and Sites of Collection in Northeast India.

States	Collection Sites	Species recorded and collected
1.Mizoram	Tanhril ,Chanmari ,Tlawng, Saiha , Lunglei, Zawlnuam ,Chawngte, Champhai , Khawzawl	1. Cruiser *Vindula erota* 2. Yellow Coster *Acraea issoria* 3. Red Lacewing *Cethosia biblis* 4. Common Leopard *Phalantha phalanta* 5. Large yeoman *Cirrochroa aoris* 6. Leopard Lacewing *Cethosia cyane* 7. Common yeoman *Cirrochroa tyche* 8. Indian Fritillary *Argynnis hyperbius* 9. Vagrant *Vagrans egista* 10. Large Silverstripe *Argynnis childreni* 11. Rustic *Cupha erymanthis*
2.Meghalaya	Barapani , Nehu Campus, Garo Hills	1. Yellow Coster *Acraea issoria* 2. Small Leopard *Phalantha alcippe* 3. Red Lacewing *Cethosia biblis* 4. Leopard Lacewing *Cethosia cyane* 5. Indian Fritillary *Arginius hyperbius* 6. Large Silverstripe *Argynnis childreni* 7. Eastern silverstripe *Argynnis laodice*
3.Tripura	Agartala	1. Red Lacewing *Cethosia biblis* 2. Common Leopard *Phalantha phalanta* 3. Vagrant *Vagrans egista*
5.Assam	Bageswari pahar , Tunia river Sunyasi pahar	1. Red Lacewing *Cethosia biblis* 2. Leopard Lacewing *Cethosia cyane* 3. Cruiser *Vindula erota*
6.Arunachal Pradesh	Chimpu , Zero point	1. Red Lacewing *Cethosia biblis* 2. Cruiser *Vindula erota*
7. Sikkim	Ranipool , Rangpo , Yuksam	1. Large Silverstripe *Argynnis Childreni* 2. Eastern silverstripe *Argynnis laodice* 3. Leopard Lacewing *Cethosia cyane* 4. Red Lacewing *Cethosia biblis* 5. Common Leopard *Phalantha phalanta*

4.2 Distribution of Nymphalid butterflies in Mizoram

4.2.1 Diversity of Nymphalidae butterflies with respect to forest types of Mizoram

During the present study, a total of 115 species of Nymphalidae family belonging to eleven subfamilies was recorded. Distribution patterns of Nymphalidae in the different forest types of Mizoram were analysed and results were shown in Table 7. It showed that they were more widely distributed in the tropical wet evergreen forest with highest diversity index (1.93 in Shannon and 74.66 in Simpsons) and lowest diversity was in Mountain sub-tropical forest (1.73 in Shannon and 44.36 in Simpsons).

In Forest type 1 which is the Tropical wet-evergreen forest, a total of 929 individuals of 108 species were recorded during the survey period. *Euploea klugii, Charaxes delphis, Faunis canens, Mycalesis francisca, Elymnias nesaea, Lexias dirtea, Tanaecia julii,* were the species recorded only from this forest type. The Diversity indices showed the highest number among the three forest type which were 1.93 and 74.66 respectively (Table 7). Shannon's Equitability also showed the highest value 0.95 meaning that the species were evenly distributed in this forest type. However, Berger-Parker dominance was the lowest among the three forest types with 3.2%.

In Tropical semi-evergreen Forest (forest type 2), a total of 837 individuals of 106 species were recorded *Vanessa cardui, Doleschallia bisaltide*, and *Cupha erymanthis* were the three species found only in this forest type 2. The diversity indices were little lower compared to forest type 1 (1.91 and 72.78 in Shannon and Simpson index respectively). Shannon's Equitability was 0.94 and Berger-Parker dominance was 3.22 %.

A total of 434 of 73 species were recorded from forest type 3, Mountain sub-tropical forest hosting *A. childreni* which was not found in other forest types. This forest type had the lowest diversity with 1.73 and 44.36 in Shannon and Simpson index respectively and also

least number 0.93 in Shannon Equitability J'. However, Berger-Parker dominance was the highest among the three forest types giving 7.37%.

From the data collected, the forest type 1 Tropical wet evergreen forest had the highest temperature (23.62°C) and humidity (78.17%) during the survey period. Forest type 2 Tropical semi-evergreen forest received the heaviest rainfall 336.32 mm.The forest type 3 Mountain Sub-Tropical was with the lowest rainfall (227.36 mm), temperature (20.63°C) and humidity (70.12%) during the period of survey. From the table of Pearson correlation coefficient of diversity and meterological data, it was shown that diversity had the highest positive correlation with humidity (0.96); temperature was also positively correlated with diversity with coefficient 0.95 but rainfall did not show a good correlation but still have a fair positive correlation with diversity (0.58) which was more than 0.5

Table 7. Diversity Indices of Nymphalidae family calculated from different forest types.

Forest type	Shannon H' log base 10	Shannon Hmax Log Base 10.	Shannon J'	Simpsons Diversity (D)	Simpsons Diversity (1/D)	Berger-Parker Dominance (d)	Berger-Parker Dominance (1/d)	Berger-Parker Dominance (d %)
Type 1	1.927	2.02	0.95	0.013	74.66	0.032	30.96	3.20
Type 2	1.914	2.03	0.94	0.014	72.78	0.032	31	3.22
Type 3	1.729	1.85	0.93	0.023	44.36	0.074	13.56	7.37

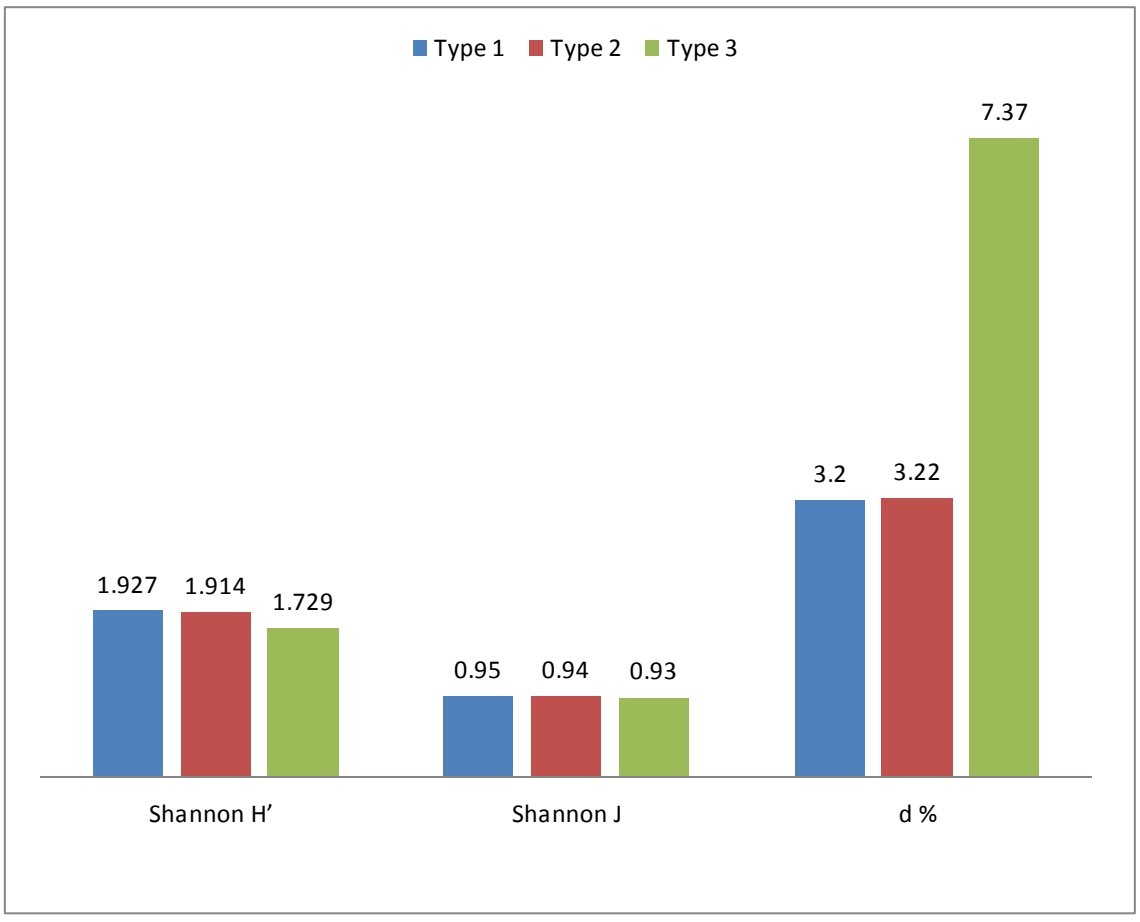


Figure 10. Histogram showing diversity of Nymphalidae family in three forest types of Mizoram.

(Type 1=Tropical Wet Evergreen Forest, type 2 = Tropical Semi-Evergreen Forest Type = Mountain Sub-Tropical Forest)

4.2.2 Distributions of Heliconiinae in Mizoram

4.2.2.1 Vertical distributions:

In Mizoram, eleven species of Heliconiinae subfamily were recorded and collected from different sites of survey. Results from the analysis of distribution patterns of Heliconiinae in different altitude ranges showed that Heliconiinae butterflies are more widely distributed in the higher elevational ranges. Only three species, *Vindula erota, Phalantha phalanta* and *Cethosia cyane* were collected from the two lowest altitudinal ranges. This pattern of distribution of each species along the vertical ranges is reflected in species richness as in Fig. 11a and the highest number of species appears at the elevation between 801 – 1001 msl. The number of individuals of all the species varied in relation to elevation (Fig. 11b) peaking at the range 601 – 800 msl where ranges of species of the lower and higher zones overlap.The biodiversity, as measured by Shannon index was maximum between 1000 – 1200 msl and highest degree of Equitability (evenness) was in the two lowest ranges which are in area <200 m and between 201 – 400 msl (Table 8). The line graph showed that the biodiversity of the butterflies increases with increase in altitudes and decreases abruptly beyond 1200 msl, while the evenness was more or less the same in all the altitudinal ranges (Fig. 12).

The species were ranked according to the abundance, the rank abundance curve for 201-400masl showed the steepest curve indicating assemblages with high dominance. Altitudinal range below 1000-1200 m ASL showed a shallower slope signifying highest evenness indicating most of the species are equally abundant in this range (Fig. 13).

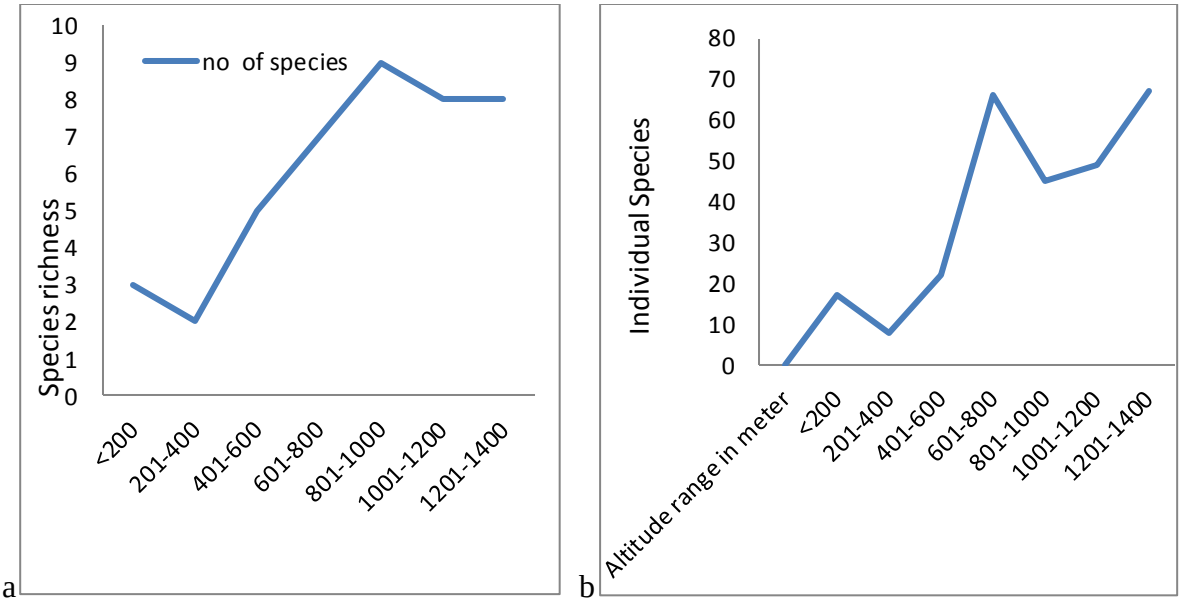


Figure 11. Graphs showing a) Species Richness and b) Individual species distribution respectively.

Table 8. Diversity indices at different altitudinal ranges of Mizoram.

Altitude range in meter	Shannon H' Log Base 10.	Shannon J'	Simpsons Diversity (1/D)
<200	0.401	0.841	2.429
201-400	0.287	0.954	2.154
401-600	0.678	0.971	5.5
601-800	0.745	0.881	4.831
801-1000	0.807	0.894	5.858
1001-1200	0.881	0.975	8.34
1201-1400	0.848	0.939	6.84

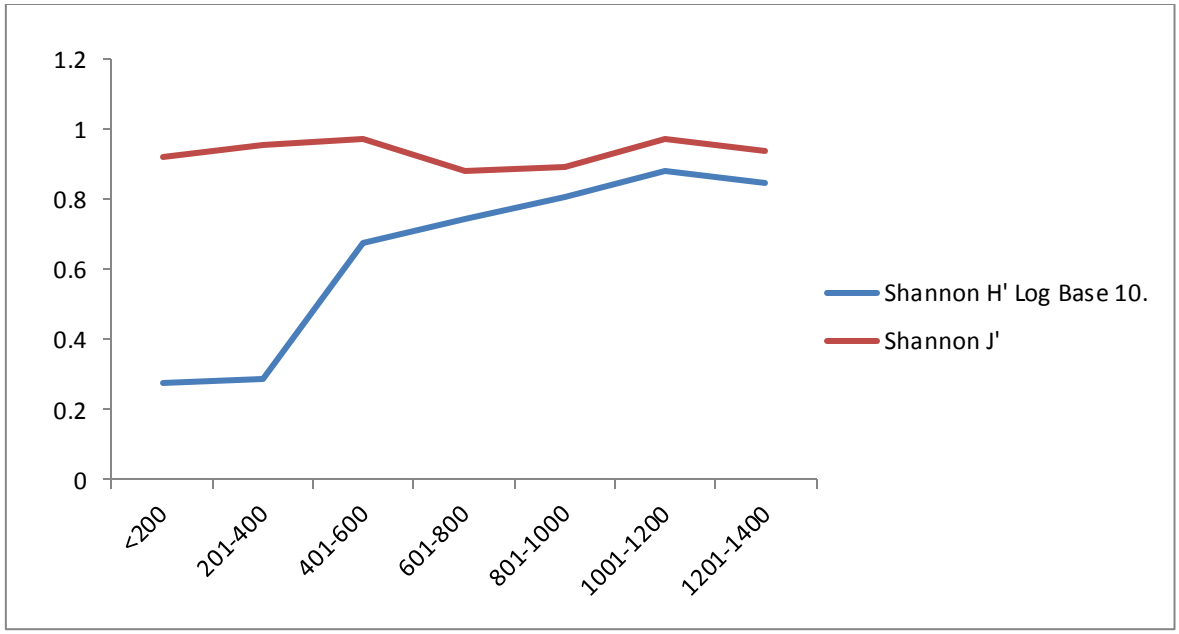


Figure 12. Graph showing the variations of Diversity Index and Evenness with increase in altitudes.

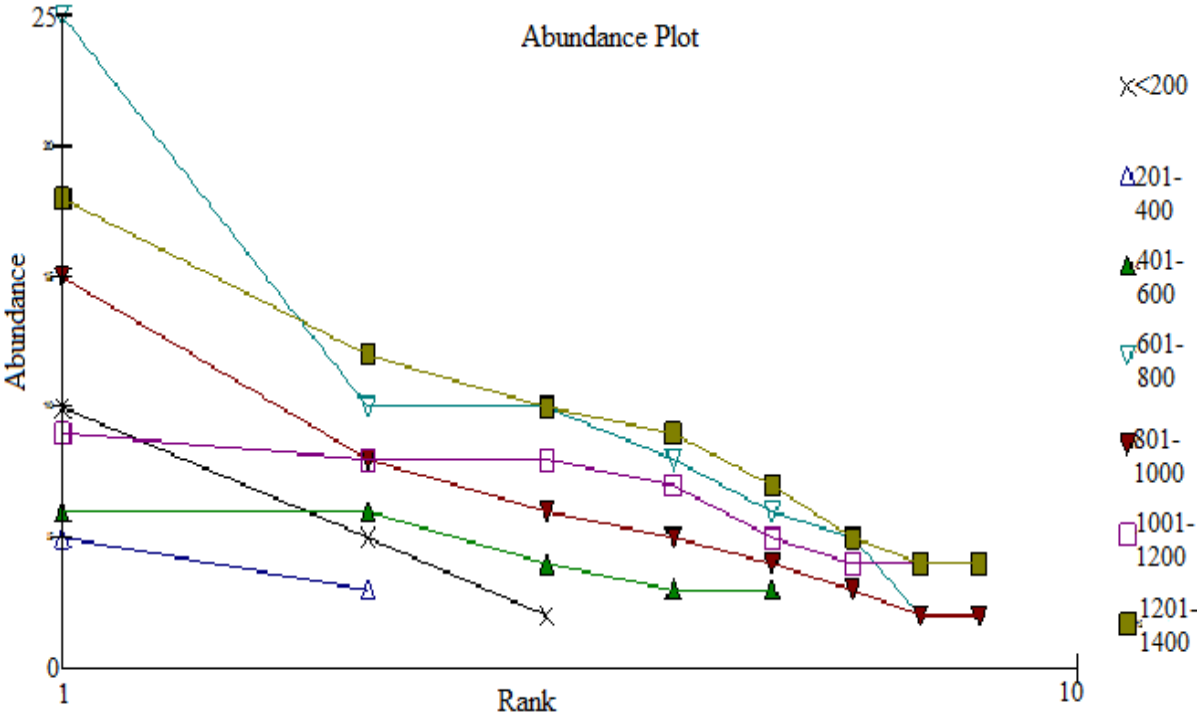


Figure 13. Species rank abundance plot for 7 altitudinal ranges The Y axis shows the relative abundance of the species (plotted using log10 scale) while the X axis rank each species in order from most to least abundant. The 7 lines show the densities of butterflies in relation to altitudinal ranges.

Table 9. List of Heliconiinae and their distribution profiles in Mizoram.

Species	Variance	Mean	Chi-sq	d.f.	Probability	Aggregation
Vindula erota	321.3333	45.6667	14.073	2	0.0010279	Aggregated
Acraea issoria	358.3333	21.6667	33.0769	2	3.00E-07	Aggregated
Cethosia biblis	8.3333	18.3333	0.9091	2	0.6406079	Random
Phalantha phalanta	75	15	10	2	0.006884	Aggregated
Cirrochroa aoris	208.3333	16.6667	25	2	8.70E-06	Aggregated
Cethosia cyane	8.3333	26.6667	0.625	2	0.7362584	Random
Cirrochroa tyche	8.3333	16.6667	1	2	0.6124016	Random
Argyrnis hyperbius	36.3333	5.6667	12.8235	2	0.0018267	Aggregated
Vagrans egista	30.3333	9.6667	6.2759	2	0.0422275	Random
Cupha	3	1	6	2	0.048449	Random
Argynis childreni	5.3333	1.3333	8	2	0.0180616	Aggregated

The results showed that majority of the Heliconiinae butterflies in Mizoram are randomly distributed in their habitats. The species; *C. biblis, C. cyane, C. tyche, V. egista* and *C. erymanthis* are found to be random in their distribution having Chi square less than 8. *V. erota, A. issoria, P. phalantha, C. aoris, A.childreni* and *A. hyperbius* were found to be aggregated, all having Chi square value more than 8 (Table 9). The species were ranked according to the abundance, the rank abundance curve for Type 1 Tropical wet evergreen forest type showed the steepest curve indicating assemblage with high dominance. Mountain sub-tropical forest showed a shallower slope signifying highest evenness indicating most of the species are equally abundant in this range (Fig. 13).

4.2.2.2 Distribution of Heliconiinae in different 3 in forest types*:*

Among the three forest types in Mizoram; Tropical wet evergreen forest type, Tropical semi-evergreen forest type and Mountain sub-tropical forest, the diversity of Heliconiinae subfamily was found to be the highest in type 3 Mountain sub-tropical forest (0.92) in Shannon H's result where type 1 Tropical wet evergreen forest type showed the lowest diversity (0.71). Simpsons Index also showed the highest diversity in forest type 3 and

lowest in type 1. The Berger-Parker dominance percentage was highest in the Tropical wet evergreen forest type (36.76%), the species dominance percentage of Tropical Semi-Evergreen Forest is 27.805 while the lowest dominance percentage (19.44%) was found in Mountain sub-tropical forest (Table 10).

From the data collected shown in Table 11, the forest type 1 Tropical wet evergreen forest had the highest temperature (23.62°C) and humidity (78.17%) during the survey period. Forest type 2 Tropical semi-evergreen forest had the heaviest rainfall of 336.32 mm and the forest type 3 Mountain sub-tropical was with the lowest rainfall (227.36 mm), temperature (20.63°C) and humidity (70.15%) during the period of survey (Table 10). From the correlation coefficient analysis, it was shown that temperature has the highest correlation with diversity (0.79); humidity is also positively correlated with diversity with coefficient 0.67 but rainfall show a negative correlation (0.41) with diversity (Table 12).

4.2.2.3 Monthly variations of Heliconiinae in Mizoram:

Butterflies in all habitats showed a highly seasonal trend. There was a significant difference in number of species in the months of the year. Species Richness was highest in the month of May (10) followed by June (9) while minimum richness was recorded in the month of January and February (2) followed by December (3) as shown in the graph. In all observed individuals from different habitats, maximum abundance of species of Heliconninae butterflies was noted in the months of June to September peaking in September. Minimum abundance was observed in the month of January and February (Fig.15).

The monthly dominance, evenness and Simpson diversity using Biodiversity Pro and PAST version 2.0 showed results as in Table 13. From the index of Berger-Perker dominance, January and February showed the maximum dominance with a percentage of 66.7% and minimum dominance was noted in the month of June with 21.8%. Maximum

Shannon Diversity was recorded in the month of May (0.907) while the mimimum diversity was in the months of January and February (0.276). Simpsons diversity index (1/d) also showed maximum in May (8.2) and minimum diversity in January and February (3). Trends in the Evenness showed that there was not much dominance all through the year as evenness ranged from 0.876 (August) to 0.961 (November).

Table 10. Diversity Indices for Heliconiinae.

Forest Types	Shannon H' Log Base 10.	Shannon Hmax Log Base 10.	Shannon J'	Simpsons Diversity (D)	Simpson Diversity (1/D)	Berger-Parker Dominance (d)	Berger-Parker Dominance (1/d)	Berger-Parker Dominance (d%)	Alpha
Type 1	0.719	0.778	0.924	0.215	4.656	0.367	2.727	36.667	1.253
Type 2	0.891	1	0.891	0.149	6.715	0.278	3.596	27.805	2.202
Type 3	0.924	1	0.924	0.127	7.866	0.194	5.143	19.444	2.284

Forest type 1: Tropical Wet Evergreen, Forest type 2: Tropical Semi-Evergreen, Forest type 3: Mountain Sub-Tropical.

Table 11. Diversity index, temperature, humidity and rainfall in the three forests types.

Forest types	Average rainfall (mm)	Average temperature (°C)	Average Humidity (%)	Shannon H' Log Base 10.
Type 1	245.67	23.62	78.175	1.927
Type2	336.32	22.53	75.409	1.914
Type 3	227.36	20.63	70.116	1.729

Table 12. Correlation between Diversity indices, temperature, humidity and rainfall in the three forests types.

	Rainfall	Temperature	Humidity	Diversity
Rainfall	1	*	*	*
Temperature	0.3054	1	*	*
Humidity	0.327	0.9997	1	*
Diversity	**0.411**	**0.7837**	**0.6677**	**1**

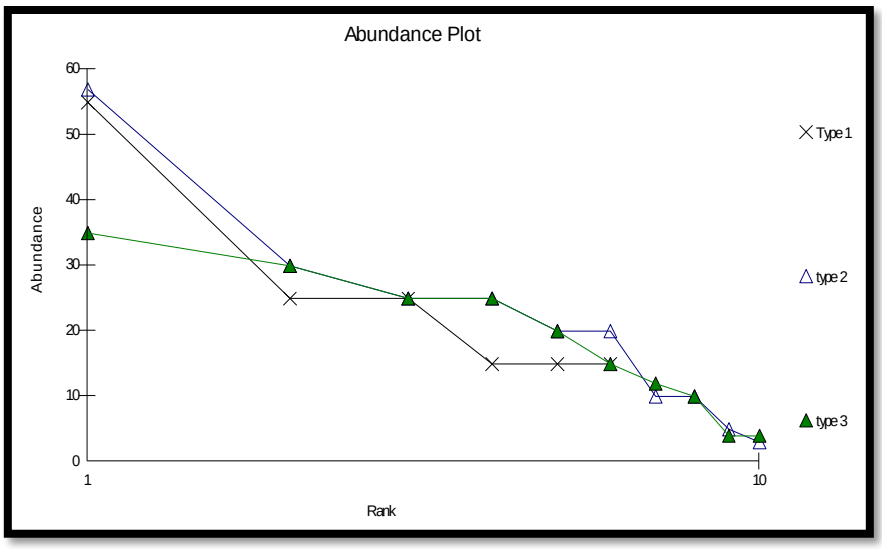


Figure 14. Species rank abundance plot for 3 forest types. The Y axis shows the relative abundance of the species(plotted using log10 scale) while the X axis rank each species in order from most to least abundant. The 3 lines show the densities of butterflies in the **3 forest types**. Species richness decreases and assemblages become less even (as indicated by steeper slopes) from forest type 1

Table 13. Diversity Indices for Heliconiinae across the year (Jan 2010-Dec 2013) in Mizoram.

Months	Berger-Parker Dominance (%)	Simpson 1/d	Simpson d	Shannon J'	Shannon D
Jan	66.7	3	0.333	0.918	0.276
Feb	66.7	3	0.333	0.918	0.276
Mar	44.4	4.25	0.235	0.898	0.628
Apr	26.7	6.9	0.145	0.945	0.799
May	25	9.2	0.122	0.907	0.907
Jun	23.8	6.8	0.145	0.915	0.82
Jul	31.9	5.1	0.196	0.909	0.715
Aug	47.2	3.5	0.282	0.876	0.689
Sep	41.7	4.2	0.237	0.886	0.568
Oct	41.7	4.4	0.227	0.944	0.568
Nov	27.3	8.87	0.145	0.961	0.568
Dec	40	5	0.2	0.96	0.458

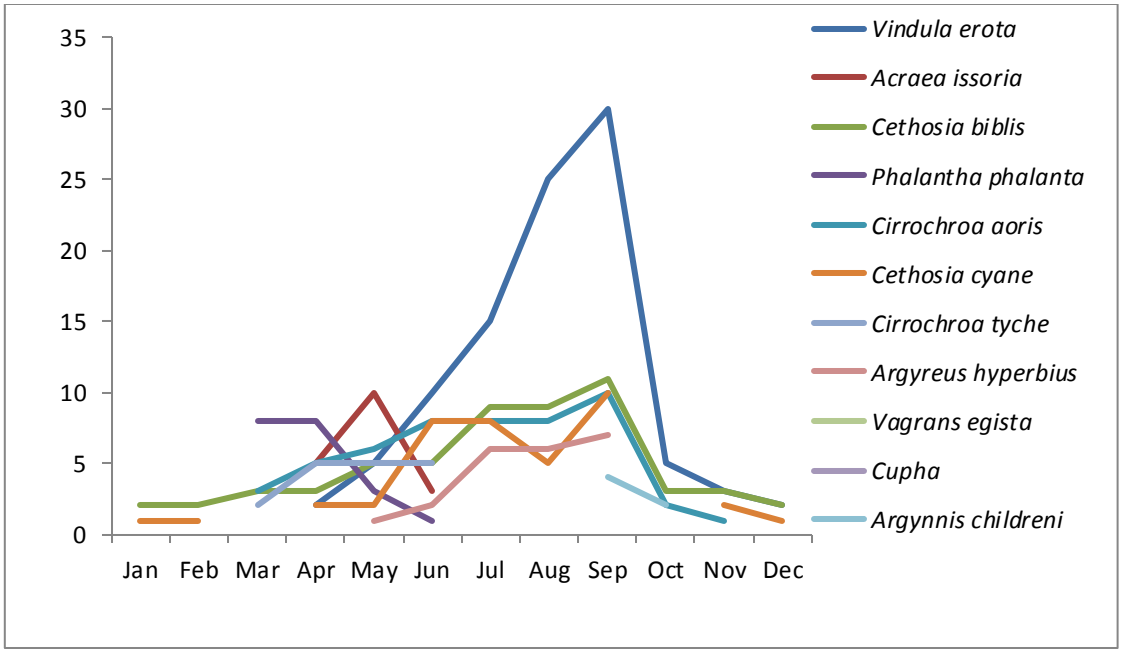


Figure 15. Species Abundance of Heliconiinae across the year (Jan 2010-Dec 2013) in Mizoram. Abundance ofeach species in terms of average number of individuals seen in a month during the study period.

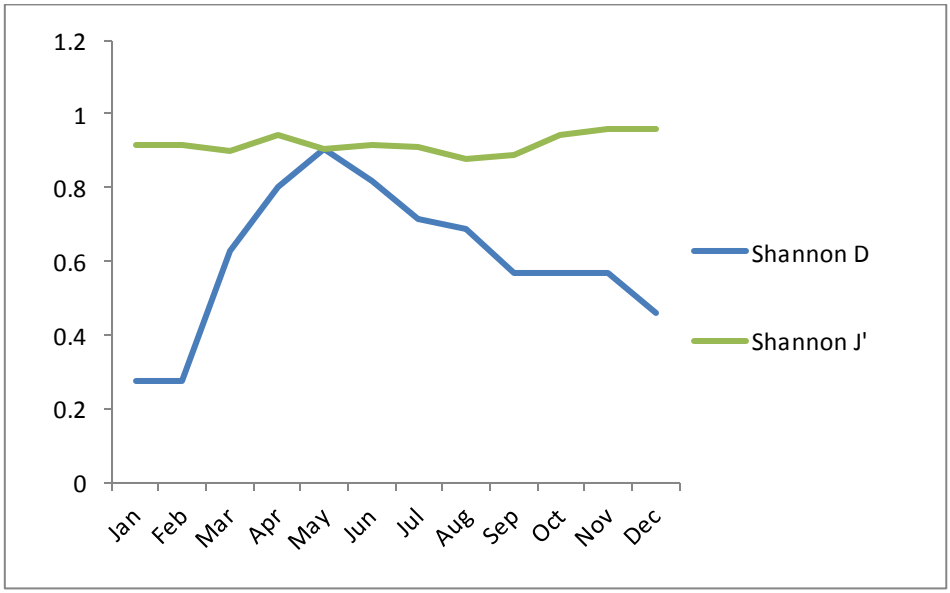


Figure 16. Graph showing maximum diversity of Heliconiinae in May, minimum in Jan and Feb with evenness in months of the year during Jan 2010-Dec 2013 in Mizoram.

4.3 Effect of leaf quality of host plant on the growth of butterfly

Acreae issoria (Yellow Coster) has a monophagous larvae feeding on *Debregeasia salicifolia*. Weekly larval observations were made at its natural sites (Durtlang, Aizawl- 1384 m ASL) and few larvae were collected for daily observations in the laboratory. In the Laboratory, the larvae were divided in groups, fed with young leaves and matured leaves separately of the host plant.

4.3.1 Biochemical analysis from the leaves of host plant *Debregeasia salicifolia*

Results from the analysis of the biochemical contents of young leaf and mature leaf were presented in Table 14. The results showed the value of total biochemical content of mature leaf was usually higher than in young leaf, except in the total water content. The average total protein value was 2.88 ± 0.22 mg/g wet wt. of the leaves, average total sugar value 20.4 ± 4.35 mg/g wet wt. of the leaves where the average total amino acids was 28.8 ±n3.9 mg/g wet wt. of the leaves. The total lipid content of the leaves ranged from 2 ± 2.28 g/g wet wt. It was also found that the larvae fed with mature leaf took lesser time for emerging into adult butterfly from the chrysalis (pupa) than the larvae fed with young leaf. The larvae feeding on the mature leaf developed and grew faster (Table 14).

The total number of days taken for emergence was correlated with the biochemical contents of the young and mature leaf separately. From the table of Linear Correlation Coefficients, the total no of days taken for emergence showed very high negative correlations with protein content (r= -0.93); carbohydrate content (r= -0.96) amino acids content (r= -0.93) and lipid content (r= -0.958), all with P values less than 0.05, where it gave a positive correlation with water content i.e. 0.411 with P value 0.238 (<0.05) showing that there was no significant relationship between the two (Table 15).

Table 14. Number of days taken for adult emergence and biochemical composition of Young and Mature leaves.

Stage of the leaves (feed)	Total Protein (mg/g wet wt.) ± SD	Total Carboydrates (mg/g wet wt.) ± SD	Total AA (mg/g wet wt.) ± SD	Total Lipids (g/g wet wt.) ± SD	Water content (%) ± SD	Days taken for adult emergence from pupa ± SD
Matured leaf	2.88 ± 0.22	20.4 ± 4.35	28.8 ±3.9	2 ± 2.28	75.6 ± 6.41	10.2 ± 1.5
Young leaf	1.56 ± 3.2	12 ± 2	14.2 ± 2.4	0.52 ± 3.6	81.2 ± 1.6	15.2 ± 2.6

Table 15. Correlation coefficients between the number of days taken for adult emergence and the biochemical contents of the leaves of the host plant.

Biochemicals	Correlation coefficient (r)	P Value
Total Protein	-0.937	0.0000643
Total Carboydrates	-0.969	0.00000397
Total Amino acids	-0.935	0.0000704
Total Lipids	-0.958	0.0000125
Water content	0.411	0.238

Pearson Product Moment Correlation.Thepair(s) of variables with positive correlation coefficients and P values below 0.050 tend to increase together. For the pairs with negative correlation coefficients and P values below 0.050, one variable tends to decrease while the other increases. For pairs with P values greater than 0.050, there is no significant relationship between the two variables

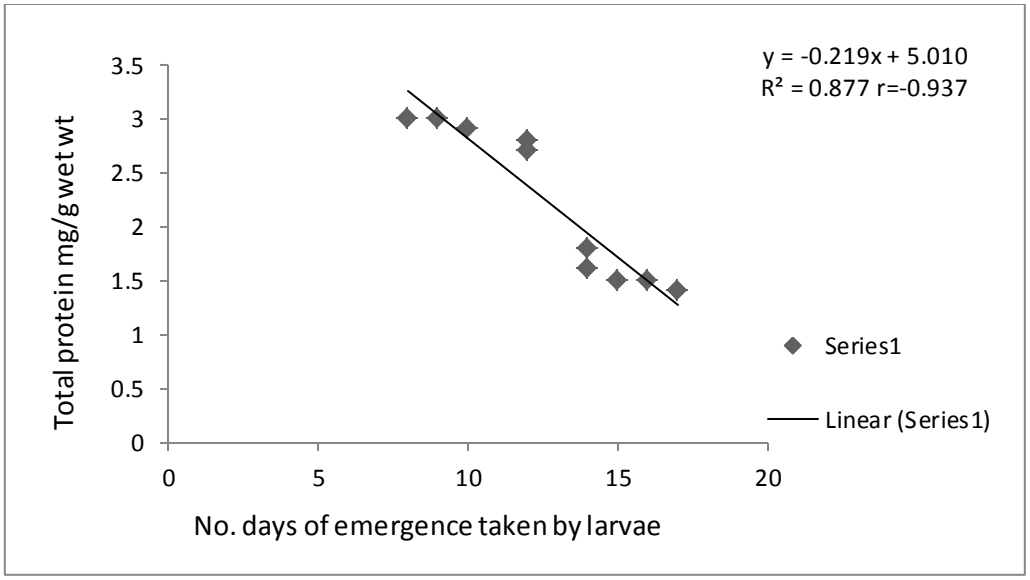


Figure 17. Regression Correlation between total protein of leaves and number of days taken for adult emergence in *A. issoria.*

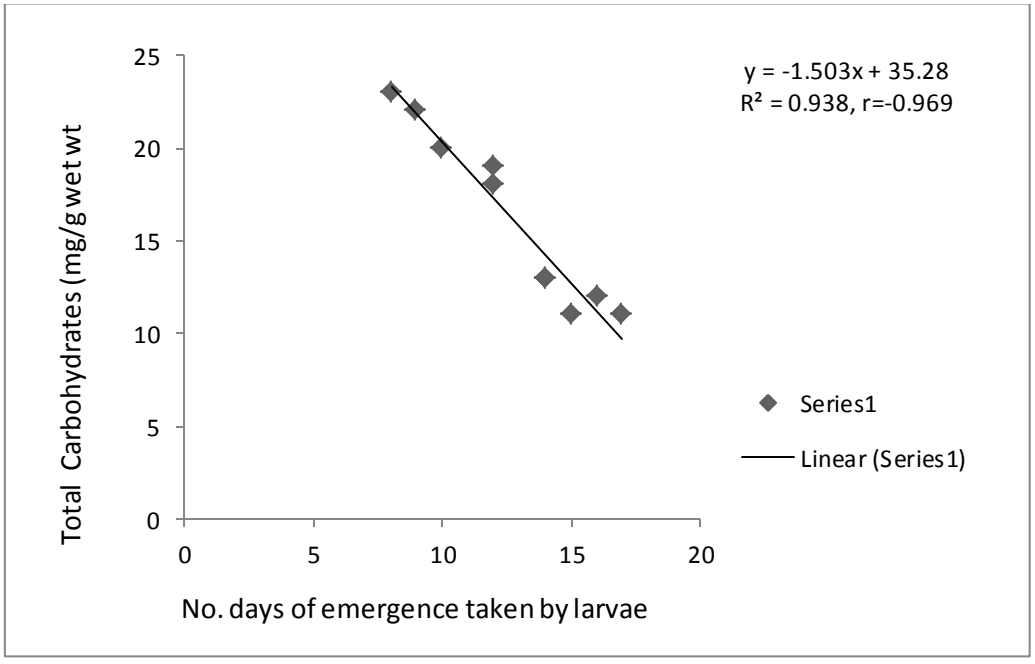


Figure 18. Regression Correlation between total carbohydrates of leaves and number of days taken for adult emergence in *A. issoria.*

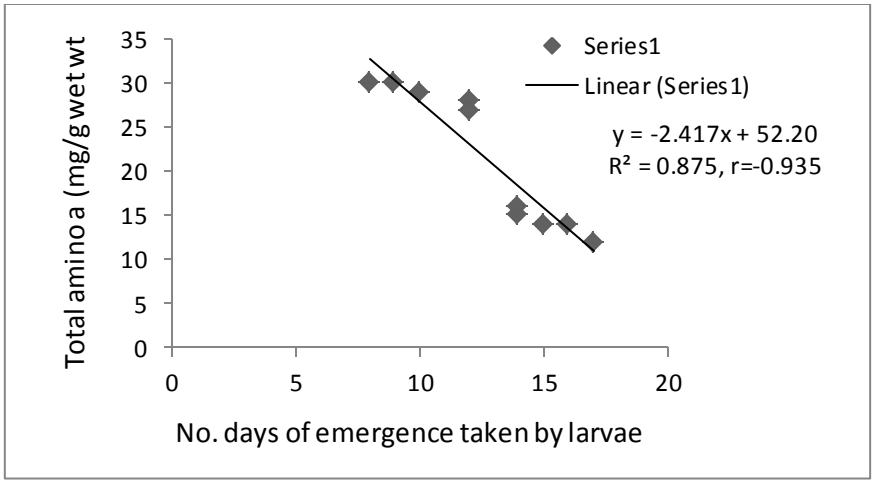


Figure 19. Regression Correlation between total amino acids of leaves and number of days taken for adult emergence in *A. issoria*.

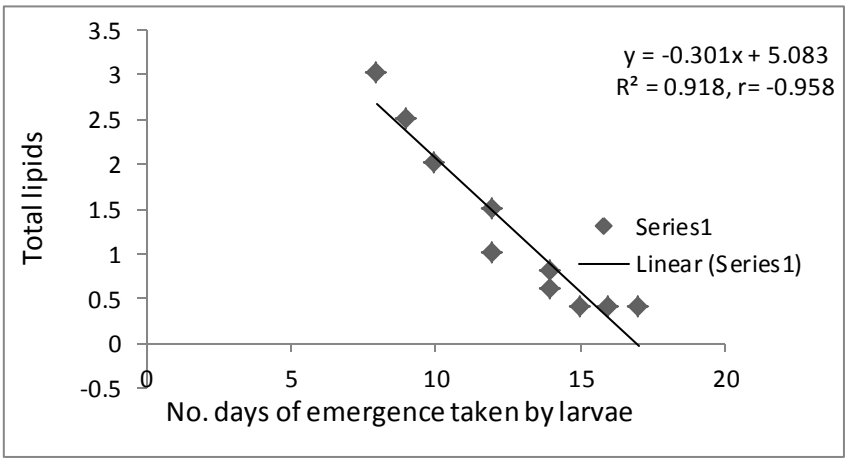


Figure 20. Regression Correlation between total lipid of leaves and number of days taken for adult emergence in *A. issoria*.

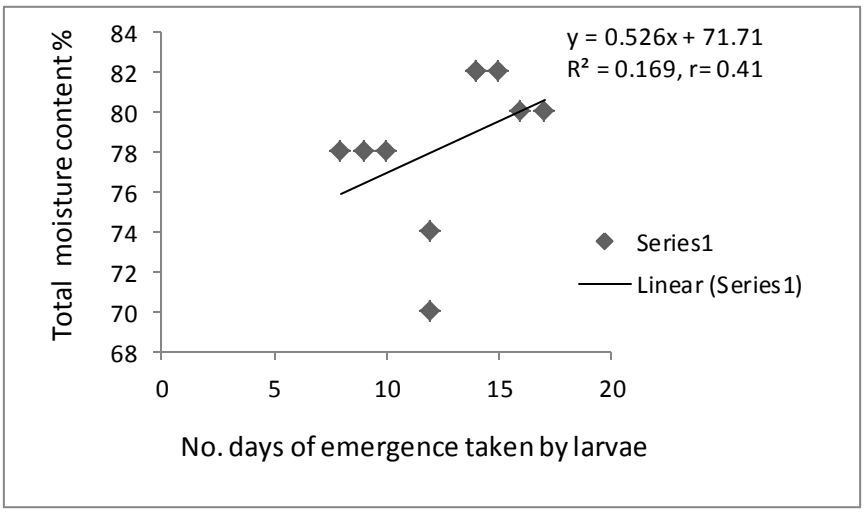
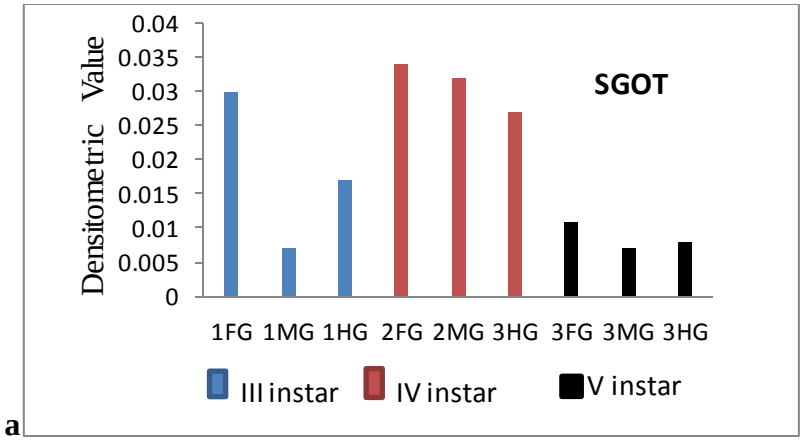


Figure 21. Regression Correlation between total water and number of days taken for adult emergence in *A. issoria*.

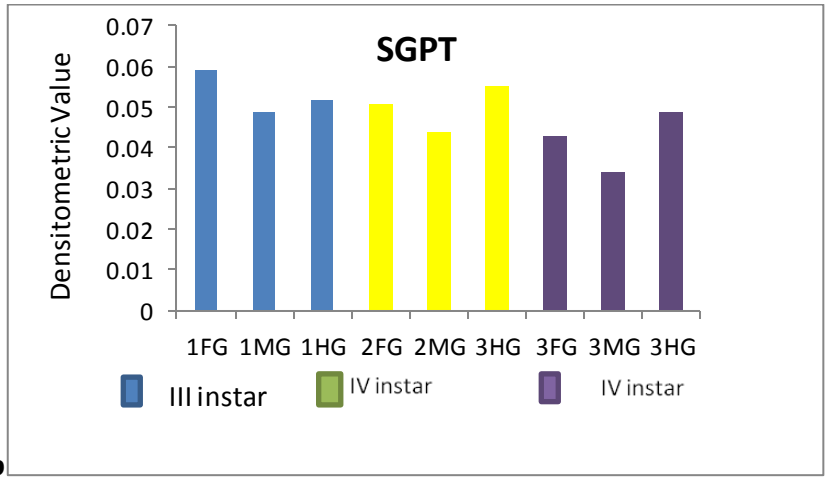
{Perfect correlations = +1 and -1; no correlation =0; very high degree of corr.=>+9 and >-9, fairly high= +0.75-0.9 and -0.75-0.9; moderate +0.5-0.75 and -0.5-0.75; low degree +0.25-0.5 and -0.25-0.5; very low degree <+0.25 and <-0.25}. The statistically significant Levene's test ($F_{11, 48=}$1592; P<0.05) on the effect of biochemical contents of the hostplant revealed the heterogeneity of variance among the biochemical under assayed on the developmental period which was taken as number of days taken for emergence.

4.3.2 Pattern of gut based enzymes in *Acraea issoria*

The pattern of the gut based enzymes SGOT (Serum Glutamic Oxaloacetic Transaminase), SGPT (Serum Glutamic Pyruvic Transaminase), ALP (Alkaline Phosphatase) from different regions of the gut (foregut, midgut and hindgut) are shown in the bar diagrams (Fig. 22). SGOT and SGPT activity pattern varied with larval instars of *Acraea issoria*. SGOT activity was high in 4th instar larvae where 3rd and 5th instars showed lower activity especially in the midgut and hindgut. Descending pattern of SGPT activity was observed in all the 3 instars in which maximum activity was in the foregut and minimum in the hindgut. However, ALP activity pattern showed maximal in the midgut of all the III and IV instars of larvae and ascending pattern was found from the 3rd, 4th and 5th instars larvae. This increase in the ALP activity with the advancement of developmental stages may be due to the increased food consumption of larval instars. The correlation coefficients calculated between the larval growth and the densitometric values of the gut enzymes showed high positive correlation (0.9) in ALP activity, high negative correlation (-0.8) in SGPT activity and no correlation in SGOT activity. The statistically non-significant Levene's test ($F_{2, 27=}$0.0078; P>0.05) for heterogeneity of variance, based on means on the growth of larvae with the enzyme activities in the gut of larvae revealed homogeneity of variance of the three enzymes activity.



a



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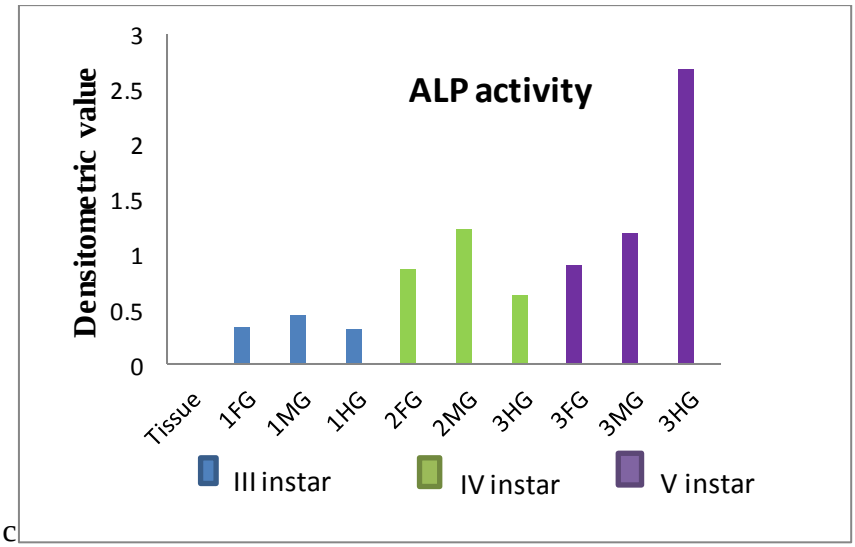


c

Figure 22. Bar diagrams showing Gut enzyme pattern in three instars larvae. a. SGOT (Serum Glutamic Oxaloacetic Transaminase) b. SGPT (Serum Glutamic Pyruvic Transaminase) c. ALP (Alkaline Phosphatase) [FG= foregut, MG= midgut, HG= hindgut]

4.4 Analysis of data from Morphological Characterization

The thirteen species of Heliconiinae butterflies used for the study were: *C. aoris A.laodice, V. erota, P. phalanta, A. issoria, V. egista, C. biblis, C. cyane, A. hyperbius, A. children, C. tyche, C. erymanthis,* and *P. alcippe.* From the binary matrix constructed on the basis of 23 characters, a dendrogram was generated using the UPGMA (Unweighted pair-group method with arithmetical averages). Neighbour Joining tree using both Jaccard and NE172 coefficients was constructed.

Jaccard's coefficients of similarity matrix determined by analysis using the morphological identification keys from thirteen species of Heliconiinae revealed maximum similarity value (0.889) between CY (*C. tyche*) and LY (*C. aoris*) which are the two species belonging to the *Cirrochroa* genus, both under the tribe Vagrantini while the minimum value (0.478) was between C (*V. erota*) and LL (*C. cyane*) belonging to tribes Vagrantini and Heliconiini respectively (Table 16).

The dendrogram based on morphology divided the subfamily into 2 major clades with different genetic distance. Cluster 1 comprised of only *Vindula erota* and cluster 2 comprised of all the other 12 species again divided into 3 sub-clades. The first subclade consisted of *Acraea issoria* which was the only species present from tribe Acraeni, the second subclade gave the two *Cethosia* species belonging to Heliconiini tribe and the third subclade comprised of the rest 9 species. In subclade 3, the three species (*A. hyperbius, A. childreni & A. laodice*) of tribe Argynnini were in one cluster the other cluster consisted of 6 species (*Vagrans egista, Cupha erymanthis, Cirrochroa aoris, Cirrochroa tyche, Phalanta phalantha* and *Phalantha alcippe*) all from tribe Vagrantini (Fig. 23).

Table 16. Jaccard's coefficients of similarity matrix from morphological data.

ROWS\COL	C	V	R	LY	CY	CL	SL	YC	RL	LL	IF	LS	ES
C	1.000												
V	0.500	1.000											
R	0.273	0.667	1.000										
LY	0.455	0.700	0.778	1.000									
CY	0.364	0.600	0.875	0.889	1.000								
CL	0.250	0.455	0.667	0.545	0.600	1.000							
SL	0.231	0.545	0.778	0.636	0.700	0.700	1.000						
YC	0.182	0.273	0.444	0.364	0.400	0.400	0.500	1.000					
RL	0.154	0.455	0.500	0.417	0.455	0.333	0.417	0.400	1.000				
LL	0.133	0.383	0.417	0.357	0.385	0.286	0.357	0.333	0.800	1.000			
IF	0.250	0.455	0.667	0.545	0.600	0.600	0.545	0.273	0.333	0.286	1.000		
LS	0.250	0455	0.667	0.545	0.600	0.600	0.545	0.273	0.333	0.286	0.778	1.000	
ES	0.250	0.455	0.667	0.545	0.600	0.600	0.545	0.273	0.333	0.286	0.778	0.778	1.000

Maximum similarity value (0.889) found between CY and LY which are the two species belonging to the *Cirrochroa* genus while the minimum value (0.133) was between C and LL, belonging to *Vindula* and *Cethosia* genera respectively [C=*V. erota*, V= *V. egista*, R= C. erymanthis, LY= *C. aoris,* CY= *C. tyche*, CL= *P. phalantha,* SL= *P. alcippe,* RL=*C. biblis,* LL= *C. cyane,* IF= *A. hyperbius,* LS=*A. childreni,* ES=*A. laodice*]

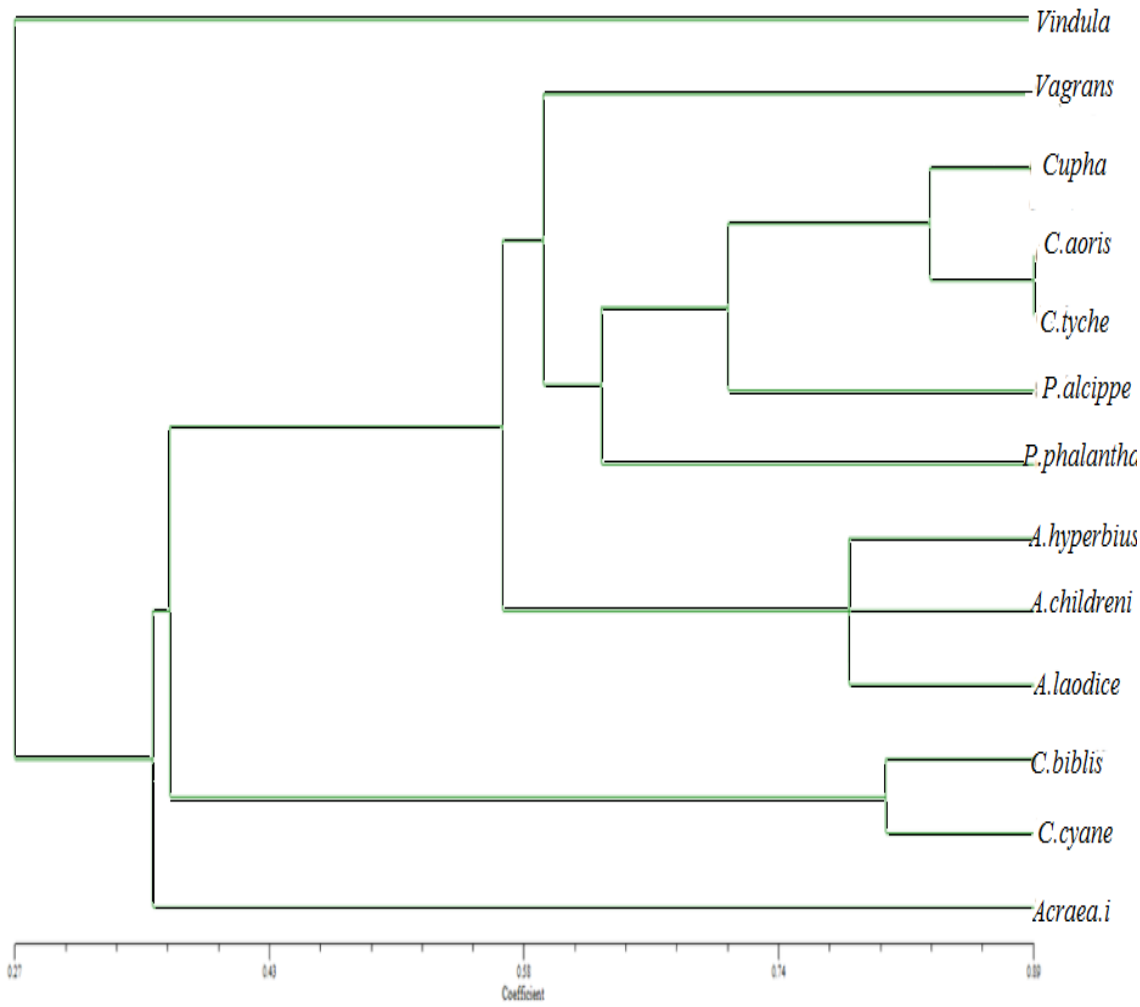


Figure 23. Dendrogram showing thirteen species of Heliconiinae based on Morphological characters.

4.5 Analysis of data from RAPD-PCR

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 Heliconiinae butterflies with six primers (OPT-1, OPT-4, OPT-5, OPT-11, OPB-12 and OPB-15). Out of the 316 total discrete fragments, Primer OPT-1 gave as many as 73 prominent bands while OPT-11 produces just 39 bands. The amplified ranges of primers was as low as 110 bp as in OPT-11 to as high as 1910 bp as in OPB-1 and there was a total of 232 (73.41%) 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. The resolving power of the primers ranged from 5 to 11, with an average of 8.01, primer OPT-1 showed the highest resolving power. The polymorphic information content (PIC) value of RAPD primers varied from 0.16 to 0.19 with an average of 0.176. For RAPD analysis, effective multiplex ratios (EMR) for all the primers were estimated at 0.12 and 0.13 while marker index (MI) was in between 0.6 to 0.8 (Table 16). For the analysis and comparison of these patterns, a set of distinct, well separated bands were selected, neglecting the weak and unresolved bands**.**The statistically significant Levene's test on the Polymorphic Information Content of the primers revealed the homogeneity of variance among the primers test on each samples ($F_{5, 24}$=890; P<0.05).

Jaccard's coefficients of similarity matrix (Table 18) determined by analysis using six RAPD primers from 13 species of Heliconiinae revealed Maximum similarity value (0.261) between MZBF75 and MZUsl which are the two species belonging to the *Phalantha* genus, both under the tribe Vagrantini while there were no similarities (0.00) between MZBF10 *(Vindula erota)* and MZBF137 (*Argynnis childreni)*, MZBF31 *(Acreae issoria)* and MZBF136 *(Cupha erymanthis)*, MZBF35 (*Cethosia biblis*) and MZBFsl *(Phalantha alcippe),* MZBF75 (*Phalantha phalanta)* and MZBF109 *(Argynnis laodice)*, MZBF98 *(Cethosia*

cyane) and MZBFsl *(Phalantha alcippe)*, MZBF116 *(Vagrans egista)* and MZBF109 *(Argynnis laodice)* MZBF137 (*Argynnis children)* and MZBF136 *(Cupha erymanthis)*.

From the RAPD data obtained dendrograms were generated using the UPGMA (Unweighted pair-group method with arithmetical averages). Neighbour Joining tree using Jaccard's coefficient was built. As informative as the bands obtained from the RAPD data may be in terms of genetic distances or the dissimilarity among the species, the dendrogram tree generated were not as realistic in terms of the tree clustering among the species in relation to tribe-wise; as species were not forming the same clade with other species of the same tribe in most cases. Two major clades were formed, one clade consisting of species from tribe Vagrantinni with Acraeini in between. The second clade consisted ofthe other species of the other two tribes, Heliconiini forming sister clade to Argynini. However, species of same genus formed monophyly in each subclade (Fig. 25).

Table 17. RAPD primers, Polymorphic bands, % Polymorphism, PIC, EMR and MI as resolved by the 6 primers among the 13 species of Heliconiinae.

Primer name	5'-3' Orientation	Total band	Polymorphic band	Polymorphism %	PIC	RP	MI	EMR
OPT-01	GGGCCACTCA	73	47	64.3	0.197	11.0	0.64	0.12
OPT-04	GTGTCTCAGG	50	38	75	0.175	7.69	0.76	0.13
OPT-05	GGGTTTGGCA	62	46	74.19	0.181	9.53	0.74	0.13
OPB-11	GTAGACCCGT	39	33	84.61	0.163	6	0.84	0.13
OPB-12	CCTTGACGCA	58	41	70.68	0.173	8.6	0.70	0.12
OPB-15	GGAGGGTGTT	34	27	79.41	0.170	5.23	0.79	0.13

PIC= Polymorphic information content, RP= Resolving power, MI= marker index EMR= effective multiplex ratios

Table 18. Jaccard's coefficients of similarity matrix determined by analysis using six RAPD primers from 13 species of Heliconiinae.

ROWS\COL	MZBF10	MZBF31	MZBF35	MZBF75	MZBF78	MZBF98	MZBF101	MZBF110	MZBF116	MZBF109	MZBF137	MZBFs1	MZBF136
MZBF10	1.000												
MZBF31	0.135	1.000											
MZBF35	0.057	0.061	1.000										
MZBF75	0.056	0.059	0.033	1.000									
MZBF78	0.071	0.049	0.056	0.114	1.000								
MZBF98	0.047	0.049	0.118	0.054	0.095	1.000							
MZBF101	0.063	0.065	0.048	0.125	0.061	0.083	1.000						
MZBF110	0.109	0.043	0.100	0.098	0.061	0.020	0.055	1.000					
MZBF116	0.205	0.071	0.111	0.108	0.091	0.067	0.059	0.125	1.000				
MZBF109	0.024	0.025	0.000	0.000	0.023	0.048	0.087	0.087	0.000	1.000			
MZBF137	0.000	0.043	0.103	0.128	0.085	0.109	0.075	0.118	0.104	0.043	1.000		
MZBFs1	0.094	0.065	0.000	0.261	0.059	0.000	0.050	0.050	0.086	0.030	0.051	1.000	
MZBF136	0.167	0.000	0.037	0.115	0.125	0.029	0.135	0.105	0.086	0.063	0.000	0.182	1.000

MZBF10*(V. erota)*, MZBF31*(A. issoria)*,MZBF35 (*C. biblis*),MZBF78 (C.aoris), MZBF75 (*P. phalanta)*,MZUBF101 (C.tyche), MZBF109*(A. laodice)*, MZBF98 *(C. cyane)*, MZBFsl*(P. alcippe)*, MZBF116*(V. egista)*,MZBF109*(A. laodice,)*MZBF137 (*A. children,)* MZBF136*(C. erymanthis)*.

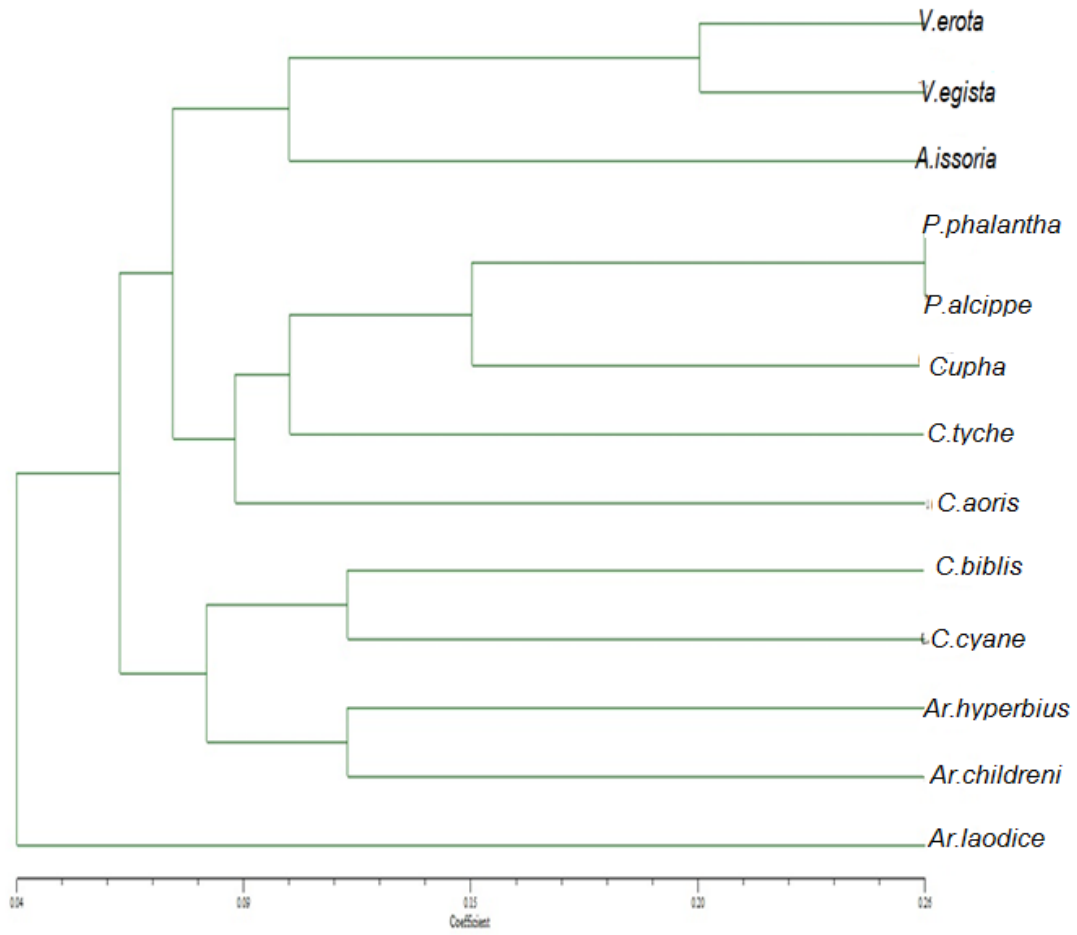


Figure 24. Dendrogram of thirteen species of Heliconiinae based on RAPD data.

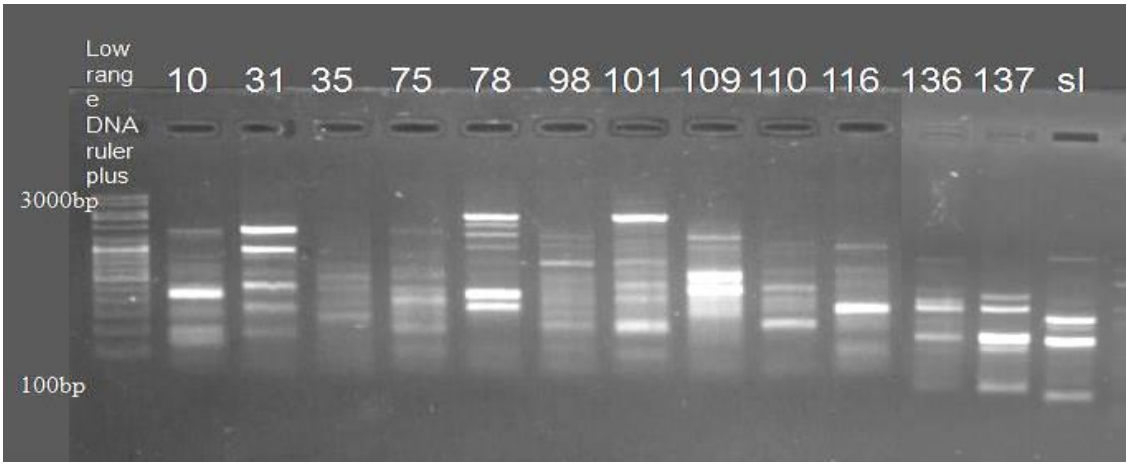


Figure 25. RAPD fragments generated by OPT- 5 primer in 13 Heliconiinae species of butterfly. (10=*Vindula erota*, 31=*Acreae issori,* 35=*Cethosia biblis*, 75=*Phalanta phalantha*, 78=Cirrochroa aoris, 98=*Cethosia cyane*, 101=*Cirrochroa tyche*, 109=*Argynnis laodice*, 116=*Vagrans egista*, 136=*Cupha erymanthis*, 137 =*Argynnis children*, sl=*Phalanta alcippe*)

4.6. Phylogenetic study inferred from Mitochondrial DNA sequence analysis

4.6.1. Characteristics of data sets

The CO1 and ND1 regions of the mitochondrial genome were amplified using PCR from the species of Heliconiinae butterflies collected from Northeast India. The size of the CO1 sequenced PCR amplified fragments were approximately 750 bp (Fig. 26) while the amplified ND1 sequenced fragments were approximately 580 bp long which includes fragments of 16S rRNA, the intervening tRNA leu region and the ND1 gene region (Fig. 27) The amplified sequences were submitted and published in NCBI genbank (Table 30).

CO1 data

The sequences of thirteen species used for the study contained no indels and there was a perfect match in the alignment. MEGA analysis of CO1 data generated the genetic distance of each of the thirteen species of Heliconiinae as shown in the Table 19. The nucleotide maximum distance (p-distance) between *Cethosia biblis* and *Cethosia cyane* was found to be lowest at 0.056 and the highest p-distance was found between *C. tyche* and *V. erota; C. cyane* and *C. aoris; C. cyane*and *V. egista* at 0.186 (Table 19).

The Maximum likelihood estimate of transitional substitution matrix between A/G = 2.84, T/C = 16, C/T = 36.47and G/A = 5.8 while those of transversionsal substitutions between T/A = 13.78, C/A = 4.03, A/T = 18, A/C = 2.31, C/G = 0.35 G/C = 0.41 (Table 21). Substitution pattern and rates were estimated under the General Time Reversible model (+G+I). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+*G*], parameter = 0.1910). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.0000% sites). Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions

are shown in normal. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 100, the nucleotide frequencies are A = 29.68%, T/U = 38.78%, C = 17.02%, and G = 14.53%. For estimating ML values, a user-specified toplogy was used. The maximum Log likelihood for this computation was -2992.787. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 613 positions in the final dataset.

The estimated Transition/Transversion bias (R) was 1.06.Substitution pattern and rates were estimated under the Kimura-2-parameter model (+G+I). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+*G*], parameter = 4.5039). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 64.0850% sites). The nucleotide frequencies are A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. For estimating ML values, a user-specified toplogy was used. The maximum Log likelihood for this computation was -3277.655. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 613 positions in the final dataset.

The Tajima test statistic (D) was 1.284120 for 13 number of sequences (m), number of segregating sites (S) was 199, and the value for p_s andΘ are 0.324633 and 0.104612 respectively with 0.165189 nucleotide diversity ($\boldsymbol{\pi}$) where p_s = S/m, $\Theta = p_s/a_1$

ND1 data

Estimates of evolutionary divergence between ND1 sequences of the ten species of Heliconiinae butterflies were generated as p-distance given in the Table 20. The nucleotide maximum distance (p-distance) between *C. biblis* and *C. cyane* was found to be

lowest at 0.050 and the highest p-distance was found *A. issoria and P. phalantha* at 0.254. The number of base substitutions per site from between sequences is shown in the Table 22. The number of base substitutions per site from averaging over all sequence pairs was calculated as 0.174. Analyses were conducted using the Maximum Composite Likelihood model and involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 310 positions in the final dataset.

The Maximum likelihood estimate of transitional substitution matrix between A/G = 14.32, T/C = 2.13, C/T = 12.27 and G/A = 37.02 while those of transversional substitutions between A/T = 12.04, T/A = 8.81, C/G = 5.93, G/T = 2.98 and G/C = 3.64 (Table 22). Substitution pattern and rates were estimated under the Tamura-Nei (1993) model. In the Table 22, rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in normal. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 100, the nucleotide frequencies are A = 33.43%, T/U = 45.69%, C = 7.95%, and G = 12.93%. For estimating ML values, a user-specified topology was used. The maximum Log likelihood for this computation was -1593.23.

According to Tajima's (1989) test of neutrality, the number of segregating sites (S) was 123 and the nucleotide diversity (π) within the sequences was 0.150, and Tajima test statistic (D) gave a value of 0.531. The estimated Transition/Transversion bias (R) under the GTR model across the sequences was 0.7. Overall codon bias usage in CO1 and ND1 are given in the Tables 23 and 24 respectively where Relative Synonymous Codon Usage (RSCU) value of each codon was given. RSCU is a simple measure of non-uniform usage of synonymous codons in a coding sequence (Sharp *et al.* 1986). A codon that is used less

frequently than expected will have an RSCU value of less than 1 and vice versa for a codon that is used more frequently than expected. If the synonymous codons of an amino acid are used with equal frequencies, their RSCU values were equal to 1.

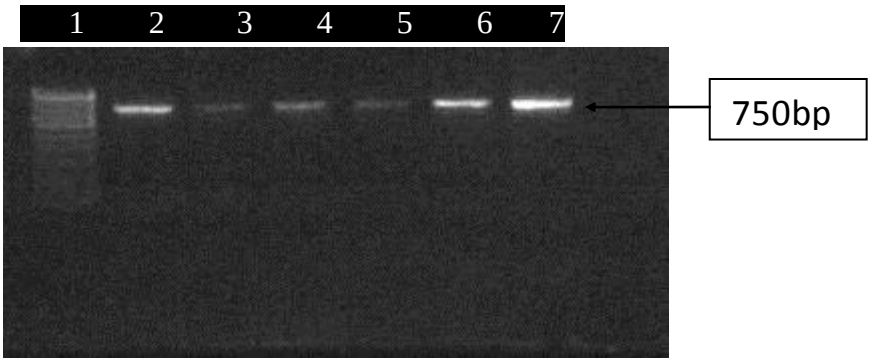


Figure 26. Gel electrophoresis representation of CO1 gene among species of Heliconninae under study. Lane 1: 100 bp ladders; Lane: Bands showing CO1 genes of 2. *Acraea issoria,* 3. *Cethosia biblis,* 4. *Phalanta phalantha,* 5. *Cirrochroa aoris,* 6. *Cirrochroa cyane.* 7. *Vagrans egista*

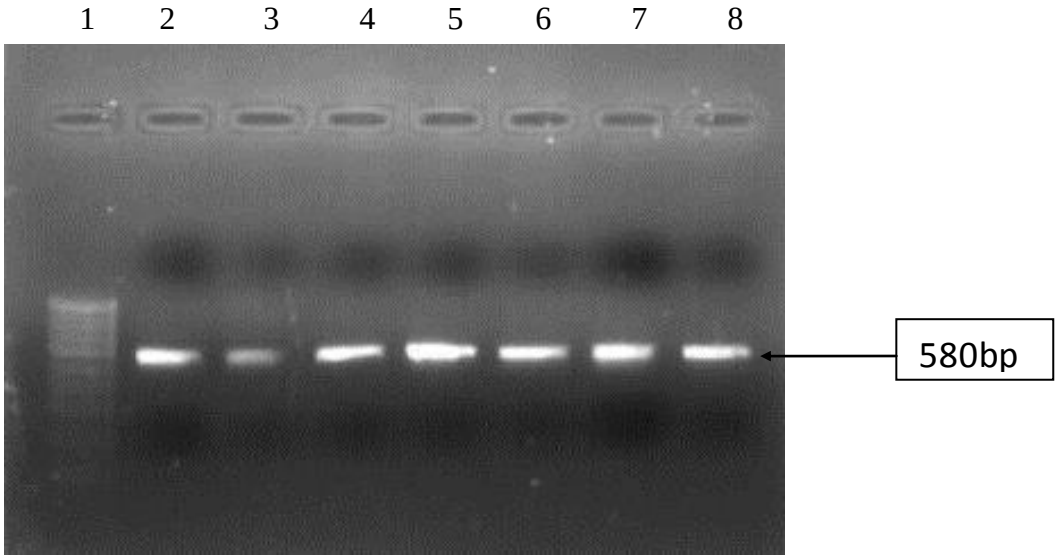


Figure 27. Gel electrophoresis representation of CO1 gene among spepies of Heliconninae under study. Lane 1: 100 bp ladders; Lane: ND1 genes of 1.*Cethosia cyane,* 2.*Cethosia biblis,* 3.*Vindula erota,* 4.*Vagrans egista,* 5.*Argynnis hyperbius,* 6.*Cirrochroa aoris,* 7.*Phalanta phalantha.*

Table 19. Estimates of Evolutionary Divergence between Sequences from CO1 data.

		P- distance												
		A	B	C	D	E	F	G	H	I	J	K	L	M
A	*Argynnis hyperbius*	0.000												
B	*Vindula erota*	0.151												
C	*Cirrochroa tyche*	0.132	0.186											
D	*Acraea issoria*	0.146	0.159	0.155										
E	*Centosia biblis*	0.170	0.181	0.158	0.149									
F	*Phalanta phalantha*	0.156	0.150	0.146	0.149	0.178								
G	*Cirrochroa aoris*	0.145	0.184	0.073	0.159	0.167	0.158							
H	*Cethosia cyane*	0.164	0.175	0.170	0.148	0.056	0.176	0.186						
I	*Vagrans egista*	0.152	0.180	0.177	0.152	0.179	0.154	0.177	0.186					
J	*Argynnis childreni*	0.084	0.148	0.151	0.130	0.177	0.172	0.155	0.178	0.163				
K	*Argynnis laodice*	0.097	0.174	0.137	0.129	0.169	0.173	0.144	0.182	0.156	0.080			
L	*Cupha erymanthis*	0.148	0.150	0.155	0.149	0.167	0.126	0.149	0.172	0.127	0.157	0.173		
M	*Phalanta alcippe*	0.133	0.143	0.123	0.135	0.151	0.134	0.143	0.149	0.155	0.133	0.135	0.124	0.000

The numbers of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 613 positions in the final dataset.

Table 20. Estimates of evolutionary divergence between sequences from ND1 data.

	P- distance										
	1	2	3	4	5	6	7	8	9	10	11
1.Argynnis hyperbius	0.000										
2.Vagrans egista	0.162										
3.Cirrochroa aoris	0.136	0.166									
4.Cethosia cyane	0.143	0.209	0.156								
5.Cethosia biblis	0.145	0.199	0.156	0.050							
6.Vindula erota	0.165	0.208	0.134	0.193	0.203						
7.Phalanta phalantha	0.156	0.165	0.181	0.167	0.163	0.178					
8.Cupha erymanthis	0.152	0.170	0.138	0.200	0.209	0.158	0.163				
9.Acraea issoria	0.189	0.217	0.207	0.200	0.196	0.213	0.254	0.204			
10.Argynnis childreni	0.088	0.166	0.141	0.159	0.165	0.162	0.160	0.153	0.189		
11.Euploea mulciber	0.162	0.203	0.169	0.177	0.191	0.195	0.221	0.168	0.224	0.183	0.000

The numbers of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 310 positions in the final dataset.

Table 21. Maximum likelihood estimate of substitution matrix for CO1.

	A	T/U	C	G
A	-	18.00	2.31	**2.84**
T/U	13.78	-	**16.00**	0.00
C	4.03	**36.47**	-	0.35
G	**5.80**	0.00	0.41	-

Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in normal.

Table 22. Maximum likelihood estimate of substitution matrix for ND1.

	A	T/U	C	G
A	-	12.04	0.00	**14.32**
T/U	8.81	-	**2.13**	0.84
C	0.00	**12.27**	-	5.93
G	**37.02**	2.98	3.64	-

Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in normal.

Table 23. Overall codon bias usage in CO1.

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	10.8	1.68	UCU(S)	8.2	3.56	UAU(Y)	1.8	1.23	UGU(C)	0	0
UUC(F)	2.1	0.32	UCC(S)	1.5	0.67	UAC(Y)	1.2	0.77	UGC(C)	0	0
UUA(L)	20.2	3.8	UCA(S)	4	1.75	UAA(*)	0	0	UGA(W)	4	2
UUG(L)	0.2	0.04	UCG(S)	0	0	UAG(*)	0	0	UGG(W)	0	0
CUU(L)	8.8	1.65	CCU(P)	5.8	1.8	CAU(H)	3.1	1.57	CGU(R)	1.2	1.15
CUC(L)	0.2	0.03	CCC(P)	2.9	0.91	CAC(H)	0.8	0.43	CGC(R)	0	0
CUA(L)	2.5	0.46	CCA(P)	3.9	1.22	CAA(Q)	2.8	1.95	CGA(R)	2.8	2.85
CUG(L)	0.1	0.01	CCG(P)	0.2	0.07	CAG(Q)	0.1	0.05	CGG(R)	0	0
AUU(I)	21.5	1.92	ACU(T)	5.1	1.65	AAU(N)	10.3	1.64	AGU(S)	2.2	0.94
AUC(I)	0.8	0.08	ACC(T)	0.5	0.15	AAC(N)	2.2	0.36	AGC(S)	0.2	0.07
AUA(M)	12.2	1.96	ACA(T)	6.8	2.2	AAA(K)	0	0	AGA(S)	2.3	1.01
AUG(M)	0.2	0.04	ACG(T)	0	0	AAG(K)	0	0	AGG(S)	0	0
GUU(V)	4.3	1.67	GCU(A)	6.6	1.87	GAU(D)	6	1.5	GGU(G)	3.4	0.68
GUC(V)	0.4	0.15	GCC(A)	2.2	0.63	GAC(D)	2	0.5	GGC(G)	0.2	0.05
GUA(V)	5	1.94	GCA(A)	5.1	1.43	GAA(E)	1.9	1.92	GGA(G)	15.5	3.13
GUG(V)	0.6	0.24	GCG(A)	0.2	0.07	GAG(E)	0.1	0.08	GGG(G)	0.7	0.14

Table 24. Overall codon bias usage in CO1.

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	8.4	1.94	UCU(S)	2.8	3.7	UAU(Y)	7	1.9	UGU(C)	0.2	2
UUC(F)	0.3	0.06	UCC(S)	0.2	0.24	UAC(Y)	0.4	0.1	UGC(C)	0	0
UUA(L)	14.9	5.53	UCA(S)	1.7	2.27	UAA(*)	0	0	UGA(W)	0.9	1.82
UUG(L)	0.7	0.27	UCG(S)	0.1	0.12	UAG(*)	0	0	UGG(W)	0.1	0.18
CUU(L)	0.3	0.1	CCU(P)	3.1	3.16	CAU(H)	0	0	CGU(R)	0.6	1.22
CUC(L)	0	0	CCC(P)	0	0	CAC(H)	0	0	CGC(R)	0	0
CUA(L)	0.3	0.1	CCA(P)	0.7	0.74	CAA(Q)	2.3	1.43	CGA(R)	1.2	2.26
CUG(L)	0	0	CCG(P)	0.1	0.09	CAG(Q)	0.9	0.57	CGG(R)	0.3	0.52
AUU(I)	14.5	1.99	ACU(T)	1.7	2.45	AAU(N)	5.5	1.94	AGU(S)	0.5	0.72
AUC(I)	0.1	0.01	ACC(T)	0	0	AAC(N)	0.2	0.06	AGC(S)	0	0
AUA(M)	8.1	1.71	ACA(T)	1.1	1.55	AAA(K)	4	1.57	AGA(S)	0.7	0.96
AUG(M)	1.4	0.29	ACG(T)	0	0	AAG(K)	1.1	0.43	AGG(S)	0	0
GUU(V)	3.7	2.25	GCU(A)	1.5	3.09	GAU(D)	2	2	GGU(G)	2.9	1.33
GUC(V)	0.1	0.05	GCC(A)	0.1	0.18	GAC(D)	0	0	GGC(G)	0.2	0.08
GUA(V)	1.9	1.15	GCA(A)	0.4	0.73	GAA(E)	1.2	1.37	GGA(G)	3.3	1.5
GUG(V)	0.9	0.55	GCG(A)	0	0	GAG(E)	0.5	0.63	GGG(G)	2.4	1.08

4.6.2 General Phylogenetic Pattern

The final dataset from CO1 consisted of 37 taxa, 624 characters including one outgroup. CO1 data contained 407 conserved sites, 217 variable sites and approximately 198 sites were parsimony informative. For certain taxa, we were unable toamplify the ND1 gene fragments. The final ND1 sequence data contained 324 nucleotides, of which 126 were variable and approximately 74 were parsimony informative (Table 25).

The evolution of the sequences was modeled under the GTR+G+I model for both CO1 and ND1 as it was considered to describe the substitution pattern the best from the Bayesian Information Criterion (BIC) scores. For CO1 data, GTR+G+I model scored 6290.8 using 33 parameters among the ML fits of 24 different nucleotide substitution models with a negative log likelihood of 2997.17. In ND1 data analysis using 29 parameters, the BIC score was 3208.278 with a negative log likelihood of 1486.189. Bayesian analysis for CO1 was set at 10 million generations resulted in 100000 trees. The effective sample size (ESS) was 2830.7236 and LnL score was -3874.5104. The first 20% trees were considered as the burn-in phase and discarded. The standard error of mean was 0.1488. Bayesian Inference analysis for ND1 was set at 8 million generations giving 800000 trees in which the effective sample size was 15015.04 and LnL score was-1334.44

For CO1 analysis, 4 tribes of the Heliconiinae were analysed where tribe Acraeni was represented by only one taxon. The tree topologies with the entire three optimal criterions revealed monophyly within each 4 tribes (Fig. 28). The tree topology of CO1 can be divided into 2 major clades. *E. mulciber,* a species from sub-family Danainae was used as outgroup. The first clade included all the taxa from the tribe Vagrantini where all the genera except *Phalanta* showed monophyly with high support value. Genus *Phalanta* occurred in two clades in which the first one (*P. phalantha)* formed a basal clade for *Vagrans* and *Cupha*

while the second genus formed a sister clade with *Cirrochroa*. The second clade was divided into 2 sub-clades, one consisting of the tribe Argynnini and the other sub-clade consisted of the two tribes Acraeini and Heliconnini as sister clade. For ND1 data analysis, only 10 nucloetide sequences were avalaible for the four tribes of Heliconninae represented by 8 genera. However, the tree topology showed monophyly within each genus. The tree topology was essentially in congruent with that generated from CO1 data (Fig. 29). The tribe Vagrantini formed the basal clade. Acraeni and Heliconiini formed a sister clade which again formed a sister clade with tribe Argynnini.

Table 25. Comparision of the mitochondrial DNA molecular diversity of CO1 and ND1 used in Maximum Parsimony.

Sl no		CO1	ND1
1	Total Characters	624	324
2	Conserved sites	407	198
3	Variable sites	217	126
4	Parsimony informative sites	198	74

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

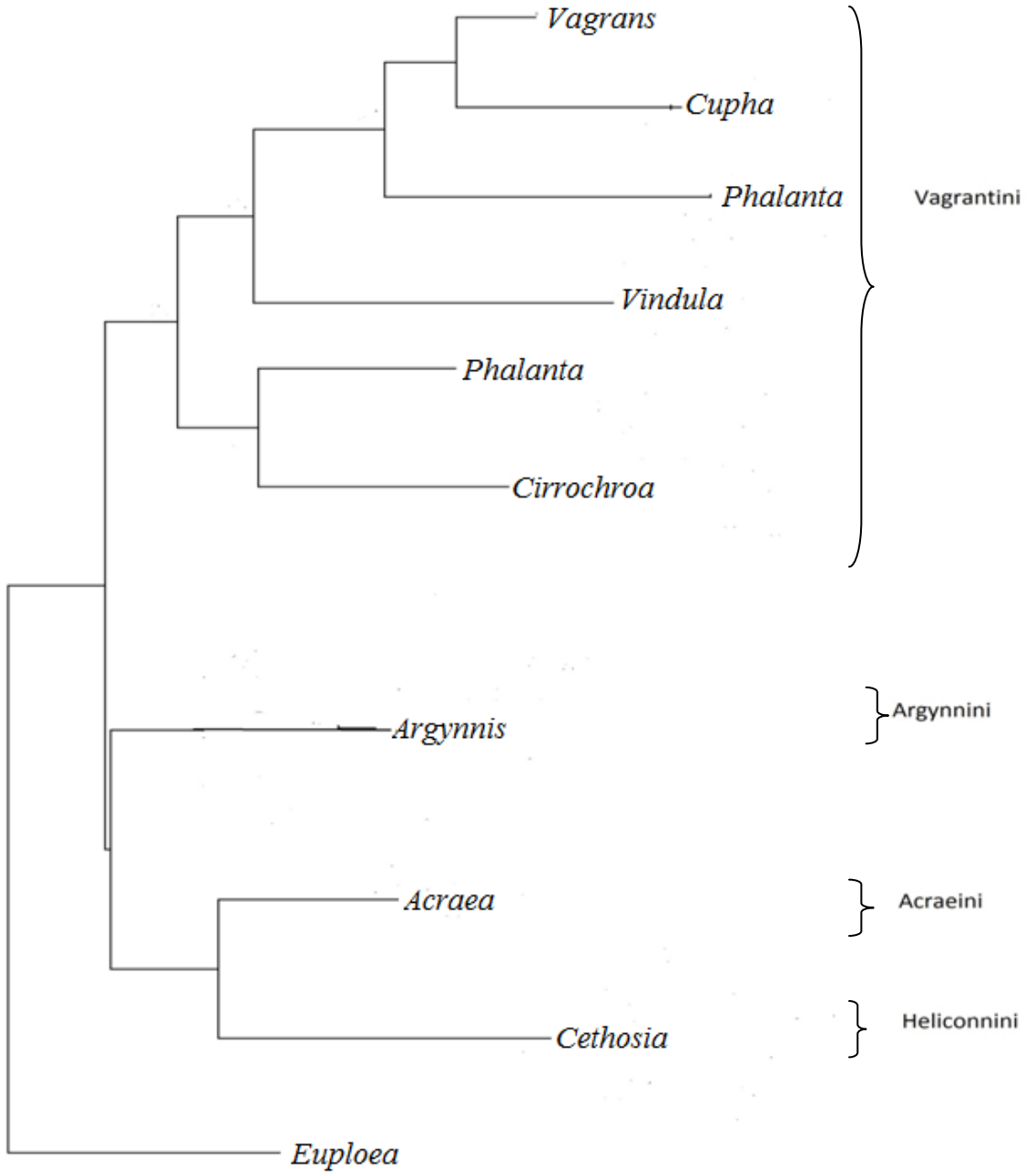


Figure 28. A reconstructed phylogenetic tree of tribes of the sub-family Heliconninae of NE India with one outgroup based on CO1 data.

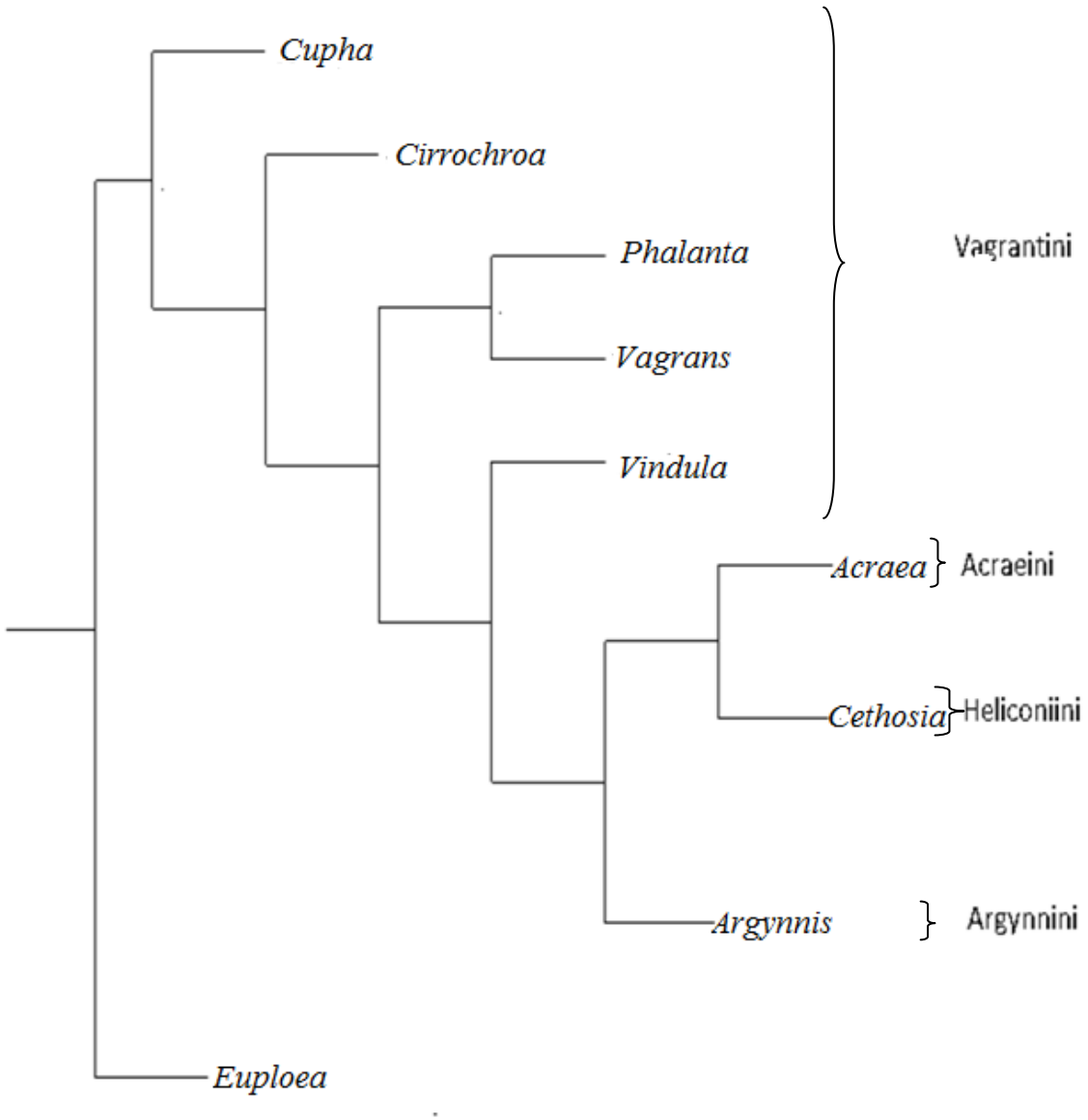


Figure 29. A reconstructed Phylogenetic tree of the sub-family Heliconninae of NE India with one outgroup based on ND1 data.

Table 26. Monophyly of the genera of Heliconninae in CO1 and ND1.

Tribe	Genus	CO1				ND1			
		No. of taxa	No. of species	Mono phyly	Occur rence of clade	No. of taxa	No. of species	Mono phyly	Occurr ence of clade
Vagrantini	*Vagran*	3	1	yes	1	1	1	yes	1
	Cupha	3	1	yes	1	1	1	yes	1
	Phalanta	4	2	no	2	1	1	yes	1
	Vindula	3	1	yes	1	1	1	yes	1
	Cirrochroa	6	2	yes	1	1	1	yes	1
Argynnini	*Argynnis*	8	3	yes	1	2	2	yes	1
Acraeni	*Acreae*	3	1	yes	1	1	1	yes	1
Heliconni	*Cethosia*	6	2	yes	1	2	2	yes	1

4.6.3 Monophyly of the genera in CO1 data analysis.

The topology obtained from Maximum Parsimony analysis with 1000 bootstrap pseudoreplicates (Fig. 30) was used as an initial estimate of the phylogeny and compared it with those obtained under alternative optimality criteria. The topologies of all the trees recovered from CO1 and ND1 using the three optimal criterions revealed the monophyly of subclades corresponding to genera and species. Only one outgroup, *Euploea mulciber* was used for all the analyses. *Cethosia* with two species and *Cirrochroa* with two species were in a monophyletic clade with 100% bootstrap support, while *Argynnis* with three species formed a monophyletic clade with 76% bootstrap support. The genus *Phalanta* occurred in two subclades where one was a basal clade for *Cethosia* (tribe Heliconnini) and *Cirrochroa* and the other formed a basal clade for *Cupha* and *Vagrans*.

The evolutionary history inferred by using the Maximum Likelihood as optimality criteria method was based on the General Time Reversal model. A tree with the highest log likelihood (-4124.3013) is shown in Fig. 31. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The clades corresponding to all the monophyletic genera showed strong support, all above 65% bootstrap support. Only the genus *Phalanta* occurred in two subclades and was recovered as a paraphyletic to the other genera within the tribe Vagrantini. All the individuals belonging to the same species are monophyletic with strong support higher than 90% bootstrap value. For the ML analyses, bootstrap support levels are generally high near the tips of the trees, typically within and among closely related species, whereas support at deeper levels among tribes or more inclusive clades is very weak, with almost no nodes reaching 50% of bootstrap value.

The Bayesian tree shown is a majority rule consensus of the trees sampled from the Markov Chain Monte Carlo (MCMC) method. The Bayesian tree was largely identical with ML tree with few discordant nodes. Tribes Argynnini and Acraeini were recovered as monophyletic clade with 100% posterior probability (PP) support value. However, a tribe Heliconini consisting of two species of *Cethosia* was paraphyletic with respect to tribe Vagrantini. In striking contrast to MP and ML analysis, support value at deeper levels in the Bayesian analysis is very high, with posterior probabilities along the backbone of the tree nearly all 100%. The tribe Argynini being the basal clade consisted of three species, each with three individuals, was recovered as a monophyletic clade with 100% posterior probability support value. The second clade corresponding to the tribe Acraeini was also a monophyly with 100% PP support value consisiting of three individuals of *Acraea issoria*. The last major clade represented the tribe Vagrantini which included all the genera from the tribe with high posterior probability support value, and also consisted of one subclade of tribe Heliconini having two species of genus *Cethosia*. *Cethosia* formed a sister clade with *Cirrochroa* arising from *Phalanta alcippe* with PP support value of 50% (Fig. 32).

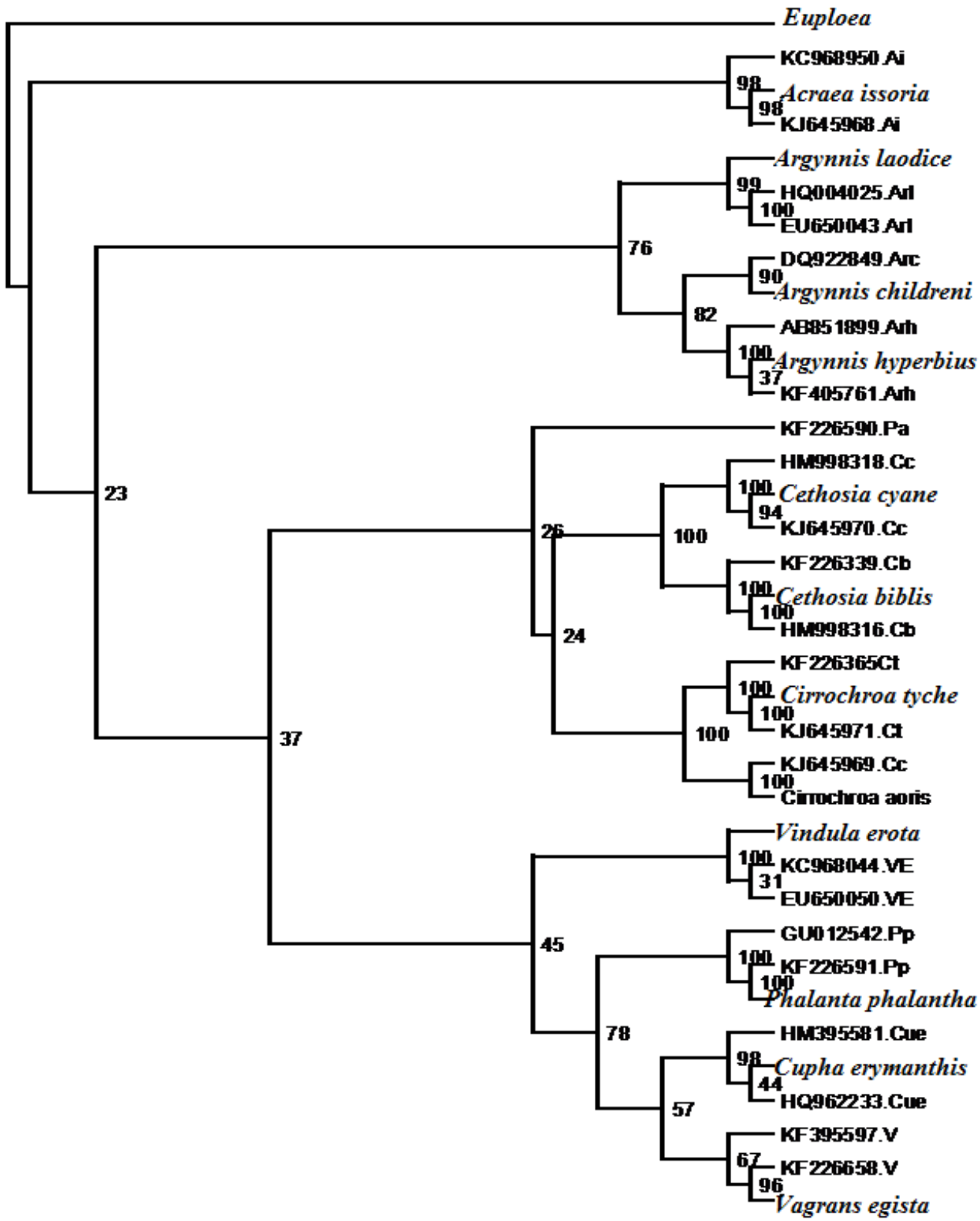


Figure 30. Consensus tree of Maximum Parsimony based on CO1 data. (The taxa with names of accession numbers are retrieved from NCBI genbank)

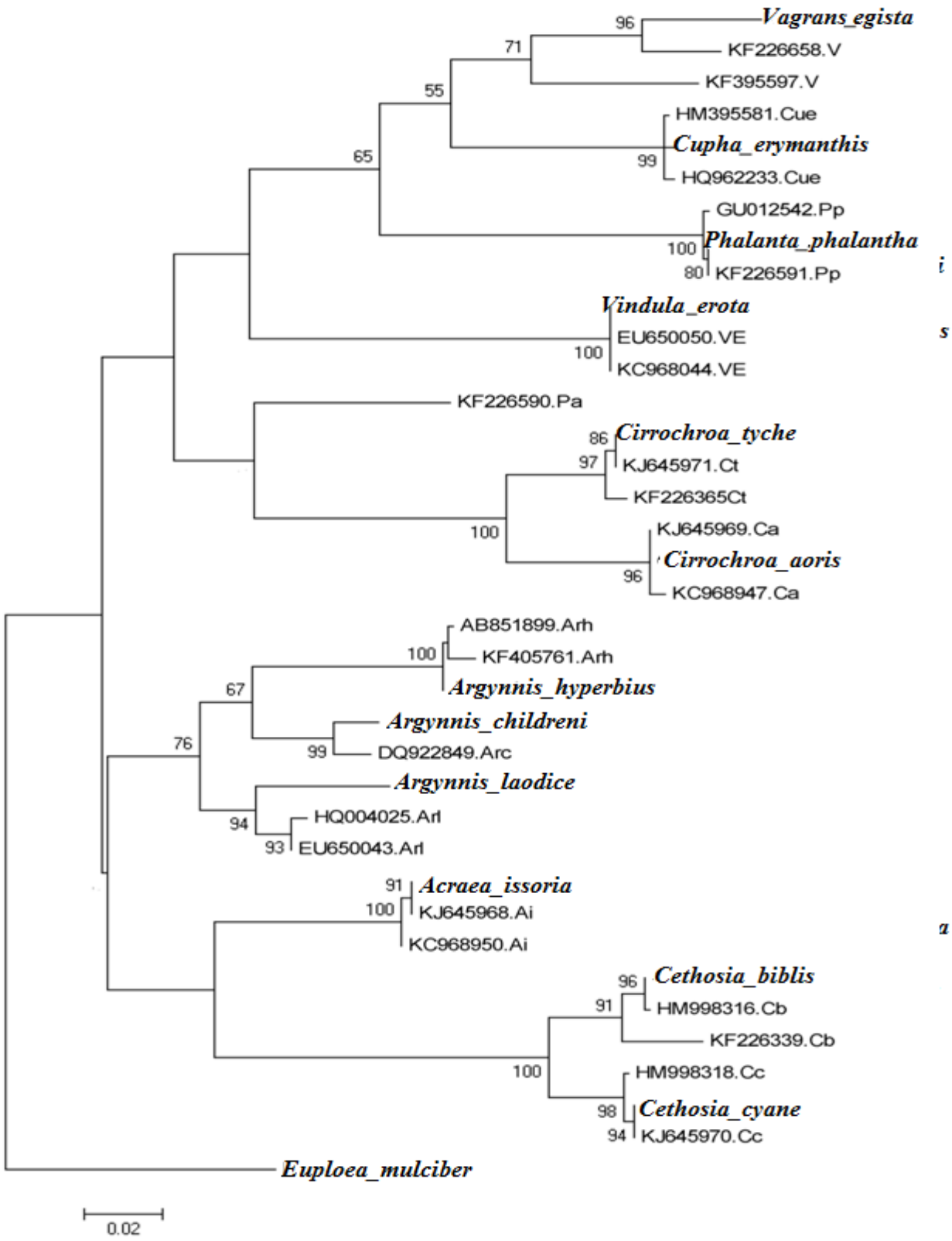


Figure 31. Relationships among sampled species of Heliconninae according to Maximum Likelihood analysis using CO1. (The taxa with names of accession numbers are retrieved from NCBI genbank)

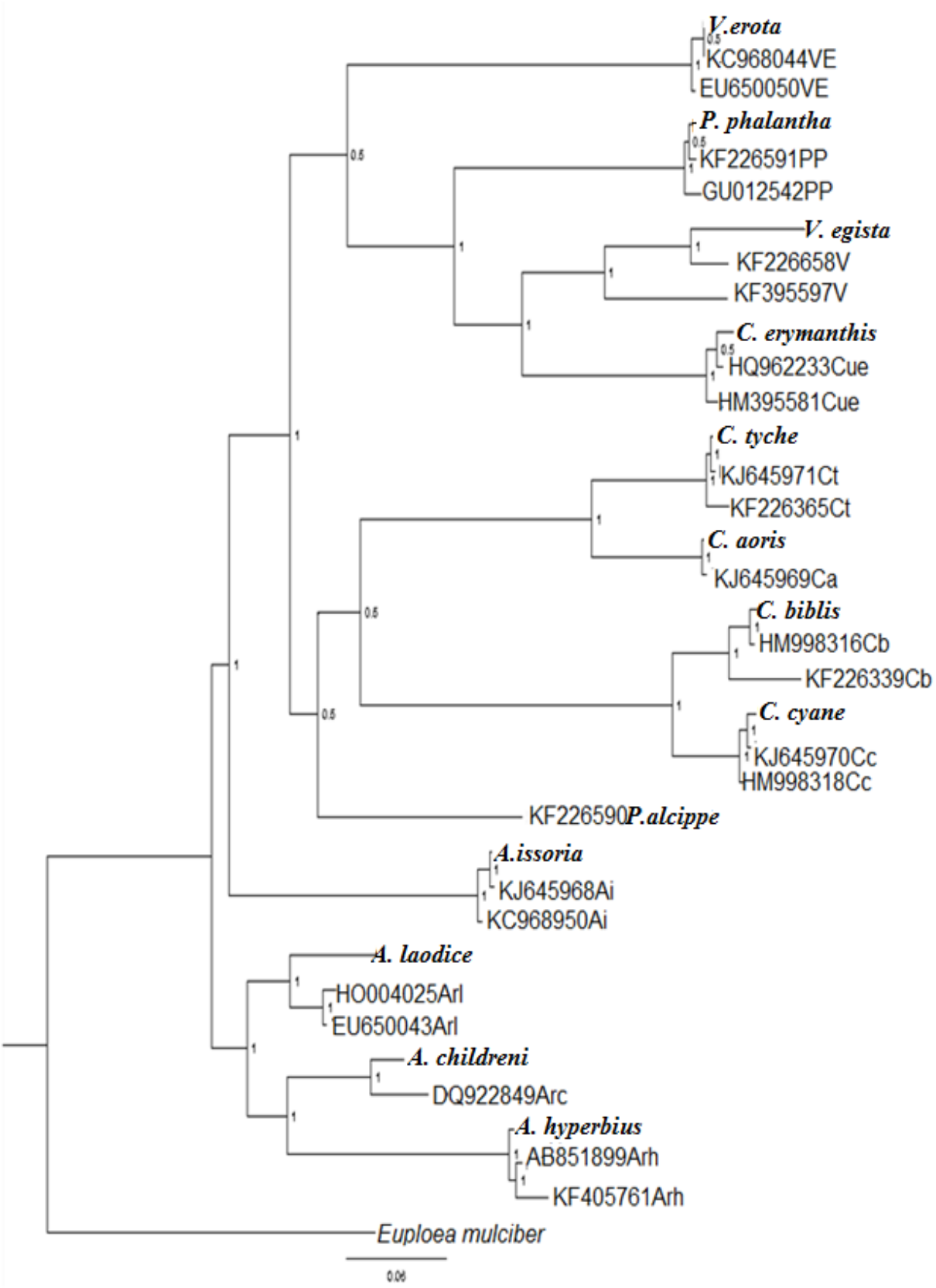


Figure 32. Bayesian probability estimate of Phylogeny of Heliconninae based on CO1 data. (The taxa with names of accession numbers are retrieved from NCBI genbank).

4.6.4 Monophyly of the genera in ND1 data analysis.

For ND1 data only ten taxa were available for analysis. *Euploea mulciber* was used as outgroup. Maximum Parsimony with 1000 bootstrap pseudo-replicates produced a tree with unweighted data. The tree consisted of two major clades. In the first clade, *Cupha* formed a sister clade to *Vindula* and *Cirrochroa*. The other clade again separated into 3 subclades. The first subclade consisted of two genera- *Vagrans* and *Phalanta*, both from tribe Vagrantini. The second subclade corresponded to tribe Argynini represented by two species of *Argynnis* with 60% bootstrap support. In the third subclade tribe Acraeini represented by Acraea issoria formed a sister clade with tribe Heliconini represented by two species of *Cethosia*having 100% bootstrap support (Fig. 33).

The evolutionary history inferred by using the Maximum Likelihood method was based on the General Time Reversal model. The tree with the highest log likelihood (-1593.2304) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree topology showed two major clades, one consisting of tribe Acraeini represented by Acraea issoria formed a sister clade with tribe Heliconini. The second clade consisted of tribe Vagrantini represented by 5 genera which is a sister clade to Argynini consisting of two species of *Argynni (*Fig. 34).

Bayesian topology also gave a monophylectic clade for tribe Heliconini and Argynini while Acraeni was paraphyly with respect to Vagrantini. Most of the nodes were supported with 50% posterior probability (Fig. 35).

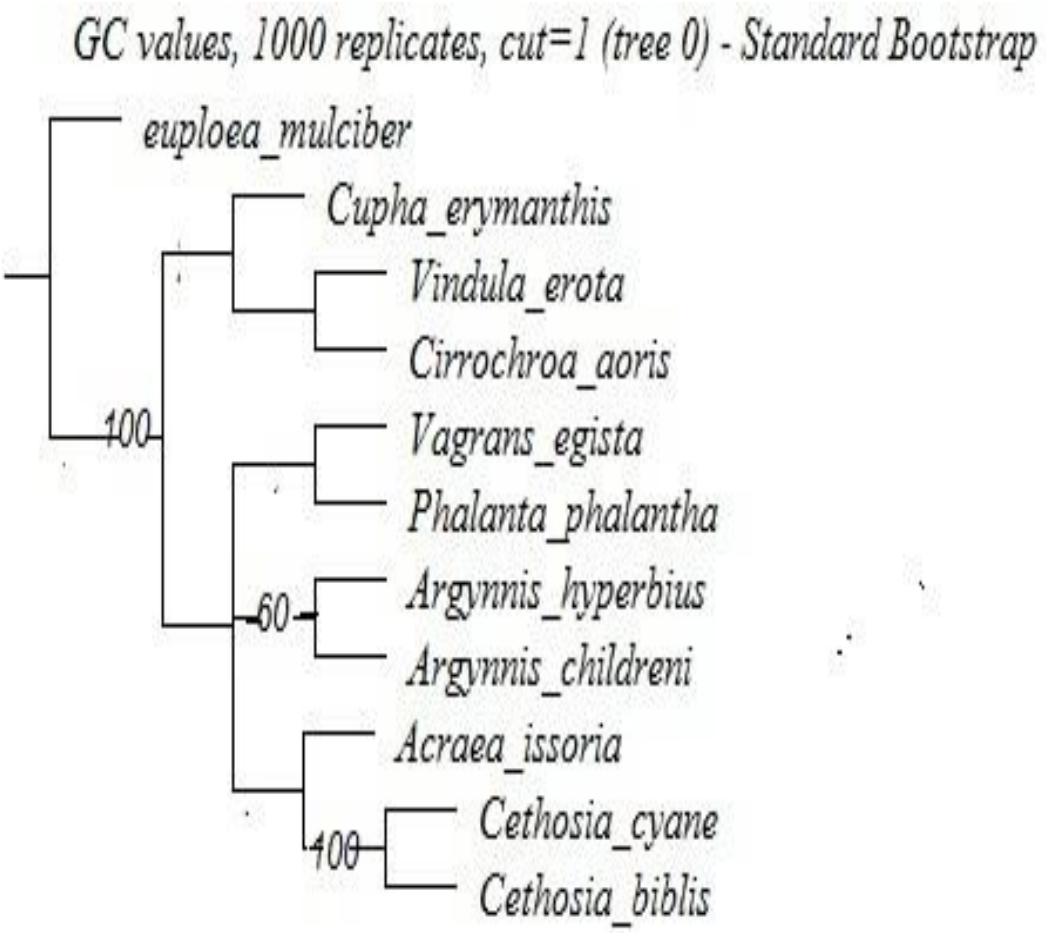


Figure 33. Tree based on Maximum Parsimony based on ND1 data.

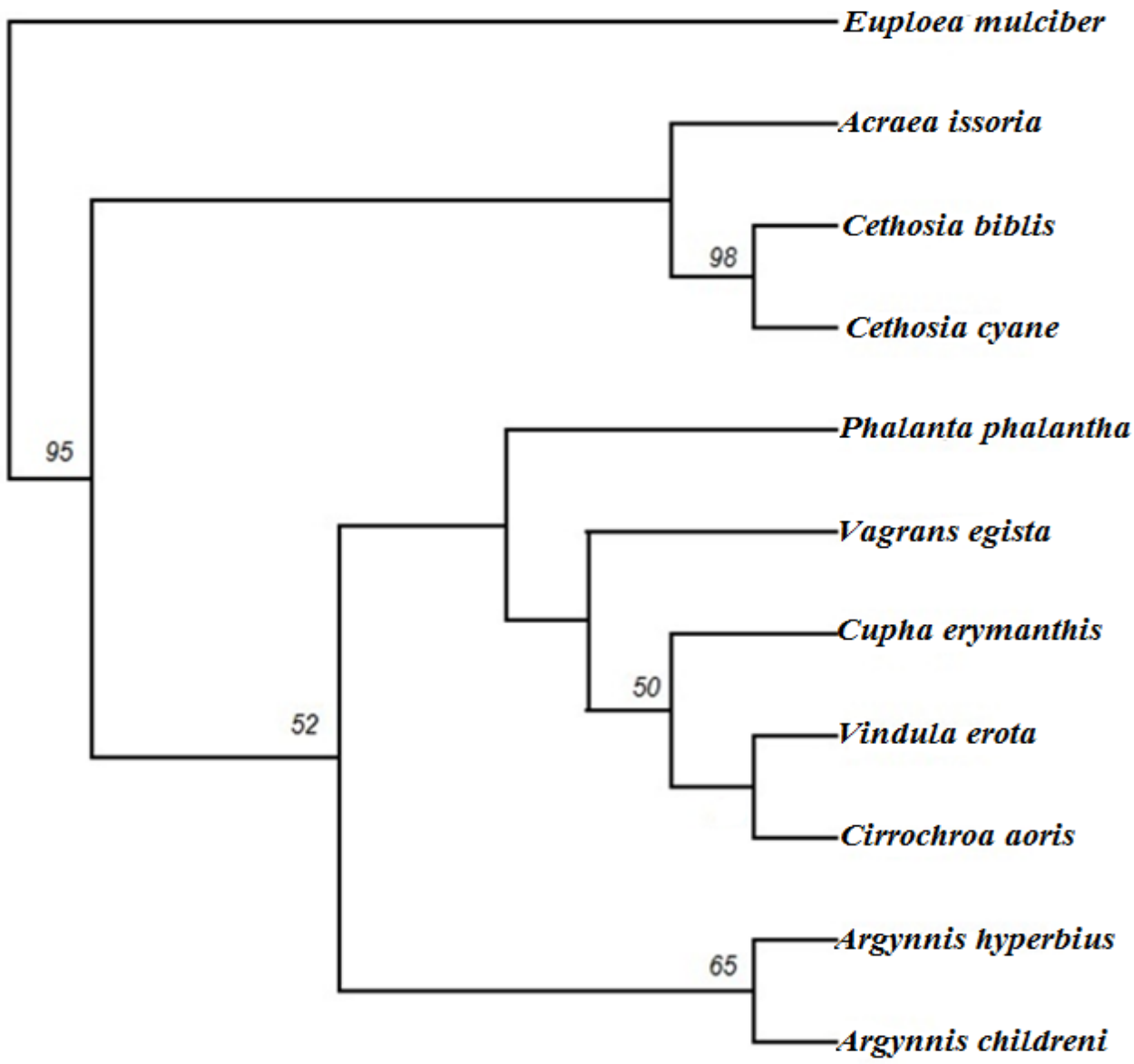


Figure 34. Relationships among sampled species of Heliconninae according to Maximum Likelihood Analysis using ND1.

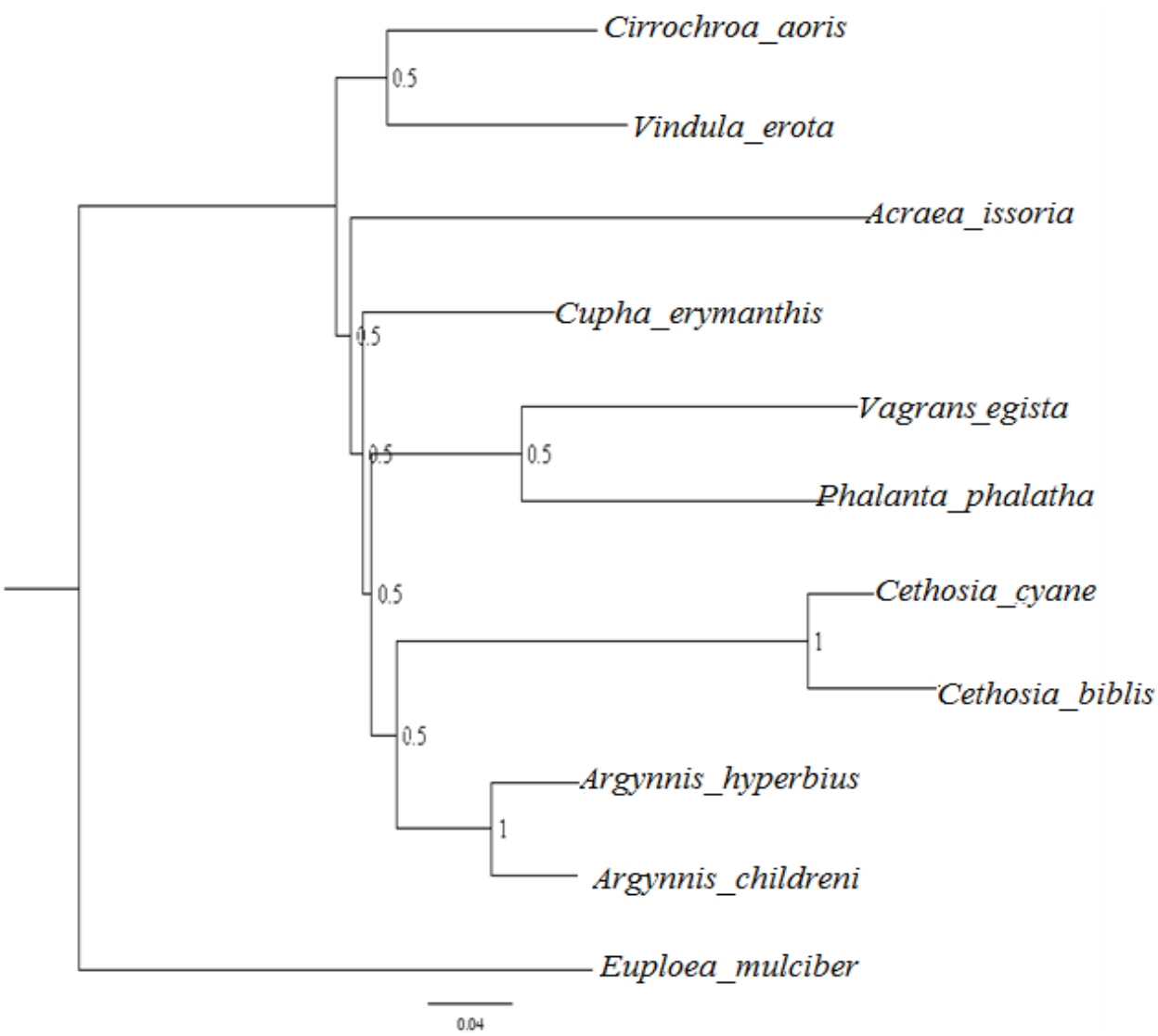


Figure 35. Bayesian probability estimate of Phylogeny of Heliconninae based on ND1 data

4.7 Physico-chemical characterization of *Distal-less (dll)* genes

Eleven distal-less protein sequences of butterflies were retrieved from the Swiss-Prot database (Table 27). The results of the primary sequence analysis of *Distal-less* genes of butterfly are given in Table 28. The computed Iso-electric point (pI) indicated that the distal-less proteins of most of the samples required an acidic buffer solution with a pH range of 4 to 6, except for those of *Heliconius erato* and *Junonia coena*. They require a basic buffer solution with a pH range of 10 to 12. On the basis of instability index, the distal-less protein of only *Heliconius erato*, with a score of less than 40 indicated it was stable in a test tube environment. Extinction coefficient of the distal-less protein at 280 nm ranged from 4470 - 19035 M–1 cm–1, *Bicyclus anynana* scoring the least and *Junonia coenia* scoring the highest. The computed aliphatic index inferred that in comparison to the other samples, the distal-less protein of *Heliconius erato* with an AI>70 may be the only sample able to withstand a wide range of temperatures. The calculated GRAVY index for all the distal-less samples, showed extremely low values. This inferred that the protein was hydrophilic (Table 28).

Proline (Pro) and Serine (Ser) comprised the highest percentage of amino acid content in all the sequences followed closely by Threonine (Thr), Glycine (Gly) and Glutamine (Gln). The average molecular weight of the distal-less protein calculated was 7670 Da (Table 29). The server SOSUI classified all the distal-less proteins as soluble proteins and therefore no trans-membrane regions were identified. Cys_Rec software computed with the exception of *Junonia coenia*, the other ten distal-less protein sequences either had only one or no cysteine residues.

The 3D structure was predicted for four of the eleven selected distal-less proteins and analyzed using Rasmol, a protein molecular viewer. It showed presence of cysteine resides in three of the samples i.e. *Heliconius cydno galanthus*, *Junonia coenia* and

Papilio xuthus (Fig. 36). However, disulphide bond (S-S) formation seems unlikely. With respect to secondary structures, the distal-less protein of *Junonia coenia* AAB32450.1 is shown to contain helix (pink) and turns (blue). There are no beta sheets and the only two cysteine molecules are spaced far apart to disallow any disulphide (SS) bond formation. *Heliconius cydno galanthus* AAW70235.1 also has a helix (pink) and turns (blue). There are no beta sheets and has only on cysteine residue. *Bicyclus anynana* AFM73657.1 has all three secondary structures i.e. helix (pink), turns (blue) and sheets (green) but no cysteine residue. The final distal-less protein of *Papilio xuthus* BAM20275.1 is shown to have all three secondary structures in addition to a cysteine residue.

A phylogenetic tree was constructed with seven sequences of distal-less gene proteins of Heliconinae (Family: Nymphalidae) butterfly using maximum likelihood method (Fig. 36) alongwith *Papilio xuthus* (Family: Papilionidae), *Bicyclus anynana* and *Junonia coenia* (Family: Nymphalidae). The tree was divided into two major clusters in which all the Heliconiinae butterflies formed one major cluster and the other three species represent another clade. However, the tree topology showed polytomy in all clades and cannot give proper resolution.

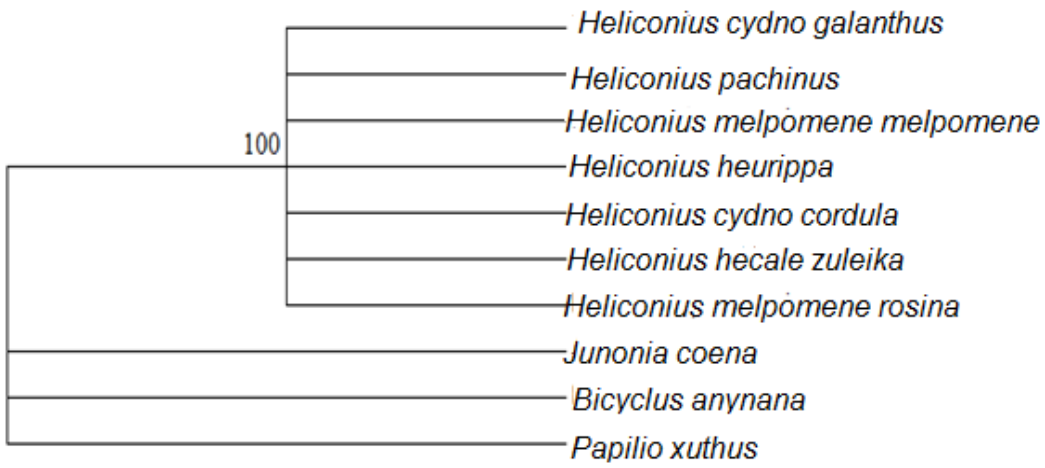


Fig. 36. A Maximum Likelihood tree constructed for Distal-less genes

Table 27. Distal-less protein sequences of butterflies retrieved from Swiss-Prot database.

Accession number	Organism name	Sequence description
AAW70235.1	*Heliconius cydno galanthus*	Distal less protein
AAW70254.1	*Heliconius melpomene rosina*	Distal less protein
AAW70243.1	*Heliconius pachinus*	Distal less protein
ABE27221.1	*Heliconius melpomene*	Distal less protein
ABE27212.1	*Heliconius heurippa*	Distal less protein
ABW74852.1	*Heliconius erato*	Distal less protein
ABE27198.1	*Heliconius cydno cordula*	Distal less protein
ABE02003.1	*Heliconius hecale zuleika*	Distal less protein
AFM73657.1	*Bicyclus anynana*	Distal less protein
AAB32450.1	*Junonia coenia*	Distal-less Dll limb-patterning gene
BAM20275.1	*Papilio xuthus*	Distal less protein

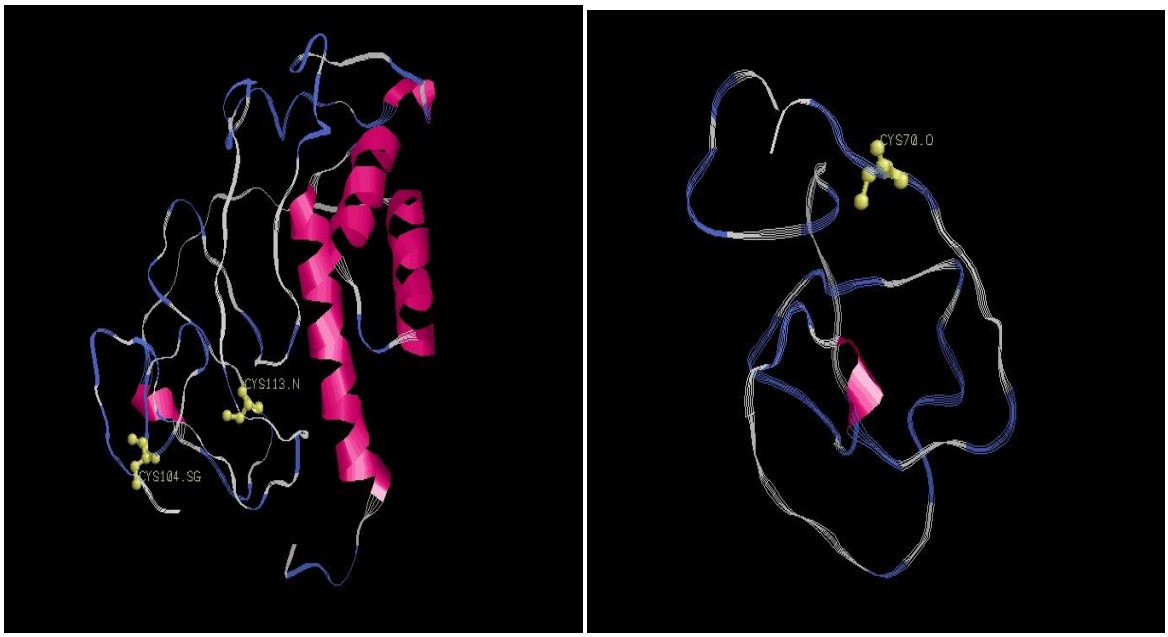
Table 28. Parameters of butterfly distal less genes computed using Expasy's ProtParam tool.

Accession number	Sequence length	M. wt	pI	– R	+ R	EC	II	AI	GRAVY
H. c. galanthus	75	7694.3	5.32	6	4	13980	89.39	33.73	-0.971
H.m. rosina	75	7670.2	5.96	5	4	13980	83.50	33.73	-0.923
H. pachinus	75	7670.2	5.96	5	4	13980	83.50	33.73	-0.923
H. melpomene	75	7752.3	5.36	6	4	13980	83.91	33.73	-0.975
H. heurippa	75	7670.2	5.96	5	4	13980	83.50	33.73	-0.923
H. erato	58	6812.8	11.42	2	11	8480	31.20	70.69	-0.948
H. c.cordula	75	7670.2	5.96	5	4	13980	83.50	33.73	-0.923
H. h. zuleika	75	7670.2	5.96	5	4	13980	83.50	33.73	-0.923
B. anynana	125	13371.4	6.61	7	5	4470	63.61	29.68	-1.082
J. coenia	197	21936.5	9.85	11	23	19035	47.92	49.09	-0.971
P. xuthus	108	11700.6	6.61	5	3	8940	58.45	33.52	-0.973

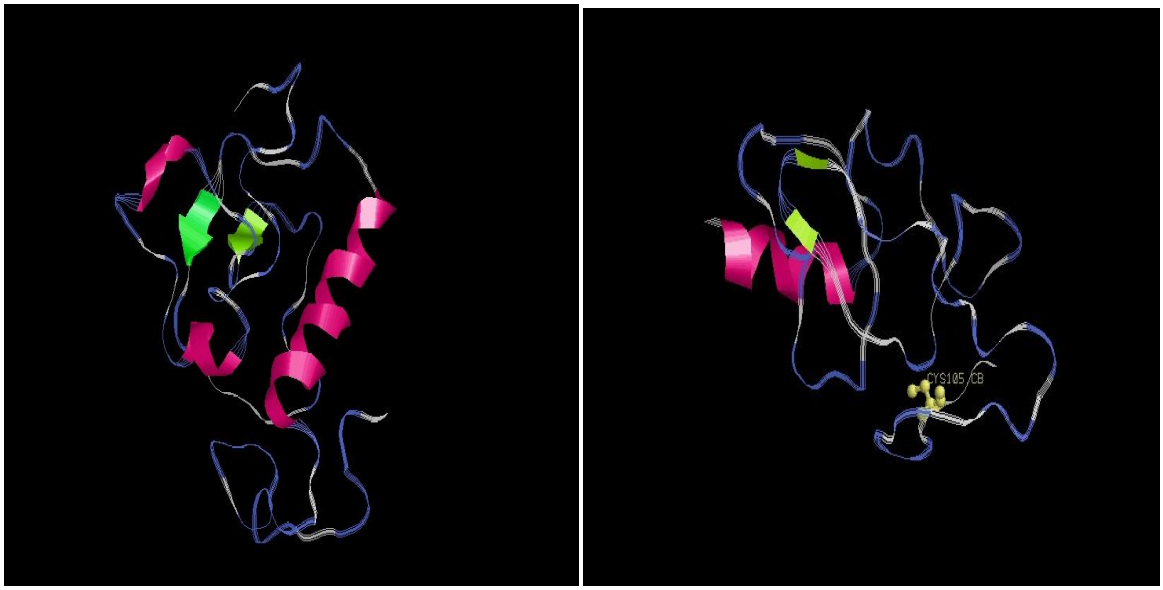
M. wt.,Molecular weight; pI, Isoelectric point; –R, Number of negative residues; +R, Number of positive residues; EC, Extinction coefficient at 280 nm; II, Instability index; AI, Aliphatic index; GRAVY,Grand Average Hydropathy

Table 29. Amino acid composition (in %) of distal less protein computed using Expasy's ProtParam tool.

Amino acids of:	**Ala**	**Arg**	**Asn**	**Asp**	**Cys**	**Gln**	**Glu**	**Gly**	**His**	**Ile**	**Leu**	**Lys**	**Met**	**Phe**	**Pro**	**Ser**	**Thr**	**Trp**	**Tyr**	**Val**
H. c. galanthus	4	1.3	0	6.7	1.3	6.7	1.3	9.3	2.7	0	2.7	4.0	0	0	21.3	18.7	8.0	2.7	2.7	6.7
H.m. rosina	4	1.3	0	5.3	1.3	6.7	1.3	9.3	2.7	0	2.7	4.0	0	0	20.0	20.0	9.3	2.7	2.7	6.7
H. pachinus	4	1.3	0	5.3	1.3	6.7	1.3	9.3	2.7	0	2.7	4.0	0	0	20.0	20.0	9.3	2.7	2.7	6.7
H. melpomene	4	1.3	0	5.3	1.3	6.7	2.7	8.0	2.7	0	2.7	4.0	0	0	21.3	18.7	9.3	2.7	2.7	6.7
H. heurippa	4	1.3	0	5.3	1.3	6.7	1.3	9.3	2.7	0	2.7	4.0	0	0	20.0	20.0	9.3	2.7	2.7	6.7
H. erato	6.9	10.3	5.2	0	0	13.8	3.4	5.2	1.7	3.4	10.3	8.6	1.7	3.4	5.2	5.2	6.9	1.7	3.4	3.4
H. c.cordula	4	1.3	0	5.3	1.3	6.7	1.3	9.3	2.7	0	2.7	4.0	0	0	20.0	20.0	9.3	2.7	2.7	6.7
H. h. zuleika	4	1.3	0	5.3	1.3	6.7	1.3	9.3	2.7	0	2.7	4.0	0	0	20.0	20.0	9.3	2.7	2.7	6.7
B. anynana	2.4	1.6	4.0	3.2	0	9.6	2.4	12.0	8.8	2.4	4.0	2.4	1.6	5.6	15.2	13.6	8.0	0	2.4	0.8
J. coenia	5.6	5.1	4.1	2.5	1.0	9.6	3.0	10.2	6.1	2.0	7.6	6.6	2.5	3.6	8.6	9.1	5.6	0.5	4.6	2.0
P. xuthus	5.6	0.9	4.6	0.9	0.9	9.3	3.7	12.0	11.1	2.8	3.7	1.9	1.9	4.6	12.0	12.0	5.6	0	5.6	0.9



Junonia coenia *Heliconius cydno galanthus*



Bicyclus anynana *Papilio xuthus*

Figure 37. RasMol (strands) representation of the homology modeled 3D structure of distal less proteins from *Junonia coenia*, *Heliconius cydno galanthus*, *Bicyclus anynana* and *Papilio xuthus* (using PDB template c2m34A, d1nk3p, c3a01A, d1ahdp, d1b72a, c2dmtA). The cysteines are shown as ball and stick models (yellow).

Table 30. GenBank accession number of the sequences submitted in NCBI.

S.N	**GenBank accession No**	**Taxon**	**Types of Genes (Partial)**
1	KJ624753.1	*Phalanta phalantha*	Cytochrome Oxidase subunit I (COI) gene
2	KM103291.1	*Phalanta phalantha*	
3	KJ624752.1	*Cethosia biblis*	
4	KM103290.1	*Cethosia biblis*	
5	KJ645970.1	*Cethosia cyane*	
6	KC968948.1	*Cethosia cyane*	
7	KM102674.1	*Cethosia cyane*	
8	KC968946.1	*Argynnis hyperbius*	
9	KJ645972.1	*Argynnis hyperbius*	
10	KC968947.1	*Cirrochroa aoris*	
11	KJ645969.1	*Cirrochroa aoris*	
12	KM102677.1	*Cirrochroa aoris*	
13	KC968944.1	*Vindula erota*	
14	KJ645967.1	*Vindula erota*	
15	JN797791.1	*Vindula erota*	
16	KM102681.1	*Vindula erota*	
17	KC968950.1	*Acraea issoria*	
18	KJ645968.1	*Acraea issoria*	
19	KM102665.1	*Acraea issoria*	
20	KM102664.1	*Acraea issoria*	
21	KJ645971.1	*Cirrochroa tyche*	
22	KC968945.1	*Cirrochroa tyche*	
23	KM102683.1	*Cirrochroa tyche*	
24	KM103293.1	*Cupha erymanthis*	
25	KM103292.1	*Argynnis laodice*	
26	KJ624751.1	*Argynnis childreni*	
27	KJ645973.1	*Vagrans egista*	
28	KC968949.1	*Vagrans egista*	
29	KC968957.1	*Cethosia cyane*	NADH dehydrogenase subunit 1 gene
30	KC968956.1	*Cethosia biblis*	
31	KC968955.1	*Vindula erota*	
32	KC968954.1	*Vagrans egista*	
33	KC968953.1	*Argynnis hyperbius*	
34	KC968952.1	*Cirrochroa aoris*	
35	KC968951.1	*Phalanta phalantha*	

V. DISCUSSION

5.1 Status of Heliconiinae in Northeast India

The present study sampled thirteen species of Heliconiinae butterflies from the Northeast region of India. Kehimkar (2008) recorded 14 species in this region; *Issoria isaea* (Himalaya queen fritillary) found in Sikkim was not sampled in this present study. Among the 13 species sampled, *Vagrans egista* (Zothansangi *et al.,* 2013), *Cupha Erymanthis* and *Argynnis Childreni* were recorded for the first time in the state of Mizoram.

Quantitative analysis of the subfamily in Mizoram revealed that the Diversity of Heliconiinae increases with increase of altitudes peaking at 1000 m – 1200 m which is the second highest zone of elevation range in the study, after which it decreases abruptly at the highest zone (1200 m-1400 m). Ghorai and Sengupta (2014) also showed highest diversity of Papilionidae in this range of altitude in West Bengal. This positive correlation of diversity and altitudes is in congruent with other studies of butterfly diversity in Columbia, Venezuela and Peru (Pyrcz and Wojtusiak, 2002).

With respect to forest types, the diversity of Heliconiinae was the highest at Type 3 i.e Mountain sub-tropical forest which happened to be located at the eastern part of the state having high elevation (above 1000 m) comparatively. Acharya *et al.,* (2011) studied the elevational gradients of butterfly species in Sikkim and found out that elevational zone between 900-1800 m contained the highest diversity. Uniyal (2007) made similar observation in his study in Himachal Pradesh. High diversity of butterflies and also plants and other animals lies at low to mid-elevation forests in the Himalayan region (Acharya, 2008; Acharya *et al.,* 2011). These forests harbor high diversity at this elevation but experiences immense anthropogenic pressures leading to much extinction (Khan *et al.,* 1997; Pandit *et al.,* 2007). In contrast with the diversity of

subfamily Heliconiinae, the diversity of the whole family Nymphalidae was found to be the highest in the forest type 1 i.e. Tropical Wet Evergreen Forest located at the lowest zone (>600 m) of elevation in the region. Negative correlation between butterflies species richness and elevation was also reported from Great Basin, USA (Fleishman *et al.,* 1998) and Spain (Sanchez-odriguez and Baz, 1995). The decline trend might be attributed to decline in temperature and rainfall towards higher elevation. Butterfly needs certain level of temperature for their activity and hence unable to cope up with the extreme climatic conditions (Fleishman *et al.,* 1998). Nevertheless, the Heliconiinae diversity widely distributed in the higher elevational ranges can be explained from the flying habits of the subfamily. These Heliconiinae butterflies are usually high elevation fliers flying up to or more than 2000 m up in the hills, common in the Himalayan and north-eastern region (Kehimkar, 2008). This may also probably associated with the availability of their food plants in suitable phenophases. In general butterfly species are found with the highest diversity in areas containing large amount of host plants (Quinn *et al.,* 1998) and butterfly diversity at local or regional scales is also closely related to their host plant density (Gutierrez *et al.,* 1995). Significant and strong correlation between host plant diversity and butterfly species richness was also observed by Ferrer-Paris *et al.* (2013).

Species Richness of Heliconninae butterflies was highest in the month of May and maximum abundance was noted in the months of June to September peaking in September. In these two peaking months, the humidity was 80-90 per cent and temperature varied between 22-27°C. Rainfall was moderate in May where September received the heaviest rainfall during the year. Humidity and Temperature showed perfect positive correlation with diversity and abundance of Heliconiinae butterflies. Rainfall did not show a significant with diversity and abundance. Species abundance and species richness were found to be lowest in January and February, the coldest and driest months of the year. In Costa Rica, Lepidoptera differed from all

other taxa, decreasing in numbers in the wet season (Boinski and Scott, 1988). Studies conducted in temperate areas, such as Texas, USA, demonstrated a positive correlation between the abundance of arthropods and the precipitation levels (Dunham, 1978). For many species of insects, abundance was independent relative to the rains. There may be several explanations for this decrease. For example, stress caused by food shortage (Janzen, 1973) or condition sunsuitable for development, which are in turn related to a series of strategies and adaptations, such as dormancy, diapause and migration (Janzen and Schoener, 1968; Denlinger, 1980; Wolda, 1988; Braithwaite 1991).

The host plants of Heliconiinae butterflies were usually distasteful. *A. issoria,* the yellow coster butterfly was used as a representative to study the butterfly life cycle and its correlation with the nutrients content of the host plant. The host plant *Debregeasia salicifolia* was found near small streams, usually on the shady side of the stream sides. Results revealed that, total protein, total sugar and amino acids were high in mature leaf than the young leaf buds. It was also found that the larvae fed with mature leaf took lesser time for developing into adult butterfly (moultimg and emerging from the chrysalis/pupa) than the larvae fed with young leaf. Many studies of host plant interaction conducted in *Pieris* butterflies showed that butterflies grew faster when fed on fertilized host plants (Myers, 1985; Chen *et al.,* 2004). Relative growth rates of larval instars were strongly affected by dietary protein in which larvae fed high protein diets grew 1.9-2.9 times as rapidly as did larvae fed low protein diets. Higher moisture content of leaves of hostplant has a direct effect on growth and development of larvae by favouring the ingestion, digestion and assimilation of nutrients. Leaves containing more water, total sugar and soluble carbohydrate and less mineral are best relished by larvae (Murthy *et al.,* 2013).

The study of pattern of gut based enzymes showed that high densitometric value of SGOT in the 4^{th} instars larvae comparing to the 3^{rd} and 5^{th} instars where SGPT showed less

variation pattern in the instar larvae. The ALP activity pattern showed an increasing densitometric value with the increase in larval stages. This increase in the ALP activity with the advancement of developmental stages may be due to the increased food consumption of larval instars. Previous works on gut based enzymes of insects focused on defensive enzymes such as polyhenol oxidase and guaiacol peroxidase (Felton *et al.,* 1989; Felton and Duffey, 1991). Many evidences demonstrate that ingested enzymes also play important roles in the gut lumen of insects. For instance, previous work on ingested fungal enzymes showed that they play an essential role in cellulose digestion in variety of plant feedinf insects (Martin, 1987). The pH of grasshopper gut fluid (ca. 6-7) (Barbehenn et al., 1996) is far more favourable for continued plant enzyme activity than the high pH conditions found in caterpillars (ca. 8-12) (Dow, 1984). However, the results of this work also suggest that these antioxidant enzymes are functional in the caterpillar gut fluids. In insect alimentary canal midgut is generally considered as a tissue where the digestive enzymes secrete and is a site for digestion and absorption of nutrients. It is also an important tissue affected by many kinds of toxicants including entomopathogens (Sutherland *et al.,* 2002). Insect gut is differentiated in three regions that include foregut, midgut, and the hindgut. Further it signifies one of the most important areas in insect physiology because of interaction between the insects and the environment. Hence it has been the focus of entomologist aiming to develop effective methods of insect pest's control. Among the three regions, the midgut region has particularly been the most studied, because alterations on it affect the growth and development of insects as a result of changes in the physiological events that depend on meal intake, absorption and transformation. The epithelium of the midgut in Lepidoptera is composed of columnar cells which are responsible for absorption and enzymes

secretion, goblet cells for ionic homeostasis, endocrine cells for endocrine function and the regenerative cells for epithelium renewal (Senthil-Nathan, 2013).

5.2 Phylogeny of Heliconiinae butterflies

Morphological characters were used to infer phylogenetic hypothesis of the Heliconninae butterflies collected. Maximum similarity value (0.889) was found between *Cirrochroa aoris* and *Cirrochroa tyche* which belong to the tribe Vagrantini. The dendrogram presented the four tribes in four separate clades which agree with the morphological based phylogenetic relationships described by Penz and Peggie (2004). The tree topology of Freitas and Brown Jr. (2004) using data from morphology and larval stage showed slight differences. (Fig.38)

Nuclear and mitochondrial genes of the thirteen species collected from the region were amplified and characterized to infer the molecular phylogeny of Heliconninae subfamily. The nuclear gene, through RAPD-PCR, presented the phylogeny of the subfamily where all the genera were recovered as monophyletic clades. The three tribes were clustered together within their respective clades but tribe Acraeini was included among the tribe Vagrantini. The similarity matrix revealed *P. phalantha* and *P. alcippe* as the most closely related species followed by *Vagrans egista* and *Vindula erota*. All the RAPD primers used in this study showed polymorphism above 60%. Galluser *et al.* (2004) also successfully used RAPD markers to differentiate two subspecies of butterflies and mentioned the high polymorphism had led to difficulty in result interpretations. Sharma *et al.* (2006) also revealed that the RAPD-PCR technique is extremely useful forrapid identification of genetic polymorphisms in Lepidopterans because of the reproducibility of the results for each of the species but suggested to use more primers to strengthen the resolution of the results. However, the ability of RAPD analysis to

detect polymorphism holds great promise for detecting population structure and genetic differentiation even within well established 'species' that would otherwise go unnoticed (Wilkerson *et al.*, 1993). RAPD markers had indeed revealed the genetic diversity and resolved the phylogenetic relationships of the genera of Heliconiinae; but it would be interesting to use more conserved markers or to investigate more conserved sequences (i.e., mtDNA) in order to better assess the genetic relationships between these butterflies.

The mitochondrial gene, CO1 data inferred the evolutionary relationship of Heliconninae dividing into four monophyletic clades corresponding to four tribes, each with monophyletic subclades of genera showing high bootstrap support value. The phylogenetic tree using ND1 data showed monophyly of the four tribes. But few discordant nodes at the level of genera was shown when compared with CO1 data. Both the p-distance matrix of CO1 and ND1 sequences showed that *Cethosia biblis* and *Cethosia cyane* were the most closely related species with p-distance value 0.056 and 0.050 respectively.

The phylogenetic trees reconstructed from all the four different data (morphology, RAPD, CO1 and ND1) revealed the monophyly of the four tribes of Heliconiinae- Vagrantini, Acraeini, Heliconini and Argynini. Most of the tree topology was similar at the tribe level with some discordant nodes at the generic level. Data of morphology and RAPD recovered monophyletic subclades corresponding to genera. However, this phylogenetic relationship of the four tribes using molecular data was incongruous with the phylogeny constructed from morphological data by Penz and Peggie (2003) and also by Freitas and Brown Jr. (2004), while the reconstructed dendrogram of morphological data in the present study was in accordance with Penz and Peggie's (2003) as in the figures below (Fig. 38).

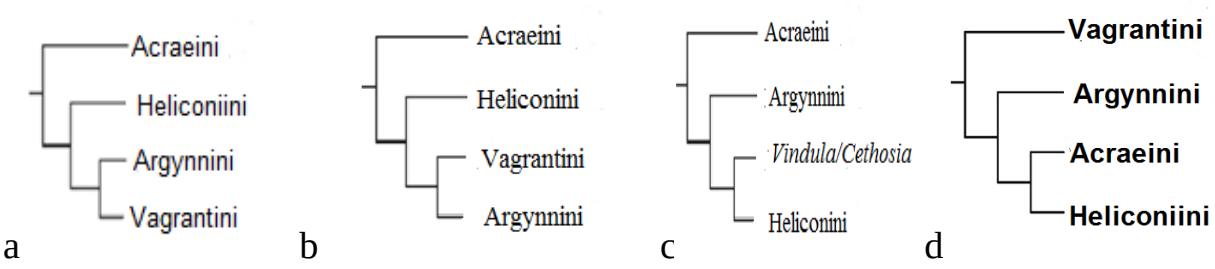


Figure 38. Reconstructed phylogenetic tree of the tribes of Heliconiinae sub-family from a) morphological data sampled from NE India; b) morphological data by Penz and Peggie, 2003; c) morphological/larval stage data by Freitas and Brown Jr., (2004); d) molecular data sampled from NE India.

The comparison of all topologies revealed that the monophyly of subclades corresponding to tribes and genera was mostly consistent among trees recovered from different data partitions. But when comparing the trees under alternative optimality criteria, slight discordances were observed in some clades corresponding to genera. This may be due to lack of comprehensive sampling. Freitas and Brown (2004) put together *Vindula* and *Cethosia* under one tribe in their study of morphological based phylogeny. The present study also revealed the inclusion of genus *Cethosia* within the clade of tribe Vagrantini from CO1 data under the optimality criteria of MP and Bayesian inference. Simonsen *et al.*, (2006) used CO1 data to study the phylogeny of tribe Argynini which gave a well-supported, robust monophyletic clade. In their classification of genera *A. hyperbius* and *A. childreni* were more closely related than with *A. laodice*, which was in congruent with the present phylogenetic hypothesis. Silva-Brandao *et al.* (2007) also used CO1 to infer the phylogeny of tribe Acraeini. Most of the species were from Africa, the species *Acraea issoria* (sampled in the present study) which along with *A.violae* is the only Asiatic representative. CO1 sequences of the present sampled species were grouped

together with species from different locations i.e. sequences from NCBI (names given in their accession numbers with abbreviation of specific names) as sister clades with strong support value mostly higher than 95%.

The rates of transitional substitution were higher than transversional substitution in both CO1 and ND1 sequences and the estimated Transition/Transversion bias (R) was higher in CO1 sequences than ND1 sequences indicating lower genetic divergence in CO1 sequences than in ND1 sequences of the Heliconiinae butterflies. The nucleotide composition was A + T rich in both CO1 and ND1 analysis, which is typical for arthropods (Simon *et al.*, 1994). The Tajima neutrality test showed a positive Tajima's D for both CO1 and ND1 signifying low levels of both low and high frequency polymorphisms indicating a decrease in population size and/or balancing selection. The Tajima's D for CO1 was more than zero signifying that multiple alleles were present, some at low, others at high frequencies while ND1 showed a value less than zero which signifies presence of rare alleles at low frequencies (Tajima, 1989). The calculated values of RSCU (Relative Synonymous Codon Usage) in CO1 genes showed that UUA (L) UCU (S) and CGA (2.85) were the most frequently used codon having RSCU more than 2.85. The codons UUA (L), CCU (P) and GCU (A) were most frequently used in the ND1 genes having RSCU value more than 3.1

The results of the present study on the hypothesis of evolutionary relationship of Heliconiinae revealed congruent phylogenetic trees using nuclear and mitochondrial genes, dividing the four tribes in monophyletic clades. Several incongruous and contradictory inferences in the node-by-node relationships were also observed between morphological and molecular data, and also among the inferences produced by the different optimality criteria. Incomprehensive sampling may probably the reason for this, as the study area included only a

small particular region. These data alone cannot strongly resolve node-by-node relationships among tribes of Heliconiinae. Such resolution will clearly require a substantial increase in both sequence and taxon sampling with stronger characters and character states.

5.3 *In-silico* characterization of Physico-chemical and structural elucidation of colour pattern genes (*Distal-less*)

An *In silico* characterization of the physico-chemical properties of eleven distal-less genes retrieved from the Swiss-Prot database revealed that the distal-less proteins of the butterflies had a low pI (Iso-electric point) while only those of *Heliconius erato* and *Junonia coena* had pI in the higher range. Since pI is actually the pH at which a protein carries no net charge, this information could be used to prepare the ideal pH of a buffer. An ideal pH would thus be at least one unit away from the calculated pI i.e. one unit above or below the calculated pI. Therefore in this case while the distal-less proteins of most of the samples required an acidic buffer solution with a pH range of 4 to 6 those of *Heliconius erato and Junonia coena* required a basic buffer solution with a pH range of 10 to 12.

The instability index which provides a measure of how stable a protein may be in a test tube condition is vital due to the fact that proteins in general are highly unstable in test tube environments. A protein with an instability index smaller than 40 is predicted as stable. In the present estimate, of the eleven distal-less protein samples only *Heliconius erato* computed an instability index of less than 40 i.e. 3.20. Thus indicating only *Heliconius erato's* distal-less protein is stable.

Extinction coefficient of a protein is a measure of how strongly it absorbs light at a given wave length. In proteins it depends almost exclusively on the number of aromatic

residues, particularly tryptophan and cystine. Cystine is the amino acid formed by the oxidation of two cysteine molecules that covalently link via a disulphide bond. *Junonia coenia* computed the highest extinction coefficient of 19035 at 280 nm while *Bicyclus anynana* computed the lowest at 4470. It's worth mentioning that observation of the 3D structure of *Bicyclus anynana* reveals the absence of cystine which might be the reason for a lower reading. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chain amino acids – alanine, valine, isoleucine and leucine. This index may be regarded as a positive factor for increased thermostability as it has been recorded that proteins of thermophilic bacteria contain high concentrations of aliphatic amino acids in comparison to other proteins. *Heliconius erato* with an aliphatic index of 70.69 may be the most thermostable protein among the eleven selected samples, thereby able to withstand a wide range of temperature conditions. GRAVY value for a protein is the sum of its hydropathy values which is nothing but the hydrophobic and hydrophilic properties of its side chains. Larger the GRAVY values more hydrophobic the protein. The distal-less proteins all computed GRAVY values of less than 1. This indicates that these proteins are hydrophilic. An analysis of the amino acid composition reveals that polar amino acids i.e. Serine (Ser), Threonine (Thr), Glycine (Gly) and Glutamine (Gln) predominate along with Proline (Pro), a non-polar amino acid. The polar amino acid although uncharged overall, they have an uneven charge distribution. Because of this, these amino acids can form hydrogen bonds with water. As a result, such amino acids are hydrophilic and are often found on the outer surface in contact with the watery environment of the cell. High number of Proline (Pro) residues on the other hand indicates presence of many conformationally rigid regions. This is because the distinctive cyclic structure of proline's side chain gives it an exceptional conformational rigidity compared to other amino acids. It therefore affects the rate of peptide bond formation between

proline and the other amino acids. Also, it is this rigid nature that affects the secondary structure of proteins and disrupts alpha helices and beta sheets. SOSUI server predicts presence and location of secondary structures of a protein. Absence or rare occurrences of secondary structure could infer that the protein is soluble and that it is not a trans-membrane protein. This is because trans-membrane proteins or trans-membrane regions in a protein are rich in secondary structures. Distal-less proteins from the eleven butterfly samples was computed by SOSUI server as soluble and also that it is not a trans-membrane protein. This can be further corroborated by observing the 3D structure which reveals almost no concrete secondary structure.

Cys_Rec software was used to identify the number of cysteine residues in each of the eleven distal-less proteins. Along with identifying these residues, the software also identifies possible or probable disulphide (SS) bond formation in any of these protein sequences. Overall only that of *Junonia coenia* contained two cysteine residues and therefore any chance of disulphide (SS) bond formation. The rest of the ten distal-less proteins had either one or no cysteine residues and thereby no chance of any disulphide (SS) bond formation.

3D structure analysis of the distal-less proteins revealed useful information. The absence or the presence of only one or two cysteine residues indicated that there was no chance of disulphide (SS) bond formation. In the case of *Junonia coenia*, even though there were two cysteine residues, Cys_Rec software analysis failed to confirm any disulphide bond formation. This was further confirmed by its 3D structure analysis where the two cysteine residues were spaced too far apart from each other to allow for any type of interaction. Proline (pro) residues were found in large number during primary sequence analysis. A 3D structural analysis revealed that these residues formed constituents of turns and if at all they formed part of a secondary structure they were situated towards the end. This confirms the fact that proline (pro)

acts as disrupters of secondary structures and are thus avoided as constituents of secondary structures, especially helices. Transmembrane proteins are generally characterized by a majority of alpha-helix structures as they form a channel connecting the extra-cellular to the intra-cellular regions. SOSUI server findings that distal-less proteins are not transmembrane proteins is confirmed by analysis of the 3D structure which reveals very less alpha-helix structures. The result giving the phylogenetic tree of distal-less genes of butterflies revealed that the gene showed minor variation and is not a good marker for butterfly phylogenetic studies.

The difference in the branching pattern between the morphological and molecular data signifies the need for using molecular tools for taxonomic classification as well as in understanding the evolutionary relationship. 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 (Vanlalruati *et al.*, 2012). However, in order to confirm the taxonomic statuts of each species, to measure the extent of hybridisation between them, and to have better resolution of phylogenetic tree, further studies with less polymorphic markers or conserved are required. The use of more specific primers (mtDNA) has proven useful for isolating small DNA fragments for some DNA sequences analysis. The present study also used 624bp CO1 and 324bp sequence which satisfied the required sequence length for DNA barcoding (Fretzel *at al.,* 2008). These mitochondrial data inferred robust and well supported relationships of the different genera and tribes of the subfamily where the present sampled species also formed strong supported clades with their corresponding species from different locations (sequences from NCBI). Nevertheless, ND1 gene being shorter and unavailability of

other sequences in gene banks, was less informative and produced more mis-estimated branches and topologies.

Although relationships among certain subgroups are widely accepted, the higher level phylogeny of Heliconiinae is still partly unresolved and a detailed analysis of Oriental genera needs to be considered. In most of the previous works on the molecular phylogeny of subfamily Heliconiinae, sampling was done mainly on species distributed from other zoo-geographical regions especially from the Neotropical and Palearctic regions. Most of the present sampled species from the Northeast region of India were found to be of Oriental origin, distributed mainly in Asia and Australia, of which majority are endemic in the regions. The present study revealed the status of the Heliconiinae butterfly, feeding habits, distributions and their correlations with the local climates in the region. It also characterized the morphology and molecular status of the Heliconiinae butterflies present in this region which enabled us to presume the evolutionary relationship within the subfamily. This study would also contribute in the classifications, systematics and evolutionary relationships of species of the oriental region in the subfamily Heliconiinae which is still in a perpetual state of refinement.

VI. SUMMARY

SUMMARY

- A systematic survey on Heliconiinae butterfly was conducted for the period of four years (July 2009-Dec 2013), During this study period thirteen (13) species of Heliconiinae belonging to the four tribes of the subfamily were collected from Northeast India. The species collected were

Acraea issoria (Hübner, 1818)
Cethosia biblis (Drury1770)
Cethosia cyane (Drury 1770)
Cirrochroa tyche (C&R felder 1861)
Cirrochroa aoris (Doubleday 1847)
Phalantha phalanta (Drury 1773)
Phalanta alcippe (Stoll 1872)
Argynnis hyperbius (Linnaeus, 1763)
Argynnis Childreni (Gray 1831)
Argynnis laodice (Pallas)
Vagrans egista (Cramer 1780)
Vindula erota (Fabricius 1793)
Cupha erymanthis (Drury 1773)

- From these thirteen species of Heliconiinae sampled, the diversity and butterfly-host plant interaction were analysed, their morphology and genes were characterized and analysed to infer the phylogenetic relationships of the subfamily. *In silico* Characterization of physico-chemical properties of distal-less genes of butterflies was also studied.
- Quantitative analysis of the subfamily in Mizoram revealed that the diversity of Heliconiinae increases with increase of altitudes peaking at 1000 m – 1200 m which is the second highest zone of elevation range in the study, after which it decreases abruptly at the highest zone (1200-1400 m ASL).

- With respect to forest types, the diversity was the highest at Type 3 i.e. Mountain sub-tropical forest which also happens to be located at the eastern part of the state having high elevation comparatively.
- The monthly variation study showed that the Species Richness of Heliconninae butterfly was highest in the month of May and maximum abundance was noted in the months of June to September peaking in September.
- Humidity and Temperature showed perfect positive correlation with diversity and abundance of Heliconiinae butterflies but no significant correlation with rainfall. Apart from this, it was concluded that the diversity and distribution pattern were also affected by the distribution of the host plants and vegetation types present in the area.
- The developmental period of the larvae (*Acraea issoria*) was also affected by the amount of biochemical contents (protein, amino acids, carbohydrates and lipids) of the host-plant with high value of positive correlation coefficients. The growth rate was found to be faster in larvae feeding on leaves with higher value of total biochemical contents.
- Morphological data based on 23 characters, constructed by focusing mainly on the wings: colour, shapes, spots and markings from the 13 sampled species were used to build phylogenetic dendrograms. It showed a perfect clustering among the genera where the four tribes formed monophyletic clades which agrees the relationship described by Penz and Peggie (2003) using morphological data. Each of the genera corresponds to the monophyletic subclades.
- Data generated by Nuclear DNA marker (RAPD) was also used to infer the phylogenetic hypothesis of Heliconiinae.The dendrograms constructed from its similarity matrix

clustered each genus in a monophyletic clade with same species closed to each other but at the tribe level, they do not show a monophyly.

- An evolutionary relationship of the Subfamily Heliconiinae was inferred from mitochondrial genes (CO1 & ND1) of the 13 samples species of Northeast India.The phylogeny was analysed using three optimality criterion (Maximum Parsimony, Maximum Likelihood and Bayesian) where it divides the tree into four monophyletic clades corresponding to 4 tribes, each with monophyletic subclades of genera showing high bootstrap support.
- The CO1 data inferred robust and well supported relationships of the different genera and tribes of the subfamily where the present sampled species also formed strong supported clades with their corresponding species from different locations (sequences from NCBI), usually higher than 95% bootstrap supportvalue.
- ND1 gene being shorter and unavailability of other sequences in gene banks, was less informative and produced more mis-estimated branches and topologies. .
- Eleven *distal-less* genes (colour pattern genes) of different butterflies were retrieved from the protein database. *In silico* characterization of their physico-chemical propertiesrevealed dominance of proline (pro) residues which are known disrupters of secondary structures.
- Polar amino acids which characterized the protein as hydrophilic also constituted a majority of the protein. This along with the fact that alpha helices were found in limited numbers categorized these proteins as non-transmembrane proteins.
- Limited cystiene residues negated the formation of any disulphide (SS) bonds which is regarded as a positive factor for stability.

- In most of the previous works on the molecular phylogeny of subfamily Heliconiinae, sampling was done mainly on species distributed from other zoo-geographical regions especially from the Neotropical, Arfrica and Palearctic regions.Most of the present sampled species from the Northeast region of India were found to be of Oriental origin, distributed mainly in Asia and Australia, of which majority are endemic in the regions.
- Being the first scientific research on the Heliconiinae from the region of Northeast India, the results and discussions revealed the status of butterfly diversity, their biology and distributions, and also their correlations with the local climates and vegetations.
- Thirty five mitochondrial sequences of the Heliconiinae butterflies were submitted and published in NCBI. It also characterizes the morphology and molecular of the species present in this region which enabled us to presume the phylogenetic status of the subfamily which will contribute in the classifications, systematics and evolutionary relationships of the subfamily Heliconiinae which is still in a perpetual state of refinement.

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VIII. APPENDIX

1. List of Acronyms

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microlitre
16S	16 Subunit
A	Adenine
ASL	above sea level
Avg	Average
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BSA	Bovine Serum Albumin
C	Cytosine
cm	Centimeter
CO1	Cytochrome *c* Oxidase subunit 1
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylene Diamine Tetra Acetic acid
EF1	Elongation Factor 1
ESS	Effective Sample Size
FW	Forewing
G	Guanine
GTR	General Time Reversal
h	Hour
ITS1	Internal Transcribe Spacer 1

ITS2	Internal Transcribe Spacer 2
leu	Leucine
LSU	Large Subunit
m	meter
M	Molar
MCMC	Metropolis-coupled Markov Chain Monte Carlo
MEGA	Molecular Evolutionary Genetic Analysis
MgCl2	Magnesium Chloride
Min	Minutes
ml	Millilitre
mm	Millimeter
mM	Millimolar
Mya	Million years ago
MZU	Mizoram University
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
nDNA	Nuclear DNA
ND1	NADH dehydrogenase subunit 1
Nm	nanometer
ORF	Open Reading Frame
PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerization Chain Reaction
pH	Negative logarithm of the hydrogen ion concentration/ the power of hydrogen
pM	picoMolar
PP	Posterior Probability

rDNA	Ribosomal Deoxyribo Nucleic Acid
RNA	Ribo Nucleic Acid
rpm	Rotation per minute
rRNA	ribosomal Ribo Nucleic Acid
s	Seconds
SDS	Sodium Dodecyl Sulfate
Sq	Square
SSU	Small Subunit
T	Thymine
TAE	Tris base Acetic acid EDTA
Taq	Thermus aquaticas
TBR	Tree Bisection Reconnection
TCA	Trichloroacetic acid
TE	Tris EDTA
Tris-HCl	Tris Hydrochloric acid
tRNA	Transfer Ribo Nucleic Acid
ts	Transition
tv	Transversion
U	Units
U	Urasil
Wt	Weight
3D	3- Dimensional

3. Conference/Seminar/ Workshop attended and participations

4.

S.No	Conference/Seminar/ Workshop attended and participations	Date
1	International Conference on Conservation and Management of Pollinators (Kuching, Malaysia)	14-17, Sep 2011
2	International Conference on Advances in Environmental Chemistry in (Aizawl)	16-18, Nov 2011
3	National Seminar on Environment, Biodiversity, Veda, and Traditional systems in (Aizawl)	10-12. Apr 2012
4	National Conference on Bioresource inventory and emerging conservation strategies with special refererence to Northeast India (Aizawl). (Awarded Best Poster Presenter)	7-8, Mar 2013
5	National Seminar on Issues of Wildlife Conservation in India with special reference to Mizoram (Aizawl)	24-25. Aprl 2014
7	Winter School on training in Insect Taxonomy (Tripura University, Agartala)	26Jan-6Feb2010
8	Workshop on Molecular phylogenetics and Evolution (Aizawl)	22-24, Nov 2010
9	UGC Networking Resource Centre Workshop on Molecular Phylogenetics (IISc, Bangalore)	1-5, Nov 2011
10	Workshop on Molecular phylogenetics and Evolution (Aizawl)	26-28, Nov 2012
11	Workshop on Random Amplified Polymorphic DNA markers and its application (Aizawl)	20-21, May 2011
12	Workshop on Bioinformatics- structure & determination of macromolecules (Aizawl)	28-29, Mar 2011
13	Training Course: Bioinformatics- Protein and their structure prediction (Aizawl)	23-24, Nov 2011
14	One week Workshop on Data analysis through SPSS (Aizawl)	27Aug-1Sep2012
15	One week Workshop on Applied statistics (Aizawl)	23-28 Jul 2012
16	Workshop on Biostatistics using Sigmaplot (Aizawl)	27 Jun, 2014

5. List of published papers

Sl.No	Papers
1	Butterfly diversity in Mizoram University Campus, Aizawl, India and their global distributions (2011). Advances in Environmetal Chemistry. ISBN : 978-93-81361-53-5. Pp 249-242
2	Genetic Variation within two species of Cirrochroa (Heliconiinae: Lepidoptera) by RAPD-PCR technique (2011). Science Vision. ISSN 0975-6175(print), 2229-6026 (online). 11(3):165-170.
3	Morphological and molecular studies of six *Junonia* species of butterflies using RAPD-PCR technique (2011). Science Vision. ISSN 0975-6175(print), 2229-6026 (online). 11(3):141-146.
4	Diversity and vertical distributions of Heliconiinae butterflies in Mizoram (2013).Bioresources and Traditional Knowledge of Northeast India.ISBN : 987-81-924321-3-7. pp 229-235.
5	A preliminary survey of butterfly dibersity in Dampa Tiger Reserve of Mizoram, Northeast India. (2014). Issuesand Trends of Wildlife Conservation in Northeast India. ISBN : 987-81-924321-7-5. Pp 256-260.