MOLECULAR CHARACTERISATION OF MITOCHONDRIAL CYTOCHROME OXIDASE SUBUNIT I GENE PARTIAL SEQUENCE OF THE PAPILIONOIDEA BUTTERFLIES OF NORTH KERALA

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CERTIFICATE

This is to certify that the thesis entitled "Molecular characterisation of mitochondrial cytochrome oxidase subunit I gene partial sequence of the Papilionoidea butterflies of North Kerala" is a bonafide record of research work done by Mr. V. Jiji joseph in the Laboratory of Molecular Biology of the Department of Zoology under my supervision and guidance, in partial fulfilment of the requirement of the Degree of Doctor of Philosophy under the Faculty of Science of the University of Calicut. I also certify that no part of this thesis has been presented before for any other Degree.

Calicut University, Dr. K.V. Lazar

January 25, 2016

DECLARATION

I, Jiji Joseph V., hereby declare that this thesis entitled "Molecular characterisation of mitochondrial cytochrome oxidase subunit I gene partial sequence of the Papilionoidea butterflies of North Kerala" is an authentic record of the work carried out by me under the supervision and guidance of Dr. K.V. Lazar, Associate Professor, Division of Molecular Biology, Department of Zoology, University of Calicut and that no part of this has been published previously or submitted for the award of any Degree, Diploma, Title of recognition before.

C.U. Campus Jiji Joseph V.

January 25, 2016

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CONTENTS

Introduction

The present study attempted to understand the cytochrome oxidase subunit I gene partial coding sequence diversity in Papilionoidea butterflies of North Kerala. The key argument is that the sequence diversity of cytochrome oxidase subunit I gene have variability to discriminate different species and their phylogeny. The samples for this study were collected from different locations of North Kerala, especially from Western Ghats, over a period of three years.

Butterflies are a most fascinating group of insects found all over the world in different intensities. Its beauty has attracted people of all walks of life inspiring thousands of stories, poems and drawings illuminating the enchanting beauty of butterflies. There are many myths and stories linking butterflies with human existence. Also the movement of wings of butterflies have become an ideal symbol and concept in chaos theory, "the butterfly effect".

Butterfly populations have significant roles on ecosystem. These involve both positive and negative roles. The beneficial roles involve pollination and biological control of weeds. The negative roles involve action of few butterflies becoming pest of some crops. The change in prevailing butterfly population of an area is a tool for monitoring environment degradation, and an indicator for intervention in the conservation and management of environment. All these indicate butterflies have close association with human beings and also a scientific example for co-evolution of animals and plants through close interaction.

Butterflies belong to the order Lepidoptera, of class Insecta of phylum

Arthropoda. The word ‘Lepidoptera’ is derived from two Greek words, *viz;* ‘Lepis’ meaning scales and ‘pteron’ meaning wings. The fragile wings have very small scales and the colour pigments of the scales give colour to the wings. The order Lepidoptera includes two sub orders - Rhopalocera comprising butterflies and Heterocera comprising moths. The two sub orders together comprise about 1,80,000 species of which 17,500 species are butterflies. Among them 1,501 species of butterflies have been discovered from India and 322 species from Kerala. Of the 322 species of butterflies identified from Kerala 19 belongs to the family Papilionidae (swallowtails), 34 to Pieridae (whites and yellows), 97 to Nymphalidae (brush-footed butterflies) and 95 species to Lycaenidae (blues) (Palot *et al.,* 2003).

Plant - butterfly co - evolution and their interdependence

The butterflies and plants have many relationships evolved through co-evolution. The larvae of each species of butterfly can grow only on a few specific larval food plants. The main food of adult butterflies is nectar. Some butterflies produce Pheromones from the alkaloids that they obtaining from the plants and in return the butterflies act as pollinating agents for these plants. Thus, the strategies for the conservation of plants and the butterflies are linked.

Conservation

Overuses of pesticides, habitat loss, widespread and illegal collection of butterflies are the threat for the existence of butterflies. The large copper butterfly, *Lycaena dispar* (Haworth, 1802) from England became extinct in 1849 due to over collection. Many Indian butterflies are endemic in North Eastern Himalayas and

Western Ghats and many of them need attention. The Indian Wild life (Protection) Act, 1972 has provisions to conserve butterflies, included one species in schedule (I), twenty two in schedule (II) and seven in schedule (IV).

Significance of Western Ghats

Western Ghats is one of the 34 biodiversity hotspots in the world. Western Ghats is the abode for many endemic plants and animals. Many butterflies of Western Ghats are endemic. Among the 24 samples used in the present study, two are rare butterflies endemic to Western Ghats viz., *Troides minos* Cramer, 1779 and *Papilio liomedon* Moore, 1874. The *P. liomedon* is a threatened species of butterfly and is protected under Schedule (I) of Indian Wild Life (Protection) Act, 1972. The *Pachliopta hector* (Linnaeus, 1758) and *Cynitia lepidea* (Butler, 1868) are also threatened species and are included in the Schedule (I) and (II) of Wild Life (Protection) Act, of 1972. In conservation, the identification of habitat disturbance is very important, to identify disturbed habitats of butterflies, the constant biodiversity monitoring is imperative. To make significant progress in the conservation of butterfly biodiversity, inventorying and monitoring must continue for ecologically sensitive areas that are under the threat of major climatic and ecological changes, baseline inventories is essential for determining if and when these changes occur.

Significance of butterflies in conservation

Monitoring of special and temporal changes in diversity of butterflies is an effective tool for understanding environmental changes, necessary for devising suitable conservation and management strategies. The recognition of habitat requirements of rare or endangered species is helpful in the manipulation of an area that may in turn increase the chances of its survival. Many of the endangered species have restricted habitat requirements involving specific host plants as food for their caterpillars. The habitats and rare host plants are often threaten by environmental encroachments by human beings through cutting down trees for timber, fire wood, industrial raw materials, urbanisation and extension of crop lands. The increased pollution also threatened the survival of many butterflies. Monitoring of butterfly population in an area is an effective tool to monitor the level of threat to the biodiversity of an area. This helps to devise necessary conservation measures before total extinction.

DNA barcoding and the present study

A DNA barcode is a short segment of nucleotide sequence of any region of genomic DNA that can discriminate species with its standardised nucleotide variabilities between species. The DNA barcoding has become a leading technique for generating comparative molecular data. The sequences of mitochondrial cytochrome oxidase subunit I (COI) gene has been determined for a large number of specimens and comparison of those DNA sequences stored in a database represents the mainstay of molecular phylogenetics, in which it allows the biologists to elucidate the evolutionary relationships among species based on sequence similarities. The mitochondrial COI DNA sequences have been used in studying the evolutionary relationship among different animal taxa owning to its conserved protein coding region, and lack of recombination. The sequence divergence accumulates more rapidly in mtDNA than in nuclear DNA due to faster mutation rate and lack of repair system.

In the present study the molecular barcoding and phylogeny analysis of Papilionoidea (Lepidoptera) from random locations of North Kerala using partial coding region of mtDNA COI gene, from 24 species of Papilionoids generated. Using the sequence data obtained, the phylogeny of each butterfly was analysed and their affinities with various butterflies was elucidated. The sequences generated in the present study were deposited in GenBank, the GenBank accession numbers with specimen numbers are given below.

S.I	**Voucher Number**	**GenBank Accession Number**	**Name**
1	CUJ1	HQ424201	*Troides minos* Cramer, 1779
2	CUJ2	HQ424202	*Atrophaneura aristolochiae* (Fabricius, 1775)
3	CUJ3	KT880639	*Graphium agamemnon* (Linnaeus, 1758)
4	CUJ4	KT880640	*Papilio polytes* Linnaeus, 1758
5	CUJ5	KT880641	*Papilio polymnestor* (Cramer, 1775)
6	CUJ6	KT880642	*Hypolimnas bolina* Linnaeus, 1758
7	CUJ7	KT880643	*Papilio clytia* (Linnaeus, 1758)
8	CUJ8	KT880644	*Pachliopta hector* (Linnaeus, 1758)
9	CUJ9	KT880645	*Atrophaneura aristolochiae* (Fabricius, 1775)
10	CUJ10	KT880646	*Papilio liomedon* Moore, 1874
11	CUJ11	KT880647	*Delias eucharis* (Drury, 1773)
12	CUJ12	KT880648	*Eurema hecabe* (Linnaeus, 1758)
13	CUJ13	KT880649	*Eurema hecabe* (Linnaeus, 1758)
14	CUJ14	KT880650	*Orsotriaena medus* (Fabricius, 1775)
15	CUJ15	KT880651	*Cynitia lepidea* (Butler, 1868)
16	CUJ16	KT880652	*Junonia iphita* (Cramer, 1779)
17	CUJ17	KT880653	*Junonia almanac* (Linnaeus, 1758)
18	CUJ18	KT880654	*Ypthima huebneri* Kirby, 1871
19	CUJ19	KT880655	*Tirumala limniace* (Cramer, 1775)
20	CUJ20	KT880656	*Melanitis leda* (Linnaeus, 1758)
21	CUJ21	KT880657	*Melanitis leda* (Linnaeus, 1758)
22	CUJ22	KT880658	*Acraea violae* (Fabricius, 1793)
23	CUJ26	KT880659	*Junonia lemonias* Linnaeus, 1758

24	CUJ27	KT880660	*Danaus chrysippus* (Linnaeus, 1758)
25	CUJ29	KT880661	*Castalius rosimon* (Fabricius, 1775)
26	CUJ31	KT880662	*Euploea core* (Cramer, 1780)
27	CUJ32	KT880663	*Troides minos* Cramer, 1779
28	CUJ33	KT880664	*Luthrodes pandava* (Horsfield, 1829)

Table1. The GenBank accession numbers of sequences generated in the present study with specimen numbers.

The major outcomes of this work were the generation of partial sequences of COI gene isolated from 28 samples belongs to 24 species of Papilionoidea butterflies and their phylogenic analysis. The sequences generated in the present study are deposited in the GeneBank with Accession numbers: KT880639 to KT880664, HQ424202 and HQ424201. The DNA barcodes of 24 Papilionoidea butterfly species developed in the present study can be used to identify them precisely and unambiguously and used for analysing their phylogeny.

The partial sequences of COI gene of 24 Papilionoidea butterfly species isolated from North Kerala also revealed the genetic diversity of butterflies in the region. The COI sequences of *Troides minos* (CUJ1) and *Papilio liomedon* (CUJ 10) generated in this study are novel. Two novel genotypes of CUJ12 and CUJ13 are also reported in the present study. It is also found that the COI sequences of *Atrophaneura aristolochiae* CUJ2 (HQ424202), contain a unique C to A transversion at 1st codon position leads in to a peptide change and the consequent taxonomic status of the same.

The Dissertation starts with a review of literature, which is presented in Chapter 1. The materials and methods are presented in Chapter 2. The results of the studies on each butterfly are presented in Chapter 3 (3.1 to 3.26). Summary of the findings are presented in Chapter 4. The Dissertation ends with a bibliography of the literature cited in the work.

Chapter 1

Review of literature

1.1 Historical aspects of the studies on butterflies

The studies on Indian butterflies first reported by Carl Linnaeus in his book "*Systema Naturae*" (1758). Fabricius and Cramer (1775) did an extensive study on butterflies of Indian forests. About 130 species of butterflies seen in North Kerala were described in Logan's "*Malabar Manual*". About 275 species of butterflies seen along Nilgiris and Wayanad were described in the article "Butterflies of Nilgiri District" (Yates, 1935; Hampson, 1989). Ferguson (1891) described 220 species of butterflies of South Kerala. About 282 species of butterflies along Coorg ranges was described by Yates (1931). A detailed description of Indian butterflies was provided in the book "*Fauna of British India*" vol. I. and vol. II. (Talbot, 1939; 1949). Sathyamoorthi (1966) published a catalogue of butterflies displayed at Madras Government Museum. Larsen (1988) studied the butterflies of Nilgiris including Wayanad and reported 299 butterflies. The diversity of butterflies of Western Ghats, India was studied by Gaonkar (1996). Kunte (1997) studied seasonal variations of butterflies along Northern Western Ghats and found their abundance in late monsoon and early winter. Arun (2003) reported the seasonal abundance of butterflies in Siruvani forest of Nilgiri biosphere reserve. Mathew and Binoy (2002) published a checklist of 282 species of migratory butterflies from Amarambalam region of Nilgiri Biosphere Reserve. Eswaran and Pramod (2005) reported 334 species of butterflies from Anaikatty Hills of Nilgiri Biosphere reserve. Padhye *et al*. (2012) identified 334 species of butterflies from Northern

Western Ghats.

1.2 Classification of butterflies based on few diagnostic characters

Several authors have classified higher groups of butterflies based on one or two diagnostic characters as reported by Warren (1947), who classified butterflies giving much emphasis on single character the prothoracic leg and suggested Nymphaloids and Libytheidae were along with the Riodinidae. He was separated Lycaenidae from Riodinidae and included it with Pieridae and Papilionidae (Warren, 1947). Later works proves this arrangement to be unnatural and unjustifiable. Since these types of classifications are often incomplete and unable to describe the correct interrelationships between the different groups. Modern taxonomists emphasized on considering as many characters as possible for taxonomic identifications.

1.3 The numeric taxonomy in the classification of butterflies

Ehrlich (1958) updated the taxonomy of butterflies using their morphology and phylogeny. The Ehrlich and Ehrlich (1967) classified butterflies with numeric taxonomy, giving numeric weightage for 96 internal and 100 external characters, and emphasised the use of numeric taxonomy for accurate results than considering few specialised features.

1.4 The various concepts about insect species

The interest over biological diversity greatly increased in the last 30 years and a major discussion about defining species was evolved. Species were considered as the primary units of classification and conservation. Many think species as only a taxonomic category but several authors criticized this view and prefer to consider species as a part of nature which exists and interacts and modified it (Wheeler, 1999; Mayr, 1963).

1.5 The concept of biological species

According to biological species concept, species are groups of interbreeding natural populations which are reproductively isolated from such other groups (Mayr, 1942). Reproductive isolation through the operation of different isolation mechanisms can be considered as the key factor delimiting species (Dobzhansky, 1937). Species were considered as populations sharing a common fertilization system, including signalling between mating partners and their calls. According to Paterson (1985), new species was evolved through the origin of new signalling system between sub groups. Many scientist support the view of Paterson and several others disagreed and are doubtful about the existence of such signalling systems (Claridge, 1988; 1995; Claridge *et al.,* 1997; Coyne *et al.,* 1988). In a broadened concept, biological species can be recognized through the presence of specific mate recognition systems, which are rarely be able to recognize in nature with certainty but remains a prerequisite for the final recognition of biological species (Claridge, 1988; 1995; 1997).

1.6 The use of morphological or biochemical or molecular markers in biological species

To overcome the difficulty of identifying specific mate recognition systems, markers that are helpful to understand the presence of reproductive isolation can be used. The markers may be based on the morphological characters or biochemical features or even molecular features of DNA involving nucleotide sequence similarities. The molecular markers can be used by a taxonomist as an indicator to identify the levels of reproductive isolation, gene flow between different groups and also to determine how far they diverge during the course of evolution (Tautz *et al.,* 2003; Blaxter 2004).

The molecular markers becomes very significant in some situations were speciation occur without much morphological changes and such species formed without recognizable changes are often called as sibling or cryptic species (Thorpe, 1940; Mayr, 1942; Claridge, 1988; 1995; Claridge *et al.,*1997). The development of molecular marker can also be used to recognise morphologically similar sibling species and morphologically dissimilar polymorphic forms (Hebert *et al*., 2004).

1.7 The concept of phylogenetic species

The diverse species we found were formed from evolution of their ancestors through several million years, and thus, the species can be defined as the groups sharing common ancestral lineages (Simpson, 1951). This is the phylogenetic species concept and many earlier workers of this species concept believe in similar ancestral lineage for diagnosing the species (Simpson, 1951; Cain and Sheppard, 1954). They believe only in cladistics and when projecting backwards in time, the species occupy a particular clade in the tree of life. While others believes in both biological and phylogenetic concepts about species (Wheeler and Nixon, 1990) and they consider species as the descendent of particular ancestor as recognised by cladistics. However they also believe it as a reproductively isolated group, distinguished from ancestors when having a specific mate-recognition system.

According to phylogenetic concept the markers considered for distinguishing a species from its ancestor is mostly morphological and in the case of a cladist, it is mostly subjective (Claridge *et al*., 1997).

1.8 Specific mate-recognition system (SMRS)

In biological species concept, the markers are specific mate-recognition system (SMRS). In a practical sense, the diagnosis of the existence of SMRS in a group is very difficult and time consuming. It is impossible in the case of asexually or parthenogeneticaly reproducing populations and in such cases phylogenetic method becomes significant (Cain and Sheppard, 1954). While considering sibling species morphological markers become less significant as there are little morphological differences, and in such cases, only biological species concept can do something, for clarification and make identification less ambiguous. In the case of allopatric populations, the conditions are opposite and have significant morphological differences but many of them are not isolated reproductively. Even though biological methods of species distinction have some solid demarcation of reproductive isolation between sexually reproducing forms, both biological and phylogenetic methods of species distinctions remain ambiguous and subjective.

1.9 Different types of insect speciation

In origin of species, there are two concepts, the allopatric speciation, taking place as a result of long term geographical isolation and sympatric speciation that takes place without any geographical isolation. According to the former concept, the ancestral population splits into two or more geographically isolated groups and they undergo independent modifications and becomes dissimilar and finally acquire

SMRSs between them (Paterson, 1985). Then the populations become reproductively isolated and they cannot interbreed even if the geographical barriers were removed (Mayr, 1942; 1963; Cain, 1959). In this concept of allopatric speciation, many intermediates were present among populations between the geographical territories at different times of speciation. The speciation taking place in the same population without geographical isolation is sympatric speciation; the theories of reinforcement of species isolating mechanisms in sympatric speciation (Wallace, 1889; Dobzhansky, 1940) supported this concept of speciation. The sympatric speciation is common among insect populations of specialist feeders like parasites, parasitoids and herbivores and they become varied with changes in their host populations (Walsh, 1864). This kind of speciation is taking place within the same home range of parent population under disruptive selection and therefore all stages of divergent population can be expected to exist in the range. These stages are sometimes described as dissimilar species when they are classified based on morphological markers, and the problems of wrong species identifications can be solved with the use of molecular markers such as DNA sequences (Hebert *et al*., 2004).

1.10 Practical problems of insect species identification

Insects are more similar in their morphology, for a common man most insects are look alike. The differences between many species involve only very minute morphological aspects and are often difficult to appreciate (Strong *et al*., 1984). Despite these morphological similarities, their ecology and habits are diverse and even species specific. Nearly half of them are herbivores and an equal number are parasitoids that depend on other insects for their food. In the case of insects, species can be differentiated and recognised from their food exploitation habits. The larval forms of many butterflies are identified from their larval food choices and there are many instances in which the butterfly species are discriminated from their feeding host plant choices (Ehrlich and Raven, 1964). This kind of species discrimination based on some morphological markers and their host preferences become difficult with the occurrence of particular host specific variants among same species with slight morphological differences. These host specific variants of a species are called 'host races' or 'biotypes' (Thorpe, 1930). The biotypes and cryptic species complexes are well studied in the case of insect pest feeding on crop plants and insect vectors transmitting human diseases. After the invention of *Anopheles* mosquito as the vector of Malaria by Dr. Ronald Ross, the effective control of malaria disease has some setbacks due to the host species specificity of Plasmodium and *Anopheles* species. Few species of mosquitoes belonging to the genus *Anopheles* can transmit the human infecting types of Malaria, and many sibling species complexes were identified with different morphological markers. The cross breeding experiments proved that they are only biotypes and not species. Later with the use of molecular markers, the *Anopheles gambiae*, was identified as the vector, and they are morphologically indistinguishable from closely related species. Thus, the eradication of other species favours their multiplication in the absence of competition (Della Torre *et al*., 2002). They are discriminated based on their ribosomal DNA sequences.

1.11 The presence of host races and biotypes

The experimental transfer of early instar nymph from one tree population to another studied genetic basis of variations seen among host races of a tree feeding leafhopper *Alnetoidia alneti* (Dlabola, 1950) showing difference in body size and colour from different tree populations. The study showed that it does not make any change to the population normally found on the same host tree and become the normal population on that tree. This experiment proved that they are only feeding variants and not sibling species, and there was no evidence for genetic variability between different host races. The Asian brown plant hopper *Nilaparvatha lugen* is a serious pest of *Oryza* species in Asian and Australian regions. The main strategy developed against their attack is the development of pest resistant varieties and they were cultivated at different regions of Asia. Certain virulent forms of the pest were identified and reared in the laboratory in separate cages as biotypes 1, 2 and 3. Each of them has the ability to attack on specific resistant host varieties (Saxena and Rueda, 1982). When infesting, all these biotypes on a susceptible variety of plant, the morphological difference between these biotypes disappear and they becomes similar and alike. This also shows that they are not new species, but only feeding variants with adaptation to feed on resistant varieties of rice (Saxena *et al*., 1983). The host races can be considered as specialised genetically distinct populations, which retain the ability for interbreeding and forming fertile offspring.

1.12 Molecular approach in insect taxonomy

Molecular methods are an alternate devise used by taxonomists to access distinct groups of organisms. The value of any character is estimated from the accuracy to distinguish taxonomic groups. The genetic, biochemical and molecular techniques used to identify and delimit species; molecular characters have varied strength and drawbacks. Molecular methods dominate in many aspects of insect taxonomy, the contributions of molecular methods to insect taxonomy is critically reviewed in annual review of entomology (Caterino *et al.,* 2000). Which concluded with a call to focus on short length DNA sequences from a small number of genes across all of insect systematics to avoid a tower of Babel in which different studies do not effectively relate to each other. Mitochondrial protein coding genes provide the most reliable genes and provide the most useful markers at the species level (Caterino *et al*., 2000). Insect species cover about three quarter of animal species and half of all described species and therefore it is the insect species to be standardise first, with suitable molecular markers then it can extended to other species easily (Tautz *et al*., 2003; Vogler and Monaghan, 2007). Molecular methods provide additional tools and characters to solve the classical problems faced in identification of specimens and also delimiting and determination of relationships of species.

The essence of correct identification needed to survey the natural range of variations in characters, well enough ahead of time, at least in the cases of insufficient morphological characters are available, to assign them to a particular species. The molecular methods provide an array of new characters that can assist correct identification of species, an enzyme allele or a nucleotide variation can serve to identify different stages of life. Therefore, the identification of a single specimen has the high chance of being correct (Medina *et al.,* 2006). Forensic entomology makes an early effort to provide a framework in a legal context for the use of molecular characters to identify insects. Different fly species are indicators of decay of dead bodies (post-mortem interval) and is reasonably accepted in court of Law, but the identification from maggots is difficult with morphological characters. This makes it necessary to rare them for many days in laboratory until emerging adults. Make delay in further activities, the molecular methods can overcome this delay and make a correct identification from any stage of life within few hours. A good classical taxonomist is particularly talented in analysis of shapes, and gradually develops a mental modelling of transformation probabilities from his several years of experiences, and correctly be applied for identifying specimens with high confidence. The need of experienced and skilled taxonomist for particular species is a limitation in many identifications, which can be overcome with the development of molecular characters and the technique can be easily taught to a technician to perform to diagnose a species at any time and from anywhere. The molecular markers need further characterisation and standardisation for using them instead of conventional morphological methods (Rubinoff *et al.,* 2006). The molecular taxonomy provides characters that show discontinuities in groups that were previously remained continuous. The molecular assaying of specimens make destruction of specimens to some extent and the continued refinement of techniques can help to minimise this damage to specimens (Hunter *et al.,* 2008). The discovery of new species of *Bemisia* whiteflies, *Archips* leaf rollers, and *Phytomyza* leaf miners was necessitated from the prior identification of ecologically distinct biotypes. The same will happen in the case of describing ten morphologically described species of skipper butterfly *Astraptes* species into one (Hebert e3t al.

2004). In the background of disappearing habitats threatening existence, the detection of species, describing and documentation of the species becomes an urgency. Which needs fast evolving gene sequences as molecular markers which make identification and documentation of species easy and efficient within short time and effort when compared with the time consuming elaborate traditional morphological methods of identifications (Godfray, 2002). The lack of visible characters in bacteria and other microorganisms lead from the development of ribosomal DNA sequence based identification system for them.

1.13 Species delimitation and phylogeny analysis

The delimitation of insect species involve determination of boundaries of genetic permeability between populations the reduced genetic permeability will leads to the absence of genetic exchange between them and make unique detectable genetic structure (Harrison, 1998; De Queiroz, 2007). Such groups come under the genomic integrity species definition (Sperling, 2003) and the unique genetic structures can be used as molecular markers delimiting species from others. The determination of genomic integrity is not meaningful in delimiting species, where the genomic differences were calibrated between populations with sister species which are either allopatric or sympatric. The different conventional markers used for delimiting a species from others such as morphological variations, allozyme variabilities and host plant specificities, were not found to be very effective. However the use of molecular data involving genomic DNA sequence features are effective in delimiting species boundaries. Use of an integrated approach using morphological, biochemical and molecular markers together can effectively solve many species delimitation problems (Cognato and Sun, 2007; Roe and Sperling, 2007; De Queiroz, 2007; Davis and Nixon, 1992). The use of mitochondrial cytochrome oxidase subunit I (COI) gene sequences by some workers gave many monophyletic groups, identify the COI gene sequences which can give many synapomorphic characters helping species delimitation and elucidating phylogenetic affinities (Burns *et al*., 2007). The large quantity of data available in the form of nucleotide sequences from DNA can be effectively used for phylogenetic reconstructions beyond species level (Felsenstein, 2004). Phylogenetic studies with DNA sequences have many challenges to reconstruct evolutionary history of a particular group involving multiple character substitutions over time. Short time period represented by internodal distance relative to total branch length and therefore molecular methods are suitable for discriminating at species level. Numerous intron less genes which do not necessitate RNA isolation and RT- PCR procedures are traced by the Leptree team (https://mitterlab.weebly.com/leptree.html) and others in 2008 and found mitochondrial COI gene as a suitable candidate. The use of mitochondrial sequence data sometimes shows faster variation rates as in *Pissodes* weevils when compared with flies and moths. However, the COI gene sequences are effective in most cases and conventionally used for insect taxonomy and phylogeny reconstruction (Avise and Mitchell, 2007). Many software applications and standardised methods are available for reconstruction of phylogeny and determination of evolutionary distance data from sequence information of a gene (Hebert *et al*., 2003). The 3% sequence divergence of COI gene between two groups can be sufficient to distinguish them as different species (Hebert *et al*., 2003), but this view was opposed by many and consider this criteria to be suitable for insect species (Cognato, 2006).

1.14 The use of different biochemical and molecular markers for species recognition

The conventional morphological markers used in species description were supplemented with many biochemical and molecular markers. They help to solve many difficulties faced with the use of morphological markers alone, in describing species such as the identification of morphologically similar cryptic species, identification of host types, biotypes and geographical races. Among them, the early ones used are biochemical markers involving structural differences of the bio molecules among different groups. The describing of wasp species by studying difference in the chemical structure of their venom and the classification of butterflies based on wing pigments (Bruschini *et al*, 2007; Ford, 1944) are examples for using biochemical markers.

1.15 The cuticular hydrocarbons

Recently the structural features of cuticular hydrocarbons and allozymes of insects were used for their classification. The cuticular hydrocarbons were studied by using methods like gas chromatography or mass spectroscopy and their differences were utilised for species discrimination. However, this method has difficulty in comparing cuticular hydrocarbons between different groups (Braga *et al*., 2013).

1.16 Allozymes

The allozymes were formed from different allelic forms of a gene in same loci having similar activity. The difference in their charge and size among different groups of insects were studied by electrophoresis in the same gel, the differences in banding patterns helped in species discrimination but their allele homology between two groups were uncertain, and they follow the Mendelian pattern of inheritance (Grill *et al.,* 2007).

1.17 Chromosome karyotypes

The chromosome karyotypes involving chromosome number, banding patterns and inversions were also used as cytological markers for understanding the relationships between different groups. They were effective tools in describing species and identifying population groups (Brown *et al.,* 2007). The method was relatively less expensive, requires only a good microscope but needs training and experience in karyotyping preparations and preservation techniques. The development of fluorescent in situ hybridisation (FISH) technique greatly enhances karyotyping to reveal the genomic architecture between different taxonomic groups.

1.18 Restriction fragment length polymorphism (RFLP)

The presence of restriction sequence homology between different phylogenic groups were compared and used as a molecular marker in Restriction fragment length polymorphism (RFLP). This method was used by many evolutionary biologists to understand phylogenetic relationships and as a technique directly utilising the sequence features of DNA molecule. The presence of a restriction sequence in the genome is a homologous character, for this study, the purified DNA isolated from organism is incubated with restriction enzyme to complete digestion, then load in agarose gel and conduct electrophoresis to separate the digested fragments into separate bands based on their size. This will generate a restriction fragment pattern on the gel for comparison. The RFLP is a relatively inexpensive and needs simple equipments and training, but it compare only few base pairs and generate few data for comparison (Nakada, 1994).

1. 19 Random amplified polymorphic DNA method (RAPD)

Arbitrarily primed PCR amplified bands of DNA from many locations of genomic DNA were produced and compared in Random Amplified Polymorphic DNA method (RAPD). In this method, several random short primers have been used which are about 10 base pairs length and these primers are called RAPD primers (Williams *et al*., 1990). The RAPD gives many short amplified bands from different regions of genome for comparison. It is a less expensive method that does not need previous genomic information, but the requirement of high quality DNA and poor reproducibility are its important demerits (Al-Barrak *et al*., 2004).

1.20 Amplified fragment length polymorphism (AFLP)

The Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) methods were combined in Amplified Fragment Length Polymorphism (AFLP). In AFLP the genomic DNA was subjected for restriction digestion before arbitrary priming and PCR amplification. The AFLP can give patterns contain far more markers than RFLP and RAPDs, but it needs good quality DNA and difficult to conduct with sub optimal DNA extractions. Therefore it is not commonly used for insect taxonomy above species level and are more useful in distinguishing host races, and small scale whole genome linkage map studies (Dopman *et al*., 2005; Mock *et al*., 2007;).

1.21 Short tandem repeats (STR)

Short tandem repeats or microsatellites were widely used for insect population studies, but there are few instances of them used for insect taxonomic studies. They are regions of DNA with short tandem repeats of two bases in sequence, giving enough allelic variations and can be easily obtained from sub optimal quantity of DNA extractions. The microsatellites are not suitable for certain groups like Lepidoptera where the flanking conserved sequences cause confusion. They are markers showing Mendelian pattern of inheritance and also occur throughout the genome. The development of microsatellite data for a particular species is expensive and the microsatellite primers developed for a species may not be useful for distinguishing another species. The microsatellite primers also have the problem of causing slippage errors during amplification (Barbara *et al*., 2007). They are very suitable in studying genetic relationships within a population and kinship relation studies.

1.22 The DNA sequencing

The direct sequencing from regions of one or two genes were used for taxonomic studies, this sequences are developed into unique barcoding sequences helping species identification. The method involves PCR amplification of regions of DNA involving particular genes and their sequencing. The candidate genes were selected in such a way that the sequence data were with enough resolution of genetic variability for distinguishing from species and homology for identifying species. It is found that the mitochondrial COI sequences are suitable among animals and are comparable for most taxa. The method is moderately expensive and therefore this method is limited to some targeted genes only. The sequence data analysis is a very suitable method and have enough strength to discriminate all taxonomic levels (Elias *et al*., 2007). The survey of barcoding sequence across large number of individuals of the same species from different populations and between related species are essential for making the barcoding data a valuable tool helping phylogenetic analysis and species identification (Rubinoff, 2006; Rubinoff *et al*., 2006).

1.23 The single nucleotide polymorphism (SNP)

The Single Nucleotide Polymorphism (SNP) present throughout the genome were used to discriminate taxonomic relationships. The method of finding SNPs need little effort and easy to automate and compare across genome, but the method generate limited information per nucleotide site moreover it consume much time for identification and comparison of numerous loci. The identification of SNPs over complete genome was moderately expensive and time consuming (Morin and McCarthy, 2007).

1.24 Expressed sequence tag libraries (ESTs)

The molecular markers having phenotypic significances are identified for taxonomic and phylogenetic studies using Expressed sequence tag libraries (ESTs), this will help to relate the marker with a functional variation in a gene. The ESTs were developed through the single direction reading of cDNA sequence cloned randomly from mRNA with the use of reverse transcriptase. The expression sequence libraries can give markers from transcriptionaly active regions of a gene, and it helps to target functional DNA across genome. This method uses single read sequencing of cDNA library which will lead to an increased probability of misreads and contaminations of bases in sequences. The method used for developing EST libraries and their screenings for molecular markers were highly expensive (Dinler and Budak, 2008).

1.25 DNA homology using microarrays

Microarrays designed by arranging several DNA sequences are in the surface of a grid along different sections of a plate (Gibson, 2002). The homology of DNA sequences from a species to several different markers can be studied from the complimentary binding pattern of DNA with the sequences fixed on grids of microarray. This will allow the comparison of large number of sequences in the genome in a short time. This method was widely used for characterisation of bacterial populations. It has also been suggested as a method for insect identification (Frey and Pfunder, 2006). The development of microarrays are highly expensive and the relatively high cost for microarrays prevent most workers from its use for classifying insects, more over the use of microarrays generate large amount of data for comparison and this make many computational complications in the analysis (Frey and Pfunder, 2006).

Among these methods, the currently most significant method is molecular barcoding which involve the sequencing of known regions of DNA that are sufficiently conserved so as to use with universal primers. At the same time it is with sufficient mutation rate that helps to distinguish one species from others. The mitochondrial gene has high mutation rate and because of poor repair mechanisms than that of nuclear genes, they are long been used to trace the genealogical histories of populations. Today the focus on mitochondrial DNA based identification, DNA barcoding gives hopes that the molecular methods will lift the perceived burden of biodiversity identification at species level (Hebert *et al*., 2003; Waugh 2007).

1.26 The insect biodiversity and DNA barcoding

Insects are the most diverse group of animals on the planet with more than 1 million described species with an equal number awaiting description and discover (Grimaldi and Engel, 2005). The insect populations affect human beings in many ways. Harmful interactions such as pest of crop plant and vectors spreading diseases. Some insect populations are helpful and beneficial helping pollination of flowers, production of some valuables like silk, honey and biological control of pests and vectors. The studies on some insects help us many ways in understanding principles of genetics, evolution, ecology and gene mechanisms of differentiation. The primary requirement for many of this kind of study is the knowledge about their taxonomic groups helping the identification, description and classification. Because of their great diversity the traditional methods of identification through morphological characters is complex and needs specialist knowledge but the number of taxonomic specialists and their work force is greatly declining (Grissell, 1999; Godfray, 2002). These situations suggest the need for development of alternate methods in taxonomic identification and description.

The agricultural pests cause immense damages to crops both in developed and developing countries, which make the importance of developing a fast accurate and unambiguous method of pest identification system for their effective control (Ball and Armstrong, 2006; Yudelman *et al*., 1998). Moreover, it has a conservational importance also, within a short period of time immense taxa were disappeared from this world because of various reasons. The development of a rapid unambiguous identification system will help the rapid formation of a species catalogue for planning and implementation of suitable conservation strategies (Myers *et al.,* 2000).

1.27 Species concept and species recognition

Species is considered as the basic units of biodiversity (Claridge *et al*., 1997) and only the real grouping in the taxonomic hierarchy. There are many species concepts and about 22 species concepts have appeared in literature, among them many are synonymous and they are based on various criteria involving ecological, mate recognition, genetic cohesion and evolutionary history. This varied species criteria lead to ambiguity resulting in widely varying estimates of taxon richness (Agapow *et al*., 2004). Among them reproductive isolation was considered as most important, but it is difficult for direct testing and cannot apply for asexual forms. Therefore, usually species were recognised from the detection of one or more apparently fixed non-overlapping diagnostic differences involving genital morphology in the last century (Eberhard, 1985; Arnqvist, 1998). This considers the rapid and prominent divergence in genitalia between species, but this has no appropriate methodology to quantify the variations in shape and number of structures. Therefore, the homology assessments are questionable. Moreover, the diagnostic criterias followed in morphological features were found only in adult stages of life and are not significant in other stages like larvae and pupae (Balakrishnan, 2005). However, in most pest attacks the affected forms are either larvae or pupal stages which cannot be identified with adult features and make the identification difficult and need rearing till emerging of adults. All this situations make identification difficult, time consuming and need specialist expert having many years of experience in identifying each group of insects. In this situation an option exists is tracing genetic variations accumulated in DNA sequences, through mutations, evolution or isolation of particular lineage. This method of tracking DNA sequences was used for the identification of least morphologically tractable groups like viruses and bacteria (Nee, 2003). This method was applied for plant identification recently, nematodes, birds, fishes and mammals. This method was very much enabled by the sequences deposited in GenBank, other databases and the development of Bioinformatic techniques for sequence comparison. Many authors referred as operational taxonomic units (OTUs) as a term delimiting taxa based on phenetic means (Sokal and Sneath, 1963). Which is not correspond to species but can be used instead of situations preferring speed and ease than theoretical considerations. The taxa delimited with phenetic DNA sequence divergence can be considered as molecular operational taxonomic units (MOTUs) (Floyd *et al*., 2002; Blaxter *et al*., 2005). This approach has become standard for environmental surveys of bacteria and other microorganisms. The MOTUs are compared with species in many ways, this can help to identify whether species or biotypes or polymorphism or sexual dimorphism. This is the case with *Astraptes fulgerator* the skipper butterfly of Costa Rica, which has 10 species with morphological differences but the molecular COI sequence data analysis shows they are alike and belong to one species (Hebert *et al*., 2004). According to Sites and Marshal (2003), there were nine methods used for delimiting species, among them seven methods were depended on Molecular markers. Thus, the use of molecular markers has an increasing acceptability among taxonomists.

1.28 DNA barcoding

DNA barcoding is the use of short standardised genomic sequences as markers for species identification. One species differ in their morphology, ecology, and behaviour as well as in their DNA sequences (Mallet and Willmott, 2003). There are many molecular studies exploring the monophyly of 'micromoths' 'macromoths' and 'butterflies' in the order Lepidoptera represent 160000 described species and with an addition of about 800 new species per year (Kristensen et al, 2007). Barcoding promises the ability to automate the identification of species by comparing the similarities in sequences of barcoding region. This will avoid the complexities of morphological identification and leads to the establishment of a simple system of identification based on DNA sequence similarity. The nucleotide databases with barcoding sequences might be applied to the identification of all life (Tautz *et al*., 2003; Blaxter, 2004). The genomic region used for barcoding has sufficient homology within species and show variation between taxa. The genomic region for barcoding also has limited sequence conservation, which allows it to amplify with a limited set of PCR primers. The first 658 base pair region of mitochondrial COI gene has this feature and is suitable for insect barcoding (Hebert *et al*., 2003). It lacks introns, simple in alignment, lack of recombination, the availability of many pairs of PCR primers make it suitable for insect barcoding (Hebert *et al*., 2003). The homogenous nature of COI sequence within species irrespective of population size makes it a suitable genetic signature of the species (Monaghan *et al*., 2005). Some authors are with an opinion that 'barcoding gaps' does not exist the 'barcoding gaps' helping species identification were the result of artefacts from a reduced number of samples deposited in the databases, further they suggest the application of other methods along with barcoding (Wiemers and Fiedler, 2007). There are studies which involve the critical evaluation of utility of DNA barcodes in species identification and phylogeny analysis (Moritz and Cicero, 2004; Aravind *et al*, 2007). The homogeneous barcoding boundaries were concordant with the species boundaries previously established through morphological or behavioural markers. The variations from homogeneity of COI sequences can be used to follow evolution and phylogenetic relationships. This can be of use in species identification for solving many problems arising through morphological, ecological or behavioural markers (Hebert *et al*., 2003). The sequence based data can be easily converted to digital form as it has the sequential feature in arrangement of four variables such as the four nitrogen bases of DNA. Furthermore, it can be applied for identification in all stages life *viz*; egg, larvae, pupae or adult and even to identify from their fragments. Additionally, it is helpful in avoiding ambiguity caused by different caste forms of social insects (Smith *et al*., 2005). The wrong species identification resulting from the high level of sexual dimorphism, where each sexual morphs were considered as different species. In such cases, molecular marker become an effective tool as in the case of butterfly *Saliana severus* (Mabille, 1895) where the two sexes were described as two different species which was solved by using barcodes of COI DNA sequence (Janzen *et al*., 2005).

The novel techniques of DNA extraction and recent advances in high throughput DNA sequencing have reduced the cost of sequencing and made the generation of large volumes of DNA data easier. This enables to produce the DNA sequences in the laboratory within few hours in an automated fashion (Shendure, *et al.*, 2004; Hajibabaei *et al*., 2005). In the near future, the attempts for developing small carbon nano tubes will give a new technology for DNA sequencing which is expected to culminate in the development of a hand held barcoder, are still in the realising of a speculation, and not become a reality. Sequencing of DNA is not a necessary step for species identification, the closely allied species of *Bombus ruderatus* and *Bombus hortorum*, are two cryptic species of Bumblebees, which were distinguished from the analysis of variations in restriction, digest of PCR product of COI genes (Ellis *et al.,* 2006). The same was done in the case of Swede midge an agricultural pest. An important challenge of DNA barcoding is the poor sequence representation of many taxa in the databases like GenBank. Some taxa have extensive works and a high percentage of sequence depositions, while some other groups are with small number of works and have no or few sequence representations in databases (Sanderson *et al*., 2003). The small number of barcode representation of taxa in databases results in poor matches and raises the possibility of incomplete phylogeny results and spurious species diagnosis (Baker *et al*., 1996). The best BLAST hit never occur to find out relations when the databases has incomplete data (Tringe and Rubin, 2005). This is suggesting the importance of extensive barcoding works in the future to complete the missing barcodes for a better species or phylogeny identification (Tautz *et al*., 2003). GenBank has insufficient data about collection details and morphology of type specimens used in barcoding (Tautz *et al*., 2003), to overcome this the GenBank organisation NCBI linked with Consortium of barcoding of life (CBOL). This enable GenBank in sharing the data with Barcode of life data system (BOLD) maintained by CBOL. The CBOL has depositions with identified name of specimen, its taxonomic status, information about collection site and photograph of voucher specimen. This is an achievement of international nucleotide database collaborative (Hanner, 2005). This approach helps the standardisation of DNA barcoding from regions that are previously lacking or hindering progress in insect molecular systematics (Caterino *et al*., 2000).

1.29 DNA barcoding of Lepidoptera

The order Lepidoptera has received significant taxonomic and systematic attention, only about 165000 species of Lepidoptera were described which is roughly about 10% of 1.5 million animal species known (Wilson, 2003). About 150000 to 1250000 of Lepidoptera species have remain undescribed. Lepidoptera becomes a model group of organisms, which are successfully described with the COI sequence (Hebert *et al*., 2003; Hebert, 2003; Hebert, 2005; Elias *et al*, 2007). The presence of morphologically dissimilar developmental stages like larvae, pupae and adult in the lifecycle of Lepidopterans make it difficult to identify all developmental stages with the same morphological marker. In polymorphism within species, the individuals occur in many morphologically dissimilar forms, here the morphologically unlike becomes same species. There are some instances where look-alike becomes dissimilar species because of mimicry and these make some ambiguity in identification based on morphological markers. The high degree of sexual dimorphism exhibited by some Lepidopterans also causes a problem in species identification. This is the case of butterfly *Saliana severus* the two sexes of them were described as two different species by morphological methods, this was solved with the use of barcodes of COI, DNA sequence and they are identified as two sex morphs of same species (Janzen *et al*., 2005). Many Lepidopterans involving butterflies are with environmental significance having plant and climatic interactions (Emelianov *et al*, 2004). Hence, the study of butterfly populations of an area can be utilised as an important tool for environmental studies, their number can be considered as an indication for habitat degradation (Scoble, 1992). During environmental surveys, the accurate taxonomic data is very essential; the development of barcode data will assist easy, rapid and accurate identification of species.

Chapter 2

Materials and methods

2.1 Collection of specimens

The butterflies, belonging to Papilionoidea superfamily were collected at random from different locations of North Kerala. The location from which the specimens were collected is given in the (table 2). They were collected by using sweep nets at different instances some were from flowers, others during mud-puddling and the rare endangered were collected as they were found hit down near roadsides by passing vehicles. The collected specimens were identified by the presence of morphological features identical to those described by Bingham C. T. (Bingham, 1907). The specimens were stored at - 20^0C freezer in sealed containers until DNA extraction was done.

S. No.	Voucher No.	Name	Common name	Family	Location of collection
1	CUJ1	*Troides minos*	Southern Birdwing	Papilionidae	Alakode, Kannur
2	CUJ2	*Atrophaneura aristolochiae*	Common Rose	Papilionidae	Alakode, Kannur
3	CUJ3	*Graphium agamemnon*	Tailed Jay	Papilionidae	Aravanchal, Kannur
4	CUJ4	*Papilio polytes*	Common Mormon	Papilionidae	Aravanchal, Kannur
5	CUJ5	*Papilio polymnestor*	Blue Mormon	Papilionidae	Payyanur, Kannur
6	CUJ6	*Hypolimnas bolina*	Great Eggfly	Nymphalidae	Aravanchal, Kannur
7	CUJ7	*Papilio clytia*	Common Mime	Papilionidae	Thalassery, Kannur
8	CUJ8	*Pachliopta hector*	Crimson Rose	Papilionidae	Aravanchal, Kannur
9	CUJ9	*Atrophaneura aristolochiae*	Common Rose	Papilionidae	cheemeni, Kasaragod
10	CUJ10	*Papilio liomedon*	MalabarBanded Swallowtail	Papilionidae	Chapparapadavu, Kannur
11	CUJ11	*Delias eucharis*	Common Jezebel	Pieridae	Peringome, Kannur
12	CUJ12	*Eurema hecabe*	Common Grass Yellow	Pieridae	Aravanchal, Kannur
13	CUJ13	*Eurema hecabe*	Common Grass Yellow	Pieridae	Thalassery, Kannur
14	CUJ14	*Orsotriaena medus*	Nigger	Nymphalidae	Aravanchal, Kannur
15	CUJ15	*Cynitia lepidea*	Grey Count	Nymphalidae	Aravanchal, Kannur
16	CUJ16	*Junonia iphita*	Chocolate Pansy	Nymphalidae	Thalassery, Kannur
17	CUJ17	*Junonia almanac*	Peacock Pancy	Nymphalidae	Aravanchal, Kannur
18	CUJ18	*Ypthima huebneri*	Common four ring	Nymphalidae	Nilambur, Malappuram

19	CUJ19	*Tirumala limniace*	BlueTiger	Nymphalidae	Kottiyoor,Kannur
20	CUJ20	*Melanitis leda*	Common Evening Brown	Nymphalidae	Nilambur, Malappuram
21	CUJ21	*Melanitis leda*	Common Evening Brown	Nymphalidae	Aravanchal, Kannur
22	CUJ22	*Acraea violae*	Tawny coster	Nymphalidae	cheemeni, Kasaragod
23	CUJ26	*Junonia lemonias*	Lemon Pansy	Nymphalidae	Thamarassery, Kozhikode
24	CUJ27	*Danaus chrysippus*	Plain Tiger	Nymphalidae	Thamarassery, Kozhikode
25	CUJ29	*Castalius rosimon*	Common Pierrot	Lycaenidae	Aravanchal, Kannur
26	CUJ31	*Euploea core*	Common Crow	Nymphalidae	Thalassery, Kannur
27	CUJ32	*Troides minos*	Southern Birdwing	Papilionidae	Aravanchal, Kannur
28	CUJ33	*Luthrodes pandava*	Plain Cupid, cycad blue	Lycaenidae	Aravanchal, Kannur

Table 2. Samples collected with their voucher numbers and collection locations.

2.2 DNA extraction

The DNA extraction was done soon after the collection before drying of the specimens. The collected specimens were kept at -20^0 C freezer until DNA extraction. About 25mg of tissues from the thoracic muscles of the body was used for DNA extraction. In the case of small sized butterflies, entire tissues were taken after removing the wings. The DNA from tissue was extracted by using the genomic DNA isolation kit, "Nucleospin Tissue" manufactured by Machery Nagel, Duren, Germany, following the Manufacturer's protocol (http//www.mn-net.com). The quality of extracted DNA was checked by loading the same in a 1% agarose gel. The specimens of butterflies were damaged during tissue extraction their remaining body with wings were kept at Entomology Museum of Govt. Brennen College, Dharmadam, Thalassery.

2.3 PCR primers

The Folmer primer (Folmer *et al*., 2004) and Lep primer (Janzen *et al*., 2005) were identified as successful primers. The other PCR primers were selected using Primer 3 software (Rozen and Skaletsky, 2000) from the COI gene sequences of related insects collected from GenBank database. The primer combinations found successful in giving amplification of COI gene were presented in table 3. Among these primers the PA1, PA2 combination and PB1, PB3 combinations were found to be very effective and good in giving maximum amplification size.

Si No.	Primer name	Sequence	Melting temperature (^{0}C)	PCR product size (bp)
1	PA1 (Folmer *et al.*, 2004)	5'- GGTCAACAAATCATAAAGATATTGG - 3'	61	658
2	PA2	5' - TAAACTTCAGGGTGACCAAAAAATCA - 3'	60	658
3	PB1	5'-ATTCAACCAATCATAAAGATATTGG -3'	66	658
4	PB2	5'- TAAACTTCTGGATGTCCAAAAAATCA -3'	65	658
5	PB3	5'- CTCCACCAGCAGGATCAAAA -3'	65	609
6	PC1	5'- CAAATCATAAAGATATTGGAAC - 3'	55	659
7	PC2	5' - TAACTTCTGGATGTCCAAAAAATCA - 3'	61	659

Table 3. The primer sequences selected with their melting temperature and PCR product size.

2.4 PCR amplification of partial sequence of the mitochondrial cytochrome oxidase subunit I (CO I) gene

The mitochondrial COI gene of each specimen was amplified separately with a distinct set of forward and reverse primers. The PCR reaction mixture consist of 2x premix of EmaraldAMP GT PCR master mix - 25 µl, Forward Primer (5 µM) - 2 µl, reverse Primer (5 µM) - 2 µl, template DNA - 2µl and then make up to 50 µl by adding 19 µl of sterile dH2O. The 50 µl of the final volume of PCR reaction contain 0.2 µM of each forward and reverse primers and 2 ng genomic DNA (1 µl). The PCR temperature profile consisted of 95^{o}C/3 minutes for initial denaturation followed by 30 cycles of 95^{0}C/10 seconds, 53^{o}C/45 seconds, 72^{o}C/45 seconds and with a final extension of 72^{o}C for 3 minutes. The reaction products were stored at 4^{0}C.

2.5 PCR product analysis

The PCR products were resolved using agarose gel electrophoresis, on 1.5% TAE-agarose gel stained with ethidium bromide (Sambrook and Russell, 2001). Electrophoresis was conducted at 90V. DC for about 20 minutes, the gel was checked in a UV-trance illuminator.

S.I. No.	Primer	Primer Direction	Sample
1	PA1	Forward	CUJ1, CUJ2, CUJ6, CUJ8, CUJ9, CUJ10, CUJ11, CUJ12, CUJ13, CUJ14, CUJ15, CUJ16, CUJ17, CUJ18, CUJ19, CUJ20, CUJ21, CUJ22, CUJ26, CUJ27, CUJ29, CUJ31, CUJ32, CUJ33
2	PA2	Reverse	
3	PB1	Forward	CUJ 3, CUJ 4, CUJ 5, CUJ7
5	PB3	Reverse	

Table 4. PCR primers used for sequencing of different samples along with the annealing conditions, used with each primer set.

2.6 PCR product clean up

The PCR products were purified using StrataPrep PCR purification kit of Agilent Technologies USA as per the Manufacturer's instruction.

2.7 DNA sequencing

The purified PCR products were sequenced from both ends using Sanger's sequencing technique, at SciGenom Laboratories Ltd., Cochin.

2.8 Sequence analysis

The forward and reverse sequences were assembled and the consensus sequence obtained was trimmed off the primer sequences and the final sequence was assembled using Sequencher 5.3 (Gene codes corporation, Ann Arbor, Michigan, USA).

2.9 Consensus sequence

The bidirectional sequencing was used for the generation of a consensus sequence with higher confidence levels for each base calls. The nucleotide consensus tool available in Sequencher 5.3 was used to align the forward sequencing chromatogram with the reverse compliment of the reverse sequence chromatogram from its complimentary strand. The consensus sequence generated from this alignment was again realigned with the forward primer sequence and the reverse compliment of reverse primer sequence and was deleted from the consensus sequence.

2.10 Nucleotide BLAST (BLASTn)

The consensus sequence obtained was searched for its sequence similarities using BLASTn programme of NCBI (Altschul *et al.,* 1990). Sequence similarity search provides information about the nucleotide sequence also give its similarity and differences with other known sequences deposited in nucleotide databases. Similar sequences were selected from NCBI, and they were aligned along with query sequence using the ClustalW.

2.11 Phylogeny analysis

The Phylogenetic and molecular evolutionary analysis was conducted using MEGA Version 6 (Tamura *et al.,* 2013). The multiple sequence alignments were used for Phylogeny analysis with Neighbour Joining method (Saitou and Nei, 1987; Felsenstein, 1985). The multiple sequence alignments were done in ClustalW for the identification of nucleotide variabilities between samples.

Chapter 3

Results and discussion

3.1. *Troides minos* (Cramer, 1779) isolates CUJ1 (HQ424201) and CUJ32 (KT880663)

The specimens CUJ1 and CUJ32 were identified as *Troides minos* (Cramer, 1779) referring to the identical morphological features of the species described by Bingham C. T. (Bingham, 1907).

Synonyms: *Papilio minos* Cramer, 1779
Troides minos; Rothschild, 1895

Troides minos is the Southern birdwing butterfly, which is endemic to Western Ghats. The *T. minos* is with yellow coloured abdomen and black dots at dorsal and ventral sides. Its characteristics include: velvety black, upper side, well-marked pale adnervular white streaks on the discal area of the forewing that reach the terminal margin (Fig. 1). The hind wing exhibit bright yellow with black along the dorsal and terminal margins both on upper and under sides of the hind wing Fig. 3. The larval host plants involving *Aristolochia indica* and *Aristolochia tagala* and *Thottea siliquosa.*

PCR amplification and sequencing of partial coding region of COI gene of *T. minos* yielded 658 bp. The chromatogram, DNA sequence obtained, its conceptual translation product, BLASTn hit table, and NJ-tree are presented below (Fig. 4 to 8).

Fig. 1. The lower side of the *T. minos* CUJ32, showing yellow abdomen with black spots, Bright yellow hind wing with black veins and markings, the fore wing with black coloured veins with white shades.

Fig. 2. The upper side of the *T. minos* CUJ1.

Fig. 3. The lower side of the *T. minos* CUJ1, White coloured lining of disco cellular veins, Black spot at inter space 7, Black spots at ventral side of abdomen.

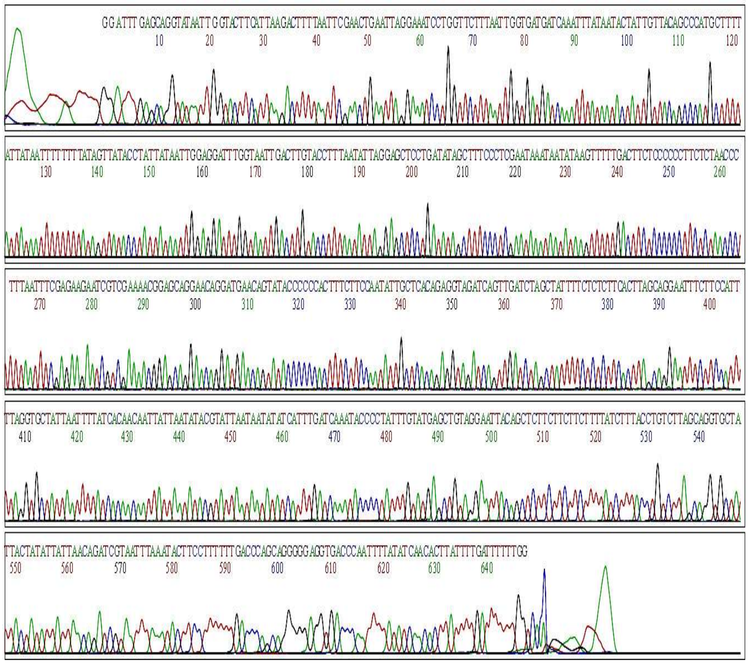

Fig. 4. The sequencing chromatogram of forward DNA strand of *T. minos* **C**UJ1 and CUJ32.

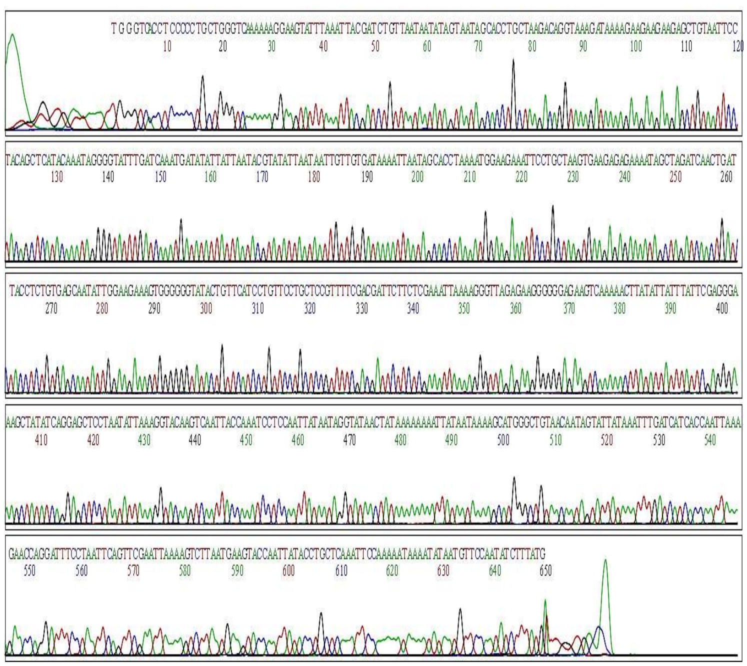

Fig. 5. The sequencing chromatogram of reverse DNA strand of *Troides minos* CUJ1 and CUJ32.

```
>Triodes minos voucher CUJ1 cytochrome oxidase subunit I
gene, partial cds; mitochondrial(658)
AACATTATATTTTATTTTTGGAATTTGAGCAGGTATAATTGGTACTTCATTAAGACTTTT
AATTCGAACTGAATTAGGAAATCCTGGTTCTTTAATTGGTGATGATCAAATTTATAATAC
TATTGTTACAGCCCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGG
AGGATTTGGTAATTGACTTGTACCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCTCG
AATAAATAATATAAGTTTTTGACTTCTCCCCCCTTCTCTAACCCTTTTAATTTCGAGAAG
AATCGTCGAAAACGGAGCAGGAACAGGATGAACAGTATACCCCCCACTTTCTTCCAATAT
TGCTCACAGAGGTAGATCAGTTGATCTAGCTATTTTCTCTCTTCACTTAGCAGGAATTTC
TTCCATTTTAGGTGCTATTAATTTTATCACAACAATTATTAATATACGTATTAATAATAT
ATCATTTGATCAAATACCCCTATTTGTATGAGCTGTAGGAATTACAGCTCTTCTTCTTCT
TTTATCTTTACCTGTCTTAGCAGGTGCTATTACTATATTATTAACAGATCGTAATTTAAA
TACTTCCTTTTTTGACCCAGCAGGGGGAGGTGACCCAATTTTATATCAACACTTATTT
```

Fig. 6. The partial sequence of mitochondrial COI gene of *Troides minos* CUJ1.

The conceptual translation of the consensus sequence of CUJ1 gave the following peptide sequence (Fig. 7)

```
>seq25 Troides minos CUJ1

TLYFIFGIWAGMIGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSSIVENGAGTGWTV
YPPLSSNIAHSGSSVDLAIFSLHLAGISSILGAINFITTIINMRINNMSFDQMPLF
VWAVGITALLLLLSLPVLAGAITMLLTDRNLNTSFFDPAGGGDPILYQHLF
```

Fig. 7. The peptide sequence obtained from conceptual translation of consensus sequence of *Troides minos* isolate CUJ1.

The nucleotide BLAST was conducted with the consensus sequence derived from *T. minos* isolate CUJ32, the BLAST give CUJ1 as the top similar sequence, the (table 5) below represent the BLAST hit table derived from it.

S. I. No.	Subject Ids	% identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	s. end	Bit score
1	*gi|312451704|gb|HQ424201.1|Triodes minos voucher CUJ1*	100.00	658	0	0	1	658	1	658	1216
2	*gi|145694555|gb|EF514455.1|Troides sp. EC-077*	98.18	604	11	0	29	632	1	604	1055
3	*gi|306407734|dbj|AB576491.1|Troides aeacus formosanus*	98.07	569	11	0	1	569	569	1	990
4	*gi|306407748|dbj|AB576498.1|Troides aeacus formosanus*	97.89	569	12	0	1	569	569	1	985
5	*gi|306407740|dbj|AB576494.1|Troides aeacus formosanus*	97.89	569	12	0	1	569	569	1	985

6	*gi|306407760|dbj|AB576504. 1|Troides aeacus formosanus*	97.72	569	13	0	1	569	569	1	979
7	*gi|545691119|gb|KF226654. 1|Troides helena cerberus*	97.47	553	14	0	1	553	1	553	944
8	*gi|829489848|gb|KM895487. 1|Troides helena*	97.35	604	16	0	2	605	1	604	1027
9	*gi|145694545|gb|EF514450. 1|Troides sp.*	97.15	596	17	0	29	624	1	596	1007
10	*gi|545691121|gb|KF226655. 1|Troides helena cerberus*	97.11	658	19	0	1	658	1	658	1110
11	*gi|9754761|gb|AF170878.1|T roides helena*	97.11	658	19	0	1	658	49	706	1110
12	*gi|145694541|gb|EF514448. 1|Troides helena*	96.99	598	18	0	37	634	1	598	1005
13	*gi|145694557|gb|EF514456. 1|Troides helena*	96.85	604	19	0	29	632	1	604	1011
14	*gi|145694551|gb|EF514453. 1|Troides sp.*	96.85	604	19	0	29	632	1	604	1011
15	*gi|145694537|gb|EF514446. 1|Troides helena*	96.81	596	19	0	37	632	1	596	996
16	*gi|193084916|gb|EU625344. 1|Troides aeacus*	96.81	658	21	0	1	658	1525	2182	1099
17	*gi|145694455|gb|EF514405. 1|Troides sp*	96.52	546	19	0	37	582	1	546	904
18	*gi|145694491|gb|EF514423. 1|Troides sp*	96.23	584	22	0	29	612	1	584	957
19	*gi|145694483|gb|EF514419. 1|Troides sp*	96.21	580	22	0	33	612	1	580	950
20	*gi|145694495|gb|EF514425. 1|Troides haliphron*	95.92	588	24	0	25	612	1	588	953
21	*gi|145694479|gb|EF514417. 1|Troides staudingeri*	95.88	582	24	0	25	606	1	582	942

Table 5. The BLAST hit Table of *T. minos* CUJ1.

T. minos used in the present study (CUJ1 and CUJ32) were showed 100% sequence similarity. While the similarity was 98.18% with *Troides sp* (EF514455) from India, the sequence similarity with *T. aeacus* from Taiwan (AB576491) was 98.07%. However, other samples of *T. aeacus* from Taiwan (AB576498, AB576494 and AB576504) were showed 97.89 to 97.72% similarity. Another species *T. helena* from Peoples Republic of China, Malaysia, India and Indonesia (KM895487, KF226655, EF514448, EF514456, EF514453 and AF170878) were with 97.47 to 96.81% similarity. The *T. haliphron* (EF514425) and *T. staudingeri* (EF514417) from Indonesia showed 95.92 and 95.88% similarities respectively.

The sequences with top similarities were identified from the BLAST and the sequences were used with the isolates CUJ1 and CUJ32 for phylogenetic analysis using MEGA6, the NJ- trees derived were represented below in Fig. 8

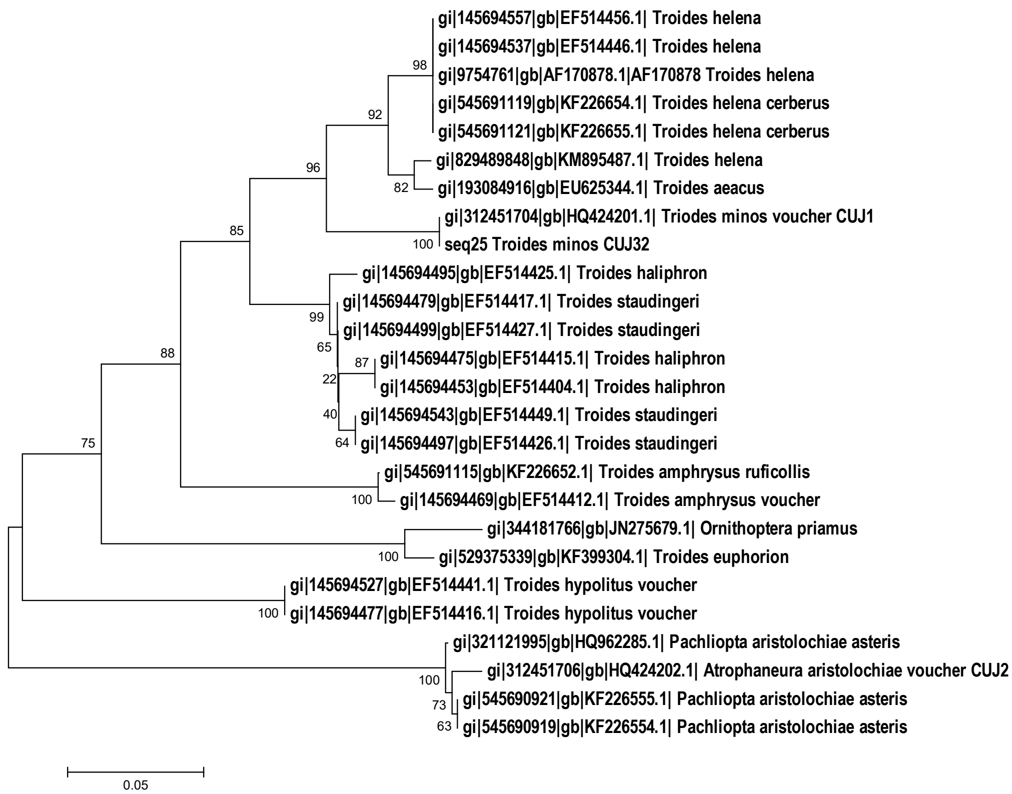

Fig. 8. The NJ-tree with phylogenetic relationships of *T. minos* CUJ1

The variations were clearly reflected in the NJ-tree developed from the sequences obtained from the database including different species of T*roides* genus from different geographical regions. The *T. minos* used in the present study (CUJ1 and CUJ32) were found sharing a single clade with 100% sequence similarity. While the *T. aeacus* and *T. Helena* were found in the next clade with the *T. aeacus* from Taiwan (AB576491, AB576498, AB576494 and AB576504) with 98.07% to

97.72% similarity. *T. helena* from Peoples Republic of China, Malysia, India and Indonesia KM895487, KF226655, EF514448, EF514456, EF514453 and AF170878 with 97.47 to 96.81% similarity were found monophyletic with the *T. aeacus* (EU625344) from P. R. China found more close to the *T. minos.* On the other side of cladogram the clade representing *T. haliphron* (EF514425) and *T. staudingeri* (EF514417) from Indonesia were monophyletic and showed 95.92 and 95.88% similarities with *T. minos*.

Discussion

The consensus sequence of *T. minos* isolate CUJ32 (KT880663) collected from Aravanchal, Kannur District, of Kerala ($12^0$13'08"N, $75^0$16'29"E), showed 100% similarity to that of *T. minos* CUJ1 (HQ424201) from Alakode, Kannur District of Kerala ($12^0$12'22"N, $75^0$27'38"E). The sequences of CUJ1 and CUJ32 were similar without any base substitutions. The *T. minos* CUJ1 (HQ424201) is an endemic butterfly of Western Ghats. This is the first reporting of its Mitochondrial COI sequence of *T. minos* in GenBank nucleotide database. The sequences of CUJ1 and CUJ32 will be useful as a molecular barcode helping identification of the species. The BLAST hit table showed many related species of the genus with close similarity involving *T. aecus* and *T. helena*. The *T. helena* with less than 97% similarity was more distant than *T. aecus* with similarities greater than 97%. Among them *Troides sp*. EC-077 (EF514455) from India was more closely related with 98.4% similarity, but the species was unidentified therefore has no significance. The *T. aeacus szhechwanus* (KT179880) from Sichuan of Peoples Republic of China showed 98.1% similarity, the *T. aeacus* (AB576491) from Taiwan has about

98.07% similarity. These suggest the close relationship of *T. minos* with them than the more South East forms. In the NJ-tree the *T. aeacus* (EU625344) from Peoples Republic of China present in the adjacent clade. The phylogenetic association of *T. minos* with *T. aeacus* from P.R. China suggested their diversification from a common ancestor. The endemism of *T. minos* in Western Ghats was arising from the isolation of a Troides ancestor in the Western Ghats. The isolation was resulted from the vicariance events like the rise of Himalayas, disappearance of the Tethys Sea and the associated climatic changes in Deccan plateau, which became arid. The arid planes of Deccan plateau isolate the *T. minos* living in the evergreen Western Ghats from their relatives of North and East. The close similarity of *T. minos* with samples from P. R. China and Taiwan than with South East Asia shows the possibility of having a common ancestor for *T. minos* and *T. aeacus* coming from North across Tethys Sea before their isolation.

3.2. ***Atrophaneura aristolochiae*** **(Fabricius, 1775) isolate CUJ2 (HQ424202)**

The specimen CUJ2 is identified as *Atrophaneura aristolochiae* (Fabricius, 1775) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio aristolochiae* Fabricius, 1775

Pachliopta aristolochiae (Fabricius, 1775)

The *Atrophaneura aristolochiae* is the common rose butterfly, a red-bodied swallowtail butterfly found in India and South East Asia. They are most common in Kerala during monsoon, pre monsoon and post monsoon periods. The upper side; velvety black, Fore wing; with well-marked pale adnervular streaks on the discal area that do not reach the terminal margin, the latter broadly velvety black; the streaks beyond end of cell extended inwards into its apex (Fig. 9A). Hind wing; elongate white discal white markings in interspaces 2–5 beyond the cell (Fig. 9A). Curved series of sub terminal lunular markings in interspaces one to seven, dull crimson irrorated with black scales (Fig. 9A). The spot in interspace one large, diffuse, irregular margined interiorly with white. Antennae, thorax and abdomen; above black, the head, sides of prothorax; above, the whole thorax and abdomen beneath vermilion-red; anal segment vermilion-red (Fig. 9A). The larval host plants include *Aristolochia indica* and *Aristolochia bracteolate.*

The PCR amplification of partial COI sequence of *A. aristolochiae* CUJ2 from Alakode, yielded a product of 658 bp. The chromatogram, DNA sequence obtained, its conceptual translation product, BLASTn hit table, and NJ-tree are presented below (Fig. 10 to 14).

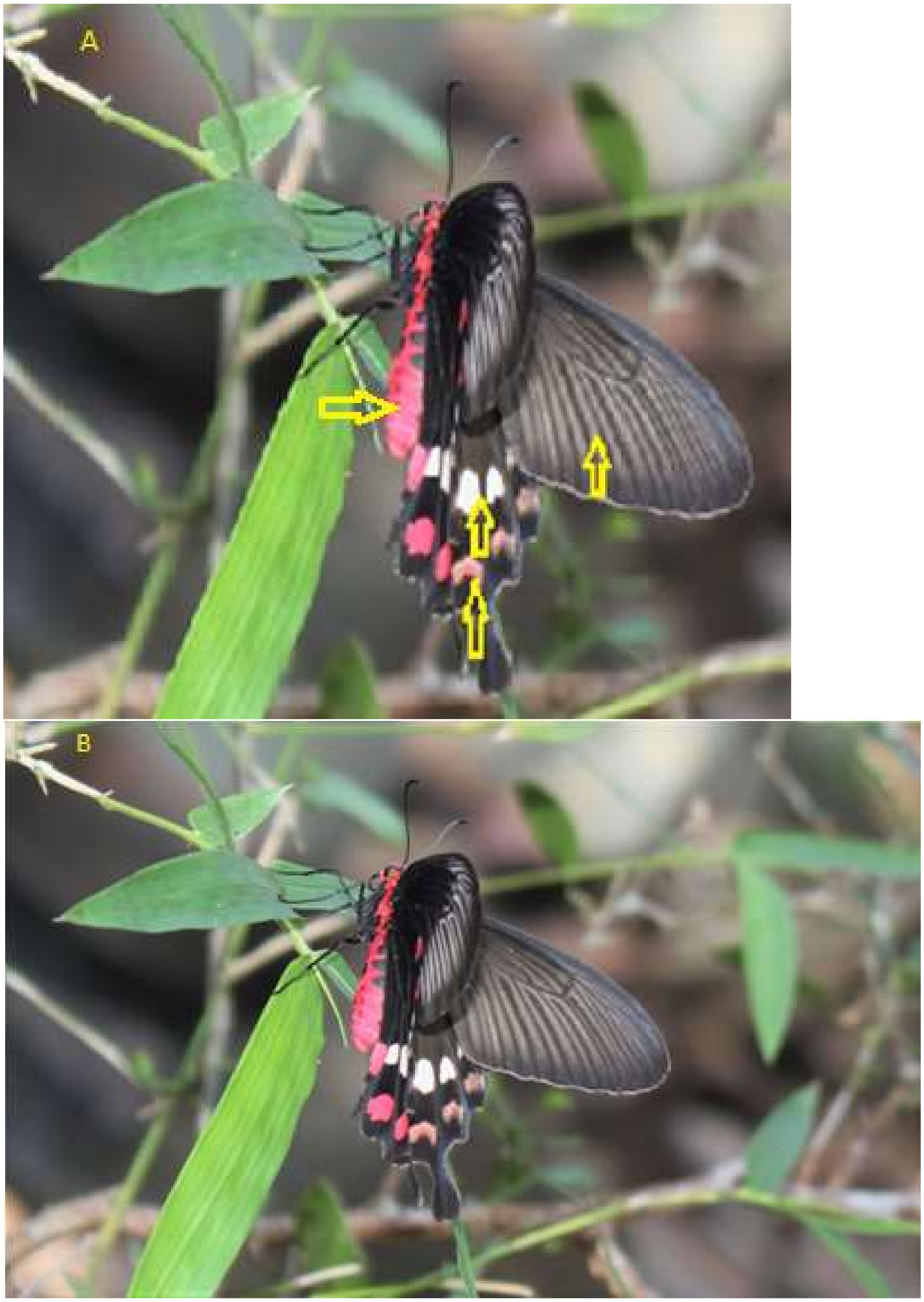

Fig. 9. The upper side of *A. aristolochiae* CUJ2, the fore wings are velvety black with adnervular pale streaks, Hind wing with elongate white discal markings in interspaces 2–5 beyond the cell, curved series of sub terminal lunular markings in interspaces one to seven crimson irrorated with black scales, vermilion-red body involving abdomen and anal segment.

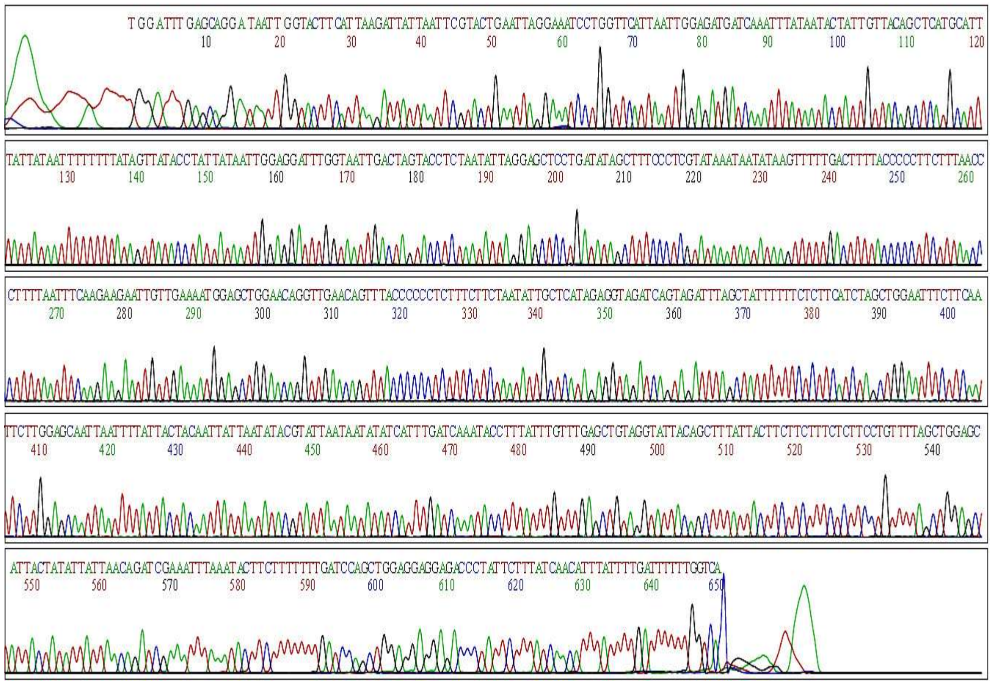

Fig. 10. The sequencing chromatogram of forward DNA strand of *A. aristolochiae* CUJ2.

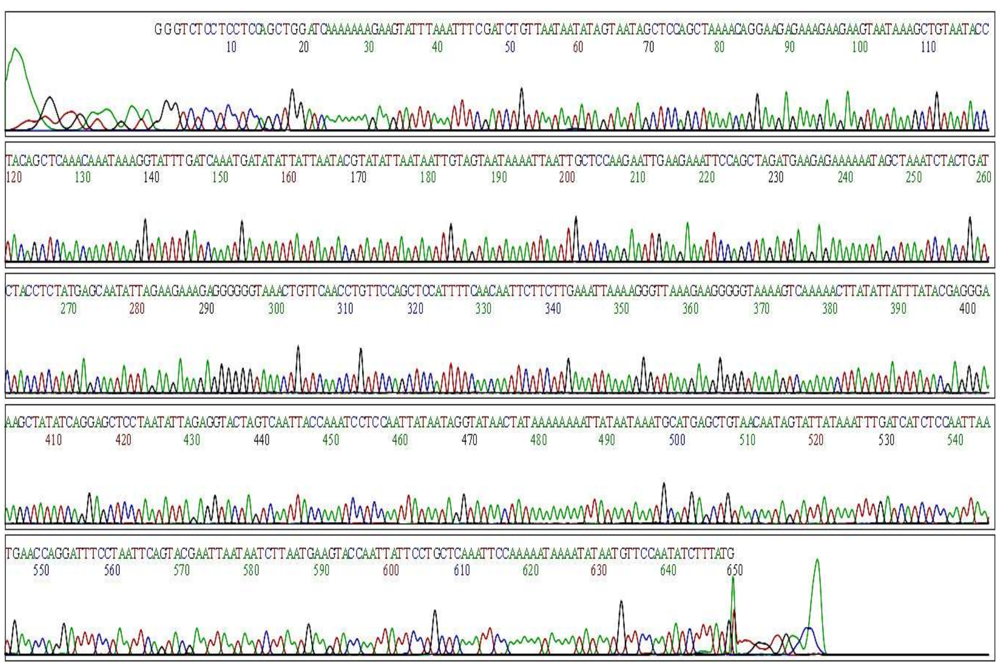

Fig. 11. The sequencing chromatogram of reverse DNA strand of *A. aristolochiae* CUJ2.

The 658 bases sequence isolated from CUJ2 deposited in GenBank with accession number HQ424202 was represented in Fig. 12.

```
>gi|312451706|gb|HQ424202.1| Atrophaneura aristolochiae
voucher CUJ2 cytochrome oxidase subunit I gene, partial cds;
mitochondrial

AACATTATATTTTATTTTTGGAATTTGAGCAGGAATAATTGGTACTTCATTAAGATTATTA
ATTCGTACTGAATTAGGAAATCCTGGTTCATTAATTGGAGATGATCAAATTTATAATACTA
TTGTTACAGCTCATGCATTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGGAGG
ATTTGGTAATTGACTAGTACCTCTAATATTAGGAGCTCCTGATATAGCTTTCCCTCGTATA
AATAATATAAGTTTTTGACTTTTACCCCCTTCTTTAACCCTTTTAATTTCAAGAAGAATTG
TTGAAAATGGAGCTGGAACAGGTTGAACAGTTTATCCCCCTCTTTCTTCTAATATTGCTCA
TAGAGGTAGATCAGTAGATTTAGCTATTTTTTCTCTTCATATAGCTGGAATTTCTTCAATT
CTTGGAGCAATTAATTTTATTACTACAATTATTAATATACGTATTAATAATATATCATTTG
ATCAAATACCTTTATTTGTTTGAGCTGTAGGTATTACAGCTTTATTACTTCTTCTTTCTCT
TCCTGTTTTAGCTGGAGCTATTACTATATTATTAACAGATCGAAATTTAAATACTTCTTTT
TTTGATCCAGCTGGAGGAGGAGACCCAATTCTTTATCAACATTTATTT
```

Fig. 12. The partial sequence of mitochondrial COI gene of *A. aristolochiae* CUJ2.

The conceptual translation of the consensus sequence of CUJ2 gave the following peptide sequence in Fig. 13.

```
>seq7Atrophaneura aristolochiae CUJ2

TLYFIFGIWAGMIGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSSIVENGAGTGWTVYPPLSSNIAH
SGSSVDLAIFSLHMAGISSILGAINFITTIINMRINNMSFDQMPLFVWAVGITALLLLLSL
PVLAGAITMLLTDRNLNTSFFDPAGGGDPILYQHLF
```

Fig. 13 The peptide sequence obtained from conceptual translation of consensus sequence of *A. aristolochiae* CUJ2.

The nucleotide BLAST was conducted with the consensus sequence derived from *A. aristolochiae* CUJ2, the Table 6 below represent the BLAST hit table derived from it.

S.I. No.	Subject Ids	% identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score

1	*gi|312451706|gb|HQ424202.1|Atrop haneura aristolochiae voucher CUJ2*	100.00	658	0	0	1	658	1	658	1216
2	*gi|584609665|gb|KJ195272.1|Pachli opta aristolochiae isolate F177*	99.83	596	1	0	35	630	1	596	1096
3	*gi|545690921|gb|KF226555.1|Pachli opta aristolochiae asteris voucher UMKL-JJW0033*	99.70	658	2	0	1	658	1	658	1205
4	*gi|584609703|gb|KJ195291.1|Pachli opta aristolochiae isolate T75*	99.66	596	2	0	35	630	1	596	1090
5	*gi|584609655|gb|KJ195267.1|Pachli opta aristolochiae isolate F317*	99.66	596	2	0	35	630	1	596	1090
6	*gi|584609635|gb|KJ195257.1|Pachli opta aristolochiae isolate F520*	99.66	593	2	0	38	630	1	593	1085
7	*gi|545690919|gb|KF226554.1|Pachli opta aristolochiae asteris voucher UMKL-JJW0035*	99.54	658	3	0	1	658	1	658	1199
8	*gi|321121995|gb|HQ962285.1|Pachl iopta aristolochiae asteris voucher YB-KHC6901*	99.54	658	3	0	1	658	1	658	1199
9	*gi|584609643|gb|KJ195261.1|Pachli opta aristolochiae isolate A57*	99.50	596	3	0	35	630	1	596	1085
10	*gi|197359542|gb|EU792486.1|Atrop haneura aristolochiae isolate SEC11CR05*	99.23	650	5	0	9	658	3	652	1173
11	*gi|564657734|gb|JF747536.1|Pachli opta aristolochiae voucher FD16*	99.06	636	6	0	23	658	3	638	1142
12	*gi|302201972|gb|HM246472.1|Pachl iopta aristolochiae adaeus*	98.93	652	5	2	8	658	653	3	1164
13	*gi|529386805|gb|KF405037.1|Atrop haneura polydorus voucher 11ANIC-07880*	96.91	550	17	0	1	550	1	550	922
14	*gi|529367643|gb|KF395456.1|Atrop haneura polydorus voucher 11ANIC-07881*	96.55	550	19	0	1	550	1	550	911
15	*gi|961438789|gb|KT879883.1|Pachli opta hector voucher RAPachec-37*	96.35	658	24	0	1	658	1	658	1083

Table 6. The BLAST hit table of *A. aristolochiae* CUJ2.

A. aristolochiae CUJ2 used in the present study was showed 99.83% sequence similarity to that of *P. aristolochiae* F177 (KJ195272) from Pune. While the similarity was 99.70%with KF226555 from Malaysia, the sequence similarity with KJ195291, KJ195267 and KJ195257 from Pune was 99.66%. However other samples of *P. aristolochiae asteris* voucher UMKL-JJW0035 (KF226554) from Malaysia were showed 99.54% similarity (Table. 6). The *A. aristolochiae* CUJ2

(HQ424202) from Alakode of Kannur District, Kerala has 99.54% identity with *A. aristolochiae* CUJ9 from Cheemeni, Kasaragod District, of Kerala.

The sequences with top similarities were identified from the BLAST and were used with the sequence of CUJ2 for phylogenetic analysis in MEGA6, the NJ-trees derived were represented below (Fig. 14).

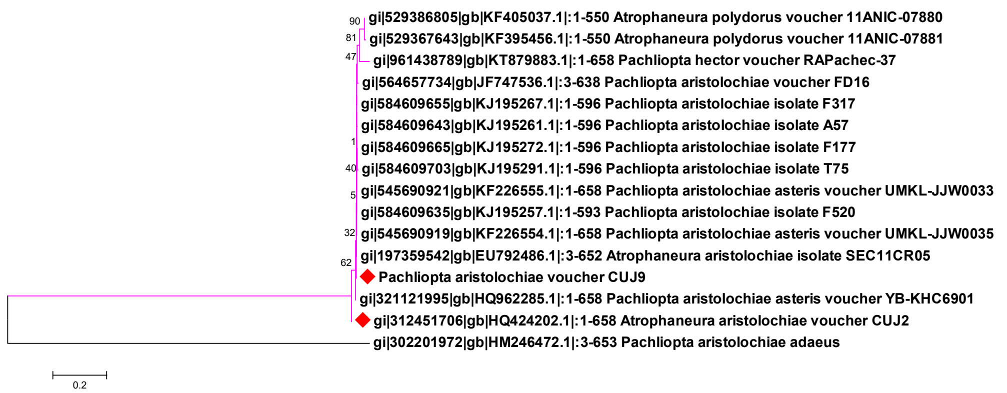

Fig. 14. The NJ-tree with Phylogenetic relationships of *A. aristolochiae* isolates CUJ2.

The phylogeny of *A. aristolochiae* isolates CUJ2 was revealed with NJ-tree developed using the related sequences from database (Fig. 14). The *A. aristolochiae* CUJ2 was placed in a separate clade with *A. aristolochiae* (KF226554) from Malaysia and *A. aristolochiae* (KJ195267, KJ195257) from Pune in the adjacent clade.

The polymorphic changes in the nucleotide positions through base substitution mutations in *A. aristolochiae* isolates CUJ9 and CUJ2 were identified with multiple sequence alignments generated with ClustalW in MEGA6.

HQ424202,CUJ2	1826	T	T	T	A	T	C	C	C	C	C	T	C	T	T	T	C	T	T	C	T	A	A	T	A	T
KJ195272		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KF226555		.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KJ195291		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KJ195267		.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CUJ9		.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HQ424202,CUJ2	1874	A	T	T	T	A	G	C	T	A	T	T	T	T	T	T	C	T	C	T	T	C	A	T	A	T
KJ195272		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
KF226555		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
KJ195291		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
KJ195267		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
CUJ9		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.

Fig. 15. The multiple sequence alignments showing nucleotide polymorphism.

CUJ2	102	I	V	E	N	G	A	G	T	G	W	T	V	Y	P	P	L	S	S	N	I	A	H	S	G	S	S	V	D	L	A	I	F	S	L	H	M	A	G	141
CUJ9		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	

Fig. 16. The peptide sequence alignment showing an amino acid substitution in the peptide of *A. aristolochiae* CUJ2 (HQ424202) from Alakode of Kannur District, Kerala, in which Methionine substitute Leucine due to C to A transversion, which occur at high constraint 1st position of codon.

Discussion

The variations found in BLAST results (table 6) were clearly reflected in the NJ-tree (Fig. 14) developed from the sequences obtained from the database. The *A. aristolochiae* CUJ2 (HQ424202) from Alakode of Kannur District, Kerala ($12^0$12’22”N, $75^0$27’38”E) has 99.54% identity with *A. aristolochiae* isolate CUJ9 from Cheemeni, Kasaragod District, of Kerala ($12^0$13’59”N, $75^0$14’52”E). The sequence of *A. aristolochiae* CUJ2 (HQ424202) was distinct in nucleotide sequence from that of CUJ9 hence it was a novel sequence. It has more similarity with *A. aristolochiae* (KF226554) from Malaysia and samples (KJ195267, KJ195257) from Pune. This was an instance of nucleotide polymorphism the mutations involved were studied with multiple sequence alignments using ClustalW (Fig. 15), two single nucleotide substitutions were involved, one with C to T transition at 1831th nucleotide of mitochondrial genome. Another with C to A transversion at 1898th nucleotide of the mitochondrial genome. The same species KJ195272 and KJ195291 from Pune were the samples from database with same mutation at 1831th nucleotide. The substitution at 1898th nucleotide of CUJ2 makes it a unique genotype. The T to C mutation is at low constraint sites and causes no peptide change but the C to A mutation is at high constraint first codon position and therefore leads to peptide change. The NJ-tree distance data revealed that the species diverged from their closely related species *A. alcinous* about 80,000 years ago. The *P. hector* was diverged from *P. aristolochiae* after 40,000 years of their evolution. The sequences isolated from samples of Malaysia (KF226554) and Thailand (HQ962285) was more similar to Indian samples than samples from P. R. China (JF747536). This suggests the distribution of ancestors of *A. aristolochiae* of Western Ghats from South East Asia. This finding supports the view of common origin and distribution of the species in South East Asia (Condamine, 2013). In the phylogenetic tree, the more closely related sequence of *A. aristolochiae* was from Pune belonging to Western Ghats with close similarity, which at the same time showed polymorphism of sequences even in the same geographical area. The distance data of the tree also revealed a recent diversification of the genus in Western Ghats about 80,000 years ago.

3. 3. *Atrophaneura aristolochiae* (Fabricius, 1775) isolate CUJ9 (KT880645)

The specimen CUJ9 was identified as *Atrophaneura aristolochiae* (Fabricius, 1775) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio aristolochiae* Fabricius, 1775

Pachliopta aristolochiae (Fabricius, 1775)

The *Atrophaneura aristolochiae* is the common rose butterfly, a red-bodied swallowtail butterfly found in India and South East Asia. They are most common in south Kerala during monsoon, pre monsoon and post monsoon periods. The upper side; velvety black, Fore wing; with well-marked pale adnervular streaks on the discal area that do not reach the terminal margin, the latter broadly velvety black; the streaks beyond end of cell extended inwards into its apex (Fig. 17). Hind wing; elongate white discal white markings in interspaces 2–5 beyond the cell (Fig. 17), curved series of sub terminal lunular markings in interspaces one to seven, dull crimson irrorated with black scales (Fig. 17), and the spot in interspace one large, diffuse, irregular margined interiorly with white. Antennae, thorax and abdomen; above black, the head, sides of prothorax; above, the whole thorax and abdomen beneath vermilion-red; anal segment vermilion-red (Fig. 17). The larval host plants include *Aristolochia indica* and *Aristolochia bracteolate.*

The PCR amplification of partial COI sequence of *A. aristolochiae* CUJ9 yielded a consensus of 609 bp. The chromatogram, DNA sequence obtained, its conceptual translation product, BLASTn hit table, and NJ-tree are presented below (Fig.19-23).

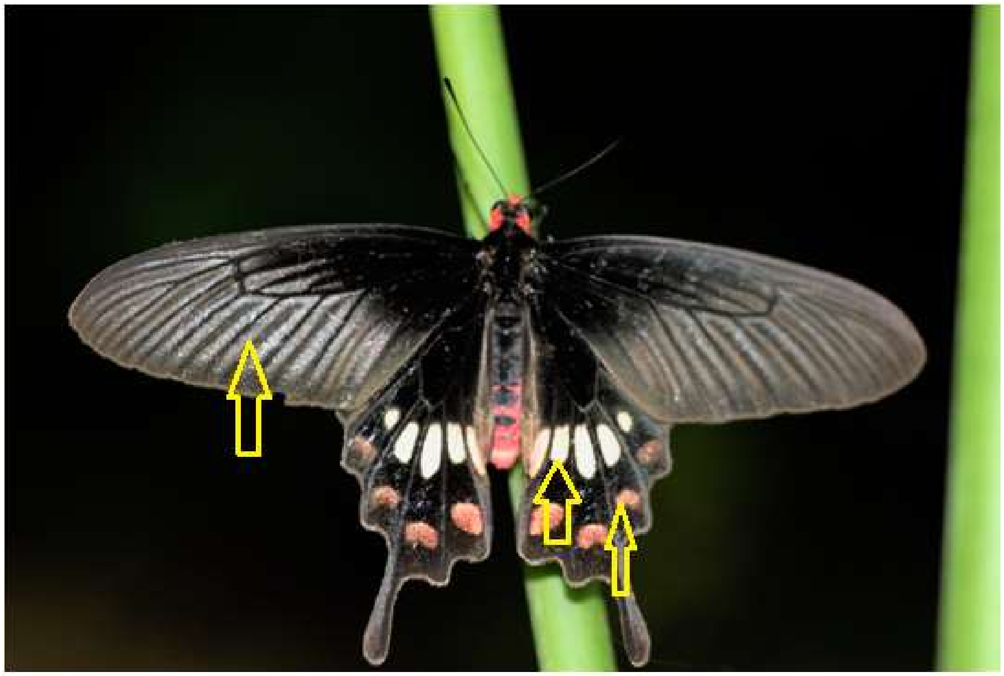

Fig. 17. The upper side of the *A. aristolochiae* CUJ9, the fore wings are velvety black with adnervular pale streaks, Hind wing with elongate white discal markings in interspaces 2–5 beyond the cell, curved series of sub terminal lunular markings in interspaces one to seven, dull crimson irrorated with black scales.

Fig. 18. The under side of the *A. aristolochiae* CUJ9

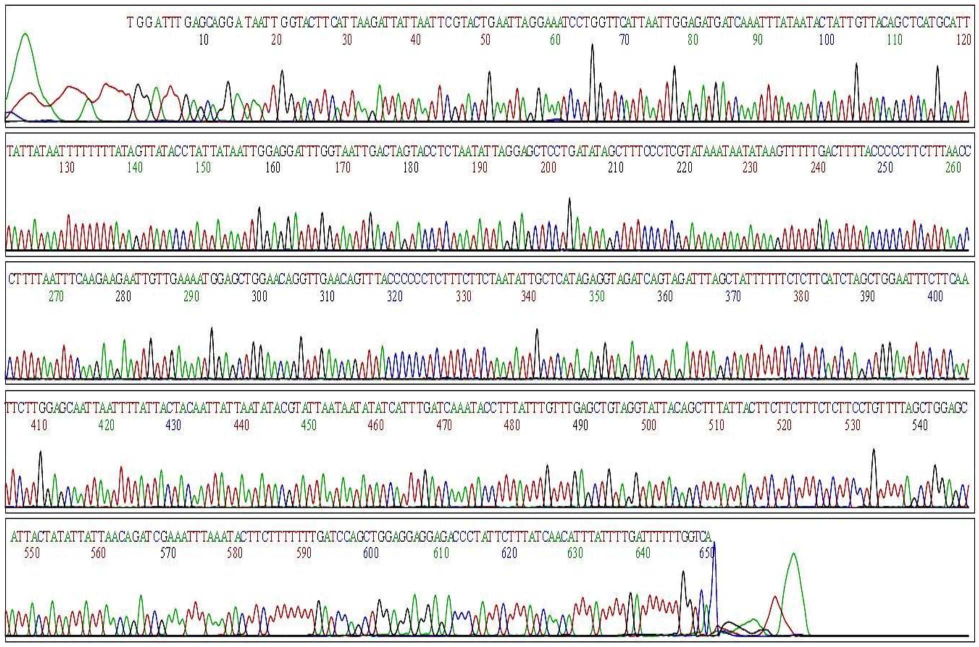

Fig. 19. The sequencing chromatogram of forward DNA strand of *A. aristolochiae* CUJ9

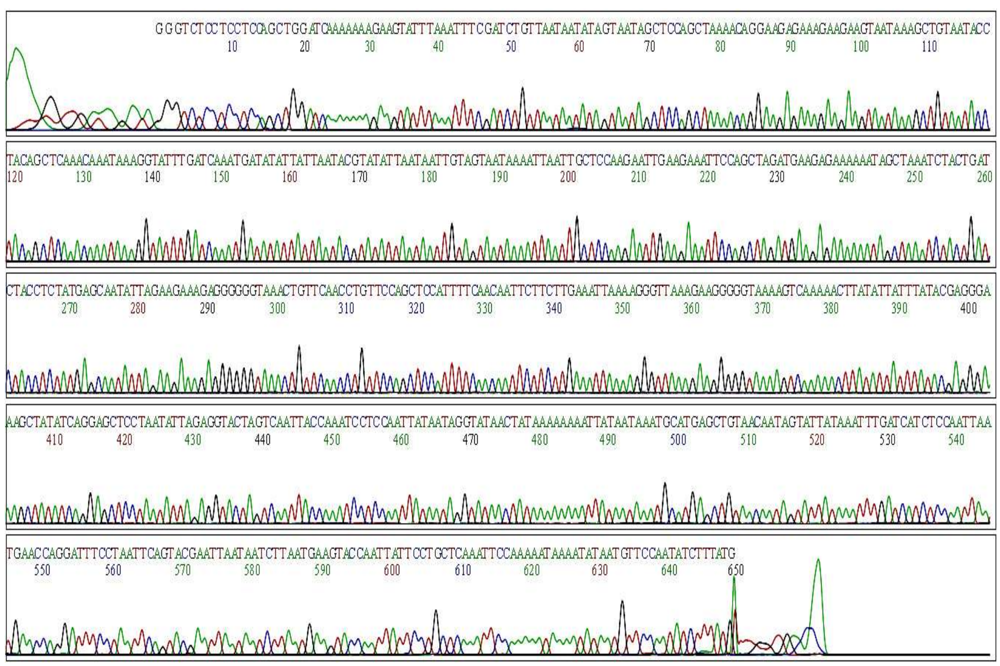

Fig. 20. The sequencing chromatogram of reverse DNA strand of *A. aristolochiae* CUJ9

```
>Pachliopta aristolochiae voucher CUJ9 cytochrome oxidase
subunit I gene, partial cds; mitochondrial (609)
AACATTATATTTTATTTTTGGAATTTGAGCAGGAATAATTGGTACTTCATTAAGATTATT
AATTCGTACTGAATTAGGAAATCCTGGTTCATTAATTGGAGATGATCAAATTTATAATAC
TATTGTTACAGCTCATGCATTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGG
AGGATTTGGTAATTGACTAGTACCTCTAATATTAGGAGCTCCTGATATAGCTTTCCCTCG
TATAAATAATATAAGTTTTTGACTTTTACCCCCTTCTTTAACCCTTTTAATTTCAAGAAG
AATTGTTGAAAATGGAGCTGGAACAGGTTGAACAGTTTACCCCCCTCTTTCTTCTAATAT
TGCTCATAGAGGTAGATCAGTAGATTTAGCTATTTTTTCTCTTCATCTAGCTGGAATTTC
TTCAATTCTTGGAGCAATTAATTTTATTACTACAATTATTAATATACGTATTAATAATAT
ATCATTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGTATTACAGCTTTATTACTTCT
TCTTTCTCTTCCTGTTTTAGCTGGAGCTATTACTATATTATTAACAGATCGAAATTTAAA
TACTTCTTT
```

Fig. 21. The partial sequence of mitochondrial COI gene of *A. aristolochiae* CUJ9.

The conceptual translation of the consensus sequence of CUJ9 gave the following peptide sequence in Fig. 22.

>seq7*Atrophaneura aristolochiae* CUJ9

```
TLYFIFGIWAGMIGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSSIVENGAGTGWTVYPPLSSNIAH
SGSSVDLAIFSLHLAGISSILGAINFITTIINMRINNMSFDQMPLFVWAVGITALLLLLSL
PVLAGAITMLLTDRNLNTS
```

Fig. 22 The peptide sequence obtained from conceptual translation of consensus sequence of *A. aristolochiae* CUJ9

The nucleotide BLAST was conducted with the consensus sequence derived from *A. aristolochiae* CUJ9, the table7 below represent the BLAST hit table derived from the BLASTn with CUJ9.

S.I. No.	Subject Ids	% identity	Alignment length	Mismatches	Gap opens	q. start	Q. end	S. start	S. end	Bit score
1	*gi|545690919|gb|KF226554.1|Pachliopta aristolochiae asteris voucher UMKL-JJW0035*	100.00	658	0	0	1	658	1	658	1216
2	*gi|584609655|gb|KJ195267.1|Pachliopta aristolochiae isolate F317*	100.00	596	0	0	35	630	1	596	1101
3	*gi|584609635|gb|KJ195257.1|Pachliopta aristolochiae isolate F520*	100.00	593	0	0	38	630	1	593	1096
4	*gi|545690921|gb|KF226555.1|Pachliopta aristolochiae asteris voucher UMKL-JJW0033*	99.85	658	1	0	1	658	1	658	1210

5	*gi|321121995|gb|HQ962285.1|Pachliopta aristolochiae asteris voucher YB-KHC6901*	99.85	658	1	0	1	658	1	658	1210
6	*gi|584609665|gb|KJ195272.1|Pachliopta aristolochiae isolate F177*	99.83	596	1	0	35	630	1	596	1096
7	*gi|584609643|gb|KJ195261.1|Pachliopta aristolochiae isolate A57*	99.83	596	1	0	35	630	1	596	1096
8	*gi|197359542|gb|EU792486.1|Atrophaneura aristolochiae isolate SEC11CR05*	99.69	650	2	0	9	658	3	652	1190
9	*gi|584609703|gb|KJ195291.1|Pachliopta aristolochiae isolate T75*	99.66	596	2	0	35	630	1	596	1090
10	*gi|312451706|gb|HQ424202.1|Atrophaneura aristolochiae voucher CUJ2*	99.54	658	3	0	1	658	1	658	1199
11	*gi|564657734|gb|JF747536.1|Pachliopta aristolochiae voucher FD16*	99.21	636	5	0	23	658	3	638	1147
12	*gi|302201972|gb|HM246472.1|Pachliopta aristolochiae adaeus*	99.08	652	4	2	8	658	653	3	1170
13	*gi|529386805|gb|KF405037.1|Atrophaneura polydorus voucher 11ANIC-07880*	97.09	550	16	0	1	550	1	550	928
14	*gi|396576492|gb|JX261944.1|Pachliopta hector voucher BUZOOUGC-Ph*	96.47	623	21	1	11	633	7	628	1027
15	*gi|572169453|gb|KF733790.1|Graphium nomius*	96.35	658	24	0	1	658	1	658	1083
16	*gi|4321340|gb|AF044023.1|Pachliopta neptunus cytochrome oxidase subunit*	94.38	658	37	0	1	658	56	713	1011
17	*gi|584609631|gb|KJ195255.1|Atrophaneura alcinous isolate GRTWM*	93.79	596	37	0	35	630	1	596	898
18	*gi|284020664|gb|GU164019.1| Parides eurimedes mylotes*	91.89	641	50	2	1	640	1	640	894

Table 7. The BLAST hit table of *A. aristolochiae* (Fabricius, 1775) CUJ 9.

The consensus sequence of *A. aristolochiae* CUJ9, BLAST against database and showed 100% similarity to that of *A. aristolochiae* KF226554 from Malaysia, KJ195267 and KJ195257 from Pune, India. While the similarity was 99.85% with KF226555 from Malaysia, and KJ195272 from Pune. However, it showed 99.54% similarity with CUJ2 HQ424202 collected from Alakode of Kannur District, Kerala (table 7).

The sequences with top similarities were identified from the BLAST and the sequences were used with the CUJ9 for phylogenetic analysis in MEGA6, the NJ-trees derived were represented below (Fig. 23).

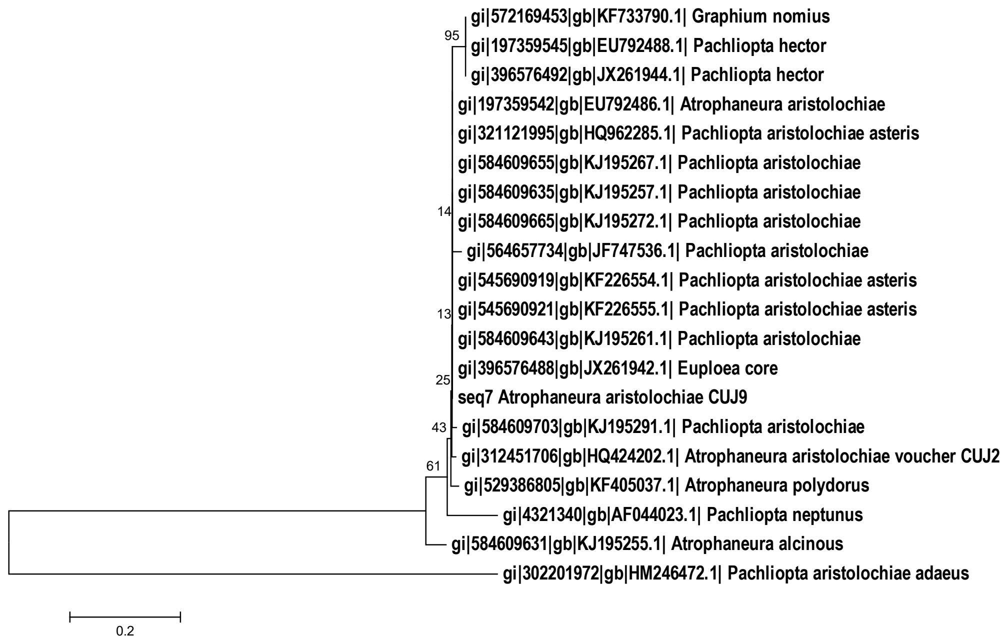

Fig. 23. The NJ-tree with Phylogenetic relationships of *A. aristolochiae* CUJ9.

The phylogeny of *A. aristolochiae* isolates CUJ2 and CUJ9 were derived with NJ-tree developed from the sequences in database with BLAST hit results along with CUJ9 (Fig. 23). The *A. aristolochiae isolate* CUJ9 was found placed at the same clade with *A. aristolochiae* KF226554 from Malaysia, KJ195267 and KJ195257 from Pune, India.

Discussion

The sequence from *A. aristolochiae* CUJ9 showed 100% similarity with that of *A. aristolochiae* KF226554 from Malaysia, KJ195267 and KJ195257 from Pune, India. Hence, the sequence is a molecular barcode for species identification. The *A. aristolochiae* CUJ2 (HQ424202) from Alakode of Kannur District (12^{0}12’22”N, 75^{0}27’38”E), has 99.54% identity with *A. aristolochiae* isolate CUJ9 from

Cheemeni, Kasaragod District (12^{0}13'59"N, 75^{0}14'52"E), of Kerala. This was an instance of nucleotide polymorphism the mutations involved were studied with multiple sequence alignments using ClustalW, (Fig.23). The NJ-tree distance data revealed that the species was diverged from their closely related species *Atrophaneura alcinous* about 80,000 years ago. The *Pachliopta hector* had diverged from *Pachliopta aristolochiae* 40,000 years after their evolution. The sequences isolated from samples of Malaysia (KF226554) and Thailand (HQ962285) had more similarity with Indian samples than samples from Peoples Republic of China (JF747536) suggesting the affinity of ancestors of *A. aristolochiae* of Western Ghats to South East Asia. This finding supports the view of common origin and distribution of the species in South East Asia (Condamine, 2013). In the Phylogenetic tree the more closely related sequence of *A. aristolochiae* was from Pune belonging to Western Ghats with 100%, similarity at the same time the population showed polymorphism of sequences even in the same geographical area. The distance data of the tree also revealed a recent diversification of the genus in Western Ghats about 80,000 years ago.

3.4. ***Graphium agamemnon*** **(Linnaeus, 1758) isolate CUJ3 (KT880639)**

The specimen CUJ3 is identified as *Graphium agamemnon* (Linnaeus, 1758) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonym: *Papilio agamemnon* (Linnaeus, 1758).

Graphium agamemnon (Linnaeus, 1758) commonly called as Green-spotted Triangle Tailed Jay, found common in Kerala (Fig. 24).

Upper side; black, fore wing with the following green markings; a spot at the extreme base of costal margin, a transverse short line near base of cell and seven spots beyond, two and two except apical spot which is single (Fig. 25). Two spots beyond apex of cell; a spot at base of interspaces 1a and 1, followed by two oblique short macular bands (Fig. 25). A discal series of spots decreasing in size towards the costa (Fig. 25).

Hind wing: three series of green-coloured markings that move transversely across the wing more or less parallel to the dorsal margin, the upper markings in interspace 7, white; a short greenish stripe at the extreme base of the wing (Fig. 25). Lover side: brownish-black, more or less submersed with pink along - costal margin, on apical area and along the outer margin of the discal markings on the fore wing, broadly along the upper and terminal margins and at base on interspaces 6 and 7 (Fig. 25). It is a common swallowtail present in North Kerala; it has a rapid flipping of wings during flight and seen hovering over flowers for nectar feeding and also found

Fig. 24. The *G. agamemnon* CUJ3

Fig. 25 The upper side of the *G. agamemnon* CUJ3 after spreading wings. The identification characters involve seven green spots in a line along the middle of fore wing from base to apex. Oblique short wavy muscular green stripes near the base of wings

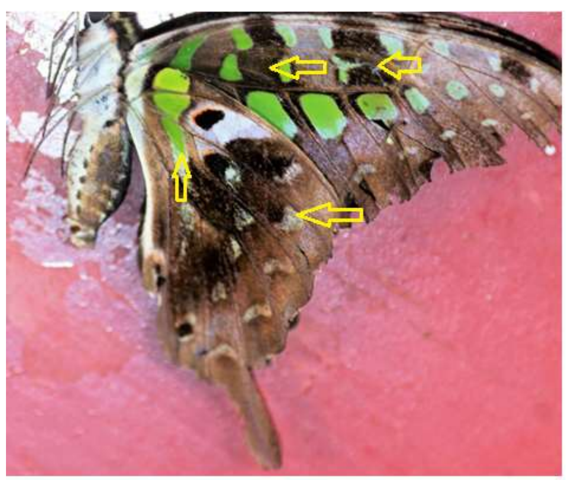

Fig. 26. The lower side of the wings of *G. agamemnon* CUJ3. Lower side of wings were brownish black green spots and pinkish linules along margins

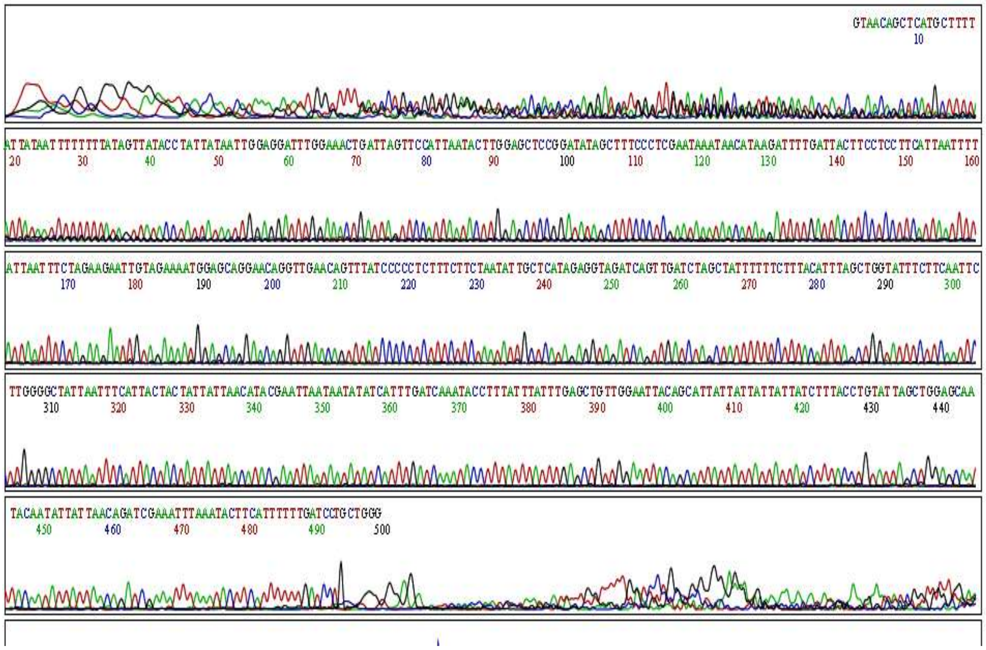

Fig. 27. The sequencing chromatogram of forward DNA strand of *G. agamemnon* CUJ3.

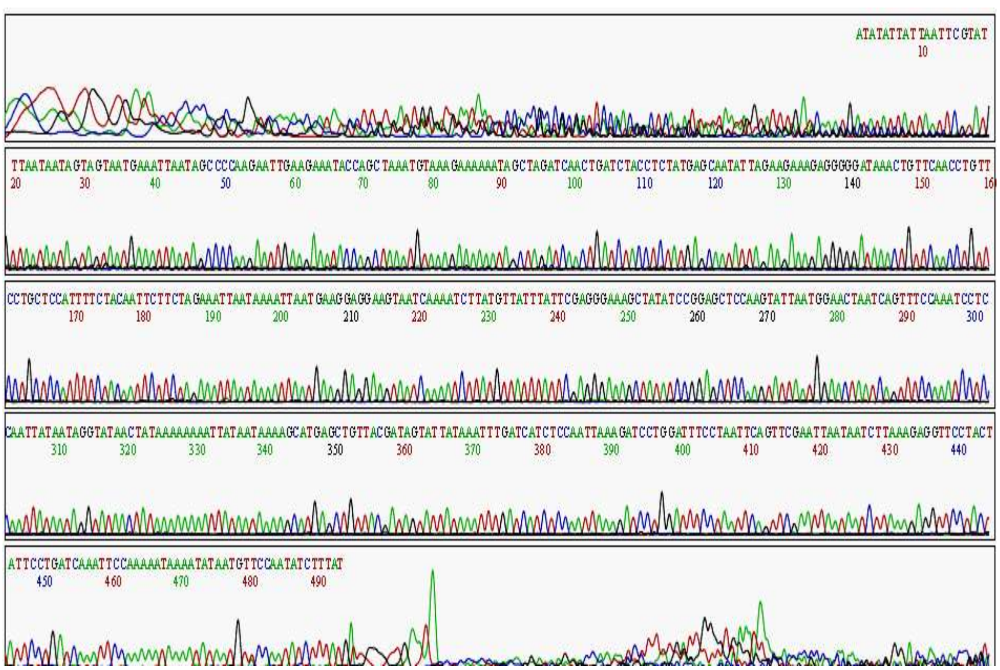

Fig. 28 The sequencing chromatogram of reverse DNA strand of *G. agamemnon* CUJ3.

performing mudpudling on moist sand of river banks at hot sunny days. The larval food plants involve *Anon reticulate, A. squamosal, Michelin champak*, *Polyalthia longifolia*, *Uvaria narum.*

The PCR amplification of partial COI sequence of *G. agamemnon* CUJ3 from North Kerala, India yielded a consensus with 609 bp. The chromatogram, DNA sequence obtained, its conceptual translation product, BLASTn hit table, and NJ-tree are presented in (Fig. 27 to 31).

```
>Graphium agamemnon voucher CUJ3 cytochrome oxidase subunit I
gene, partial cds; mitochondrial, (609bp)
AACATTATATTTTATTTTTGGAATTTGATCAGGAATAGTAGGAACCTCTTTAAGATTATT
AATTCGAACTGAATTAGGAAATCCAGGATCTTTAATTGGAGATGATCAAATTTATAATAC
TATCGTAACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGG
AGGATTTGGAAACTGATTAGTTCCATTAATACTTGGAGCTCCGGATATAGCTTTCCCTCG
AATAAATAACATAAGATTTTGATTACTTCCTCCTTCATTAATTTTATTAATTTCTAGAAG
AATTGTAGAAAATGGAGCAGGAACAGGTTGAACAGTTTATCCCCCTCTTTCTTCTAATAT
TGCTCATAGAGGTAGATCAGTTGATCTAGCTATTTTTTCTTTACATTTAGCTGGTATTTC
TTCAATTCTTGGGGCTATTAATTTCATTACTACTATTATTAACATACGAATTAATAATAT
ATCATTTGATCAAATACCTTTATTTATTTGAGCTGTTGGAATTACAGCATTATTATTATT
ATTATCTTTACCTGTATTAGCTGGAGCAATTACAATATTATTAACAGATCGAAATTTAAA
TACTTCATT
```

Fig. 29. The partial sequence of mitochondrial COI gene of *G. agamemnon* CUJ3.

The conceptual translation of the consensus sequence of CUJ3 gave the following peptide sequence in Fig. 30.

```
>seq1Graphiumagamemnon
CUJ3TLYFIFGIWSGMVGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMPI
MIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLILLISSSIVENGAGTGWTVYPPLSS
NIAHSGSSVDLAIFSLHLAGISSILGAINFITTIINMRINNMSFDQMPLFIWAVGITALLL
LLSLPVLAGAITMLLTDRNLNTS
```

Fig. 30. The peptide sequence obtained from conceptual translation of consensus sequence of *G. agamemnon* CUJ3.

The nucleotide BLAST was conducted with the consensus sequence derived from *G. agamemnon* CUJ3, the (Table. 8) below represent the BLAST hit table derived from the BLASTn with CUJ3 sequence.

S. I. No.	Subject Ids	% identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|584609661|gb|KJ195270.1|Graphium agamemnon isolate F174*	100.00	575	0	0	35	609	1	575	1062
2	*gi|525346357|gb|KC970100.1|Graphium agamemnon agamemnon voucher KC0001*	97.54	609	15	0	1	609	1	609	1042
3	*gi|302201960|gb|HM246466.1|Graphium agamemnon*	97.37	609	16	0	1	609	660	52	1037
4	*gi|9754749|gb|AF170874.1|Graphium agamemnon*	97.37	609	16	0	1	609	48	656	1037
5	*gi|564657713|gb|JF747522.1|Graphium agamemnon voucher FD1*	97.04	609	18	0	1	609	24	632	1026
6	*gi|584609697|gb|KJ195288.1|Graphium agamemnon isolate T58*	97.04	575	17	0	35	609	1	575	968
7	*gi|529369807|gb|KF396538.1|Graphium macfarlanei voucher 11ANIC-07846*	95.24	609	29	0	1	609	1	609	965
8	*gi|529373113|gb|KF398191.1|Graphium macleayanus voucher 11ANIC-07837*	92.55	604	43	2	1	603	1	603	865
9	*gi|284019876|gb|GU162809.1|Ministrymon phrutus*	91.95	609	49	0	1	609	1	609	854
10	*gi|525346429|gb|KC970136.1|Lamproptera meges virescens voucher UMKL-JJW0386*	91.78	608	50	0	2	609	2	609	846

Table 8. The BLAST hit table of *G. agamemnon* CUJ3.

The consensus sequence of CUJ3 BLAST against the database showed 100% similarity to that of *G. agamemnon* KJ195270 from Pune (Table 8).

The sequences with top similarities were identified from the BLAST and the sequences were used with the *G. agamemnon* isolate CUJ3 for phylogenetic analysis in MEGA6, the NJ - trees derived were represented below (Fig. 31).

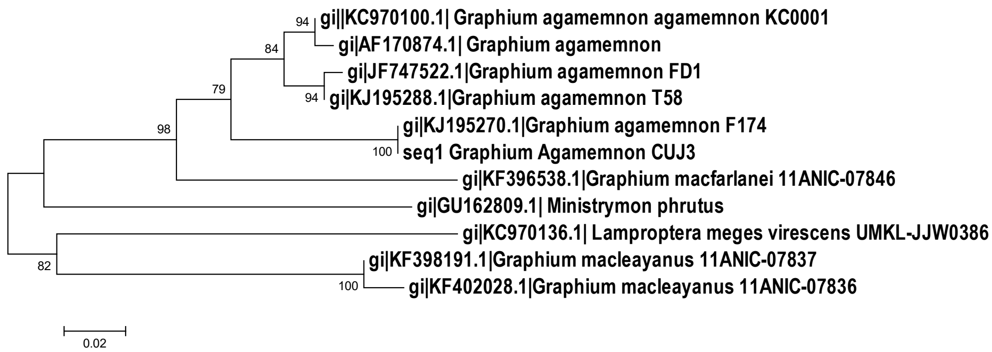

Fig. 31. The NJ-tree with Phylogenetic relationships of *G. agamemnon* CUJ3.

The phylogeny of CUJ3 was derived with NJ-tree developed from the top 10 related sequences along with CUJ3 in MEGA6 application (Fig. 31). The CUJ3 was found placed at the same clade with KJ195270 *G. agamemnon*. The *G. macfarlanei* (KF396538) from Australia with 95.24% identity was found in the adjacent clade more close to the species.

Discussion

The *G. agamemnon* CUJ3 BLAST results showed 100% similarity to that of *G. agamemnon* isolate KJ195270 from Pune. Therefor the COI sequence from CUJ3 can be useful as a molecular barcode for species identification. The NJ-tree distance data revealed that the species *G. agamemnon* diverged from their closely related *G. macfarlanei* KF396538 from Australia about 74,000 years ago and the CUJ3 was diverged about 56,000 years from *Graphium agamemnon* KJ195288 from Pune, India. The sequences isolated from samples of Malaysia, New Guinea, Philippines and Australia were found more closely related to the genus in the phylogenetic tree. This finding supports the view of common origin and distribution of the species in South East Asia (Condamine, 2013) including Myanmar, India and

Sri Lanka. In the Phylogenetic tree the more closely related sequence of *G. agamemnon* KJ195270 was from Pune belonging to Western Ghats with 100% similarity with CUJ3. The distance data of the tree also revealed a recent diversification of the genus in Western Ghats about 56,000 years ago.

3.5. *Papilio polytes* Linnaeus, 1758 isolate CUJ4 (KT880640)

The specimen CUJ4 is identified as *Papilio polytes* Linnaeus, 1758 referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonym: *Papilio walkeri* Rothschild, 1895

The *Papilio polytes*, is the Common Mormon, which is a common swallowtail butterfly widely distributed across Asia. They are frequent visitors of flowers with long corolla tube the males commonly seen as engaged mudpudling on moist sand. This butterfly is known for the Batesian mimicry displayed by the two forms of its females, which mimic non palatable less predated red-bodied swallowtails, such as the common rose and the crimson rose.

Jet black butterfly with row of white spots along the middle part of hind wing; The male has single morph only, dark-coloured swallowtailed, The upper side of fore wing ; series of white spots decreasing in size towards the apex The upper side of hind wing; complete discal band of elongated white spots, marginal red lunules

The female of the Common Mormon is polymorphic, it has three forms or morphs, Cyrus, Stichius and Romulus forms, the CUJ4 was identified as a Romulus form (Figures 32 and 33). Romulus Form; This female form mimics the crimson rose and is common over its range. It is not such a close mimic as the previous form being duller than its model. It is easy to differentiate the mimics from models by the colour of their body the models are red-bodied and the mimics are black-bodied (Fig. 33).

Fig. 32. The upper side of the *Papilio polytes* CUJ4

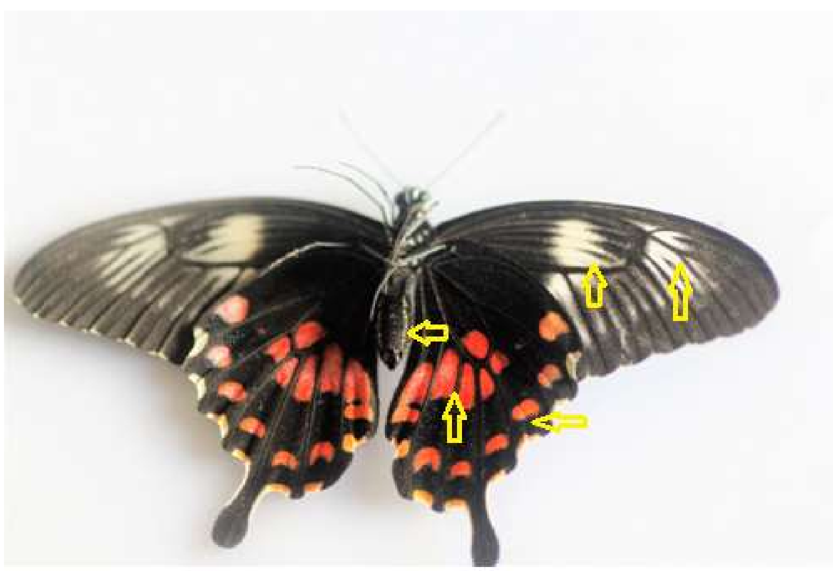

Fig. 33. The under side of the *P. polytes* CUJ4, 1. black, Fore wing with broad white interrupted band from the subcostal nervure opposite the origin of veins 10 and 11, extended obliquely to the tornus, and a second short pre-apical similar band; both bands composed of detached irregularly indented broad streaks in the interspaces, 2. Hind wing; with a discal posteriorly strongly curved series of seven crimson spots followed by a sub terminal series of crimson limulus, 3. Black coloured body.

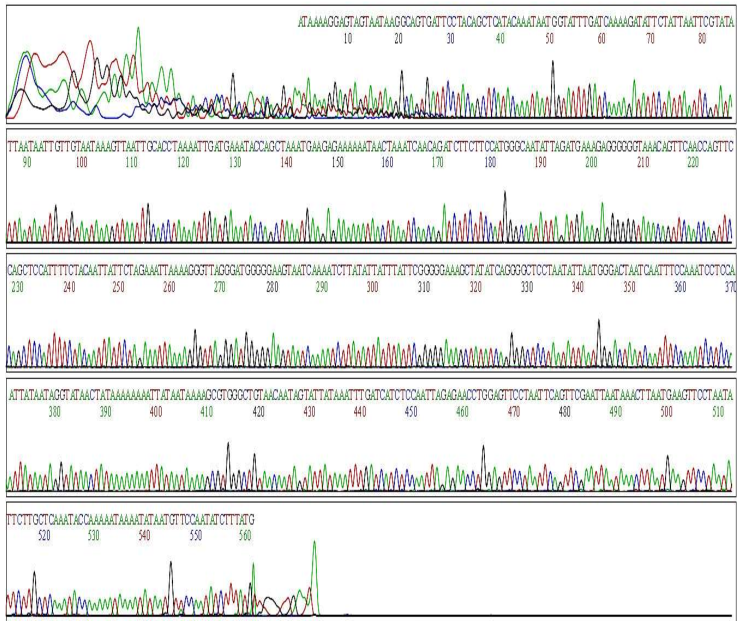

Fig. 34. The sequencing chromatogram of forward DNA strand of *P. polytes* CUJ4.

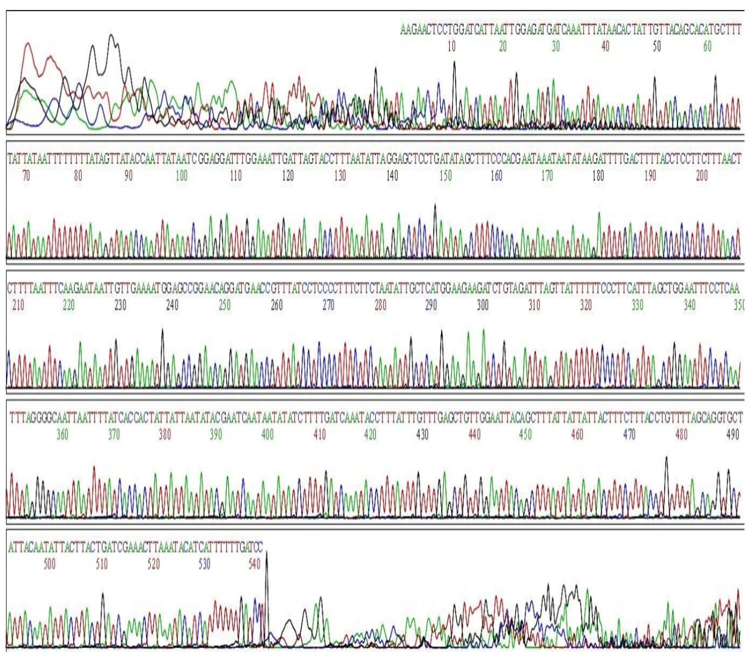

Fig. 35. The sequencing chromatogram of reverse DNA strand of *P. polytes* CUJ4.

The larval food plants involve plants *Rutaceae* family involving *Citrus spp.*, *Clausena lansium*, *Fortunella japonica*, *Glycosmis citricola*, *Zanthoxylum avicennae*, *Z. nitidum*, *Toddalia asiatica*, *Murraya koenegii*, *Aegle marmelos*, *Atalantia racemosa*, *Glycosmis arborea*, *Triphasia sp.*, *Zanthoxylum rbetsa*, the larvae are pale green in colour.

The PCR amplification of partial COI sequence of *P. polytes* CUJ4 yielded a consensus with 603 bp. The chromatogram, DNA sequence obtained, its conceptual translation product, BLASTn hit table, and NJ-tree are presented in (Fig. 34 to 38).

```
>seq2 Papilio polytes voucher CUJ4 cytochrome oxidase
subunit I gene, partial cds; mitochondrial, (603bp)
AACATTATATTTTATTTTTGGTATTTGAGCAAGAATATTAGGAACTTCATTAAGTTTATT
AATTCGAACTGAATTAGGAACTCCAGGTTCTCTAATTGGAGATGATCAAATTTATAATAC
TATTGTTACAGCCCACGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGG
AGGATTTGGAAATTGATTAGTCCCATTAATATTAGGAGCCCCTGATATAGCTTTCCCCCG
AATAAATAATATAAGATTTTGATTACTTCCCCCATCCCTAACCCTTTTAATTTCTAGAAT
AATTGTAGAAAATGGAGCTGGAACTGGTTGAACTGTTTACCCCCCTCTTTCATCTAATAT
TGCCCATGGAAGAAGATCTGTTGATTTAGTTATTTTTTCTCTTCATTTAGCTGGTATTTC
ATCAATTTTAGGTGCAATTAACTTTATTACAACAATTATTAATATACGAATTAATAGAAT
ATCTTTTGATCAAATACCATTATTTGTATGAGCTGTAGGAATCACTGCCTTATTACTACT
CCTTTCTTTACCTGTATTAGCAGGAGCTATTACTATATTATTAACAGATCGTAATT
TAAATAC
```

Fig. 36. The partial sequence of mitochondrial COI gene of *P. polytes* CUJ4.

The conceptual translation of the consensus sequence of CUJ4 gave the following peptide sequence in Fig. 37.

```
>seq2 Papilio polytes cuj4

TLYFIFGIWASMLGTSLSLLIRTELGTPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSMIVENGAGTGWTV
YPPLSSNIAHGSSSVDLVIFSLHLAGISSILGAINFITTIINMRINSMSFDQMPLF
VWAVGITALLLLLSLPVLAGAITMLLTDRNLNTS
```

Fig. 37. The peptide sequence obtained from conceptual translation of consensus sequence of *P. polytes* CUJ4.

The nucleotide BLAST was conducted with the consensus sequence derived from *P. polytes* CUJ4, the table 9 below represent the BLAST hit results.

S. I. No.	Subject Ids	% identity	Alignment	Mismatches	Gap o	Q. start	Q. end	S. start	S. end	Bit score

			leng th		**p e n s**					
1	*gi|658132119|dbj|AB969795.1|Papilio polytes*	100.00	609	0	0	1	609	1	609	1125
2	*gi|442535942|gb|KC158441.1|Papilio polytes*	100.00	609	0	0	1	609	1	609	1125
3	*gi|42405489|gb|AY457580.1|Papilio polytes*	100.00	609	0	0	1	609	49	657	1125
4	*gi|584609657|gb|KJ195268.1|Papilio polytes*	100.00	575	0	0	35	609	1	575	1062
5	*gi|62318323|dbj|AB192474.1|Papilio polytes*	100.00	555	0	0	55	609	1	555	1026
6	*gi|302201944|gb|HM246458.1|Papilio polytes*	99.84	609	1	0	1	609	660	52	1120
7	*gi|584609691|gb|KJ195285.1|Papilio polytes*	99.83	575	1	0	35	609	1	575	1057
8	*gi|584609653|gb|KJ195266.1|Papilio polytes*	99.83	575	1	0	35	609	1	575	1057
9	*gi|584609659|gb|KJ195269.1|Papilio polytes*	99.83	572	1	0	38	609	4	575	1051
10	*gi|654200397|gb|KJ636441.1|Papilio polytes*	99.82	550	1	0	60	609	1	550	1011
11	*gi|197359541|gb|EU792485.1|Papilio polytes*	99.67	601	1	1	9	609	1	600	1098
12	*gi|485475952|gb|KC810960.1|Papilio polytes*	99.51	609	3	0	1	609	12	620	1109
13	*gi|564657729|gb|JF747533.1|Papilio polytes*	99.34	609	4	0	1	609	1	609	1103
14	*gi|584609667|gb|KJ195273.1|Papilio polytes*	99.30	575	4	0	35	609	1	575	1040
15	*gi|681877193|gb|KM215138.1|Papilio polytes*	99.18	609	5	0	1	609	1484	2092	1098
16	*gi|442535944|gb|KC158442.1|Papilio polytes*	99.18	609	5	0	1	609	1	609	1098
17	*gi|584609687|gb|KJ195283.1|Papilio polytes*	99.13	575	5	0	35	609	1	575	1035
18	*gi|321121885|gb|HQ962230.1|Papilio polytes*	99.02	609	6	0	1	609	1	609	1092
19	*gi|545690957|gb|KF226573.1|Papilio polytes*	98.52	609	9	0	1	609	1	609	1075
20	*gi|672106604|gb|KM014701.1|Papilio polytes*	97.03	606	18	0	1	606	1490	2095	1020
21	*gi|302202014|gb|HM246453.1|Papilio memnon*	94.58	609	33	0	1	609	660	52	942
22	*gi|302201948|gb|HM246460.1|Papilio protenor*	94.58	609	33	0	1	609	660	52	942

Table 9. The BLAST hit table of *P. polytes* CUJ 4.

The consensus sequence of CUJ4 was BLAST against database showed

100% similarity to that of six samples among twenty of *P. polytes* sequences deposited in GenBank (table 9). Involving KT879884 from Bangalore, India, AB969795 from Japan, Okinawa, KC158441 from Pakistan, AY457580 from Malaysia, KJ195268 from India Pune and AB192474 from Japan, (table 9).

The sequences with top similarities were identified from the BLAST and the sequences were used with the *P. polytes* CUJ4 for phylogenetic analysis in MEGA6, the NJ- tree derived was represented in Fig. 38.

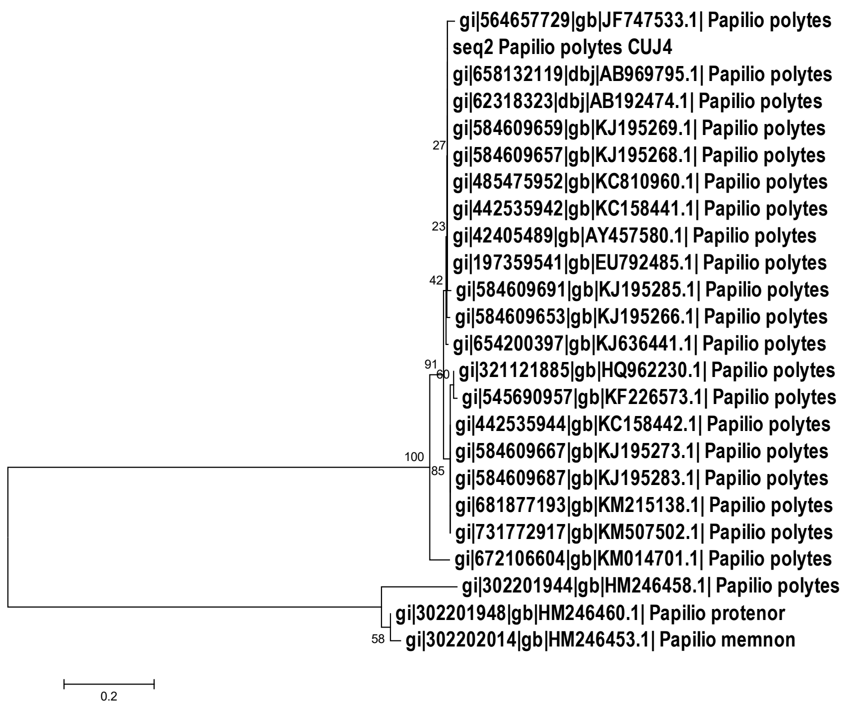

Fig. 38. The NJ-tree with Phylogenetic relationships of *P. polytes* CUJ4.

The phylogeny of CUJ4 was derived with NJ-tree developed from the top similar sequences along with CUJ4 in (Fig. 38). The CUJ4 was placed at the same clade with the above six *P. polytes* isolates. The *P. memnon* (HM246453) from P. R. China and *P. protenor* (HM246460) from P. R. China with 94.58% identities were found in the adjacent clade more close to the species.

Discussion

The BLAST results showed 100% similarity to that of six samples among twenty of *P. polytes* sequences deposited in GenBank (Table 9). Among them the two sequences of *P. polytes isolated* from Western Ghats were more closely related with CUJ4, hence it can use as a molecular barcode identifying *P. polytes*. The variations were clearly reflected in the NJ-tree developed from the sequences obtained from the database. The CUJ4 was found placed at the same clade with the six *P. polytes* isolates with 100% similarities. The *P. memnon* (HM246453) from P. R. China and *P. protenor* (HM246460) from P. R. China were the clsely related species in the adjacent clade. The NJ-tree distance data revealed that the species was diverged from their closely related species about 1,50,000 years ago. The sequences isolated from samples of Malaysia, Pakistan, Japan and India were found closer in the genus. This findings supports the common origin and distribution of the species in South East Asia (Condamine, 2013) including India, Japan, Malaysia and Pakistan. In the Phylogenetic tree the two sequences of *P. polytes* isolated from Western Ghats were more closely related with 100% similarity with CUJ4. The distance data of the tree also revealed a recent diversification of the genus in South East Asia about 1,50,000 years ago.

3.6 *Papilio polymnestor* (Cramer, 1775) isolate CUJ5 (KT8806341)

The specimen CUJ5 is identified as *Papilio polymnestor* (Cramer, 1775) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonym: *Menelaides polymnestor*

Papilio polymnestor is the blue mormon butterfly, common in South India and Sri Lanka and is endemic to the region.

Upper side of fore wings; rich velvety black, post discal band composed of internervular broad blue streaks gradually tapering and obsolescent anteriorly, not extended beyond interspace 6, (Fig. 39). Hind wing; terminal three-fourths beyond a line crossing the apical third of the cell pale blue, with superposed post discal, sub terminal and terminal series of black spots, post discal spots elongate, inwardly conical; the sub terminal oval, placed in the interspaces, the terminal irregular, placed along the apices of the veins and anteriorly coalescing more or less with the sub terminal spots (Fig 40).

The underside of fore wing; black, an elongate spot of dark red on the base of the cell; the post discal transverse series of streaks as on the upper side but grey tinged with ochraceous and extended up to costa. Hind wing; five irregular small patches of red at base, the outer three-fourths of the wing grey touched with ochraceous, but generally narrower than the blue on the upper side; the inner margin of the grey area crosses the wing beyond the cell; the post-discal and sub terminal black spots as on the upper side, inner margin crossing the wing well beyond the

Fig. 39. The upper side of the *P. polymnestor* CUJ5. 1. Upper side of fore wings; velvety black, post discal band composed of internervular broad blue streaks, 2. Hind wing; terminal three-fourths beyond a line crossing the apical third of the cell pale blue, 3. Sub terminal and terminal series of black spots, post discal spots elongate, inwardly conical; the sub terminal oval, placed in the inter spaces, the terminal irregular.

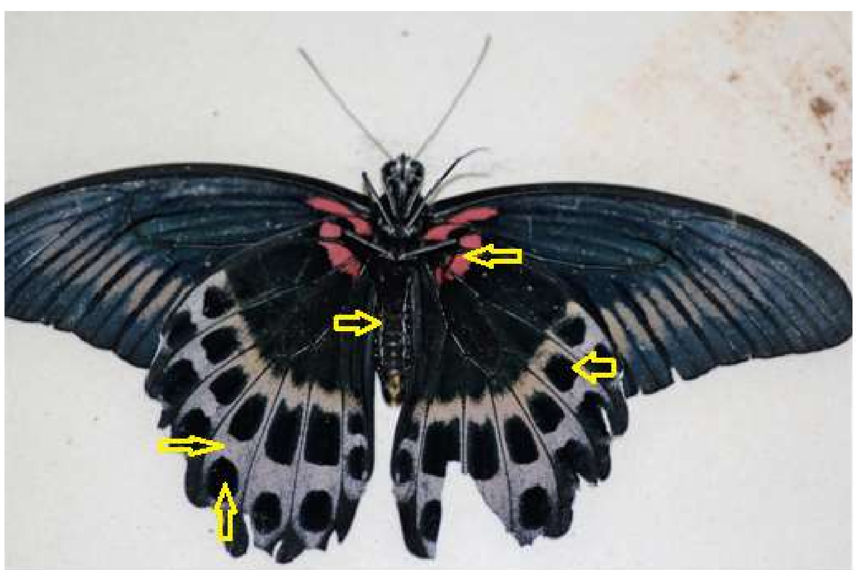

Fig. 40. The under side of the *P. polymnestor* CUJ5. 1. Under side of fore wing; black, an elongate spot of dark red on the base of the cell, 2. Antennae, head, thorax and abdomen blackish brown.

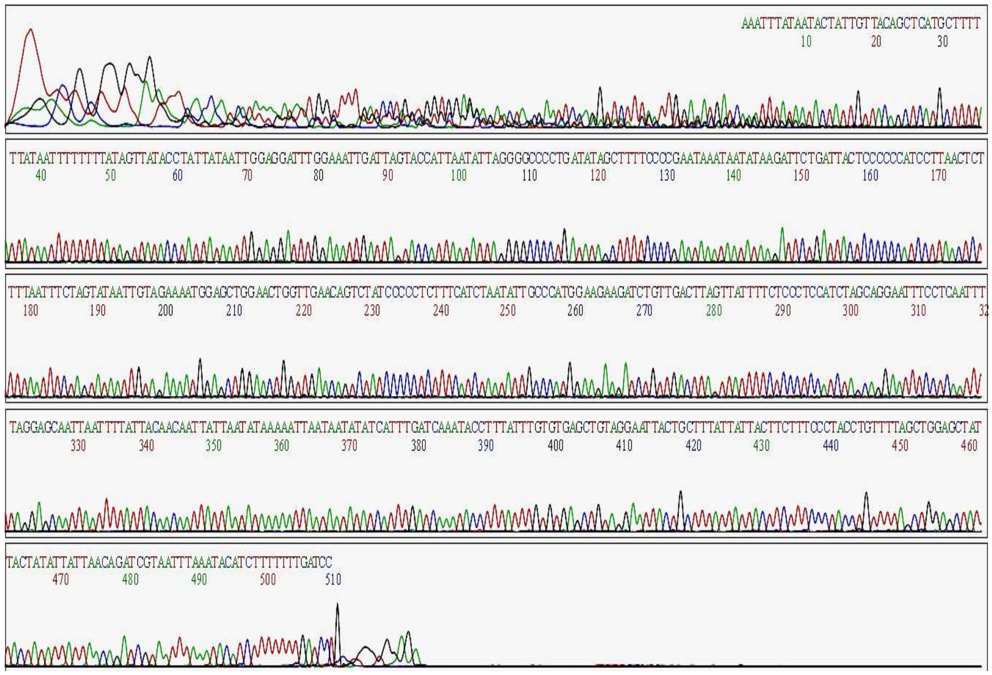

Fig. 41 The sequencing chromatogram of forward DNA strand of *P. polymnestor* CUJ5.

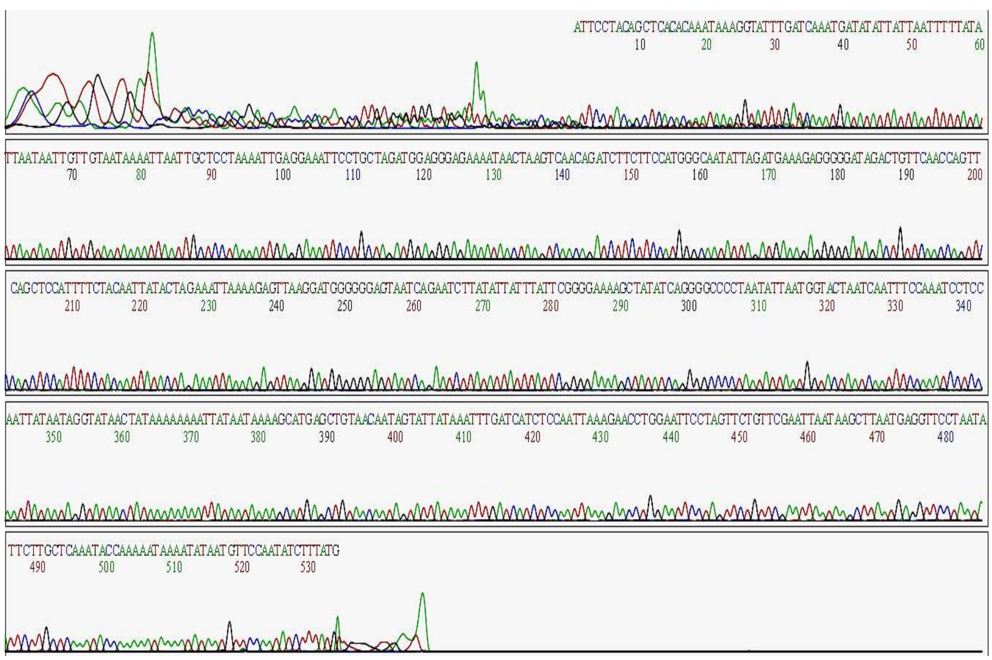

Fig. 42. The sequencing chromatogram of reverse DNA strand of *P. polymnestor* CUJ5.

apex of the cell; the sub terminal spots merged completely with the terminal spots and form a comparatively broad terminal black band; Antennae, head, thorax and abdomen blackish brown (Fig. 40).

Their larval food plants involve plants of *Rutaceae* like *Atalantia racemosa* and *Atalantia wightii· Glycosmis arborea*, *Paramigyna monophylla*, *Citrus grandis*, *Citrus limon* and other Citrus cultivars, the larvae of this butterfly is dull colored at early stages but becomes green colored few day after hatching.

The PCR amplification of partial COI sequence of *P polymnestor* CUJ5 from North Kerala, India yielded a consensus of 603bp. The chromatogram, DNA sequence obtained, its conceptual translation product, BLASTn hit table, and NJ-tree are presented (Fig. 41 to 45).

```
>Papilio polymnestor voucher CUJ5 cytochrome oxidase subunit
I gene, partial cds; mitochondrial (603bp)
AACATTATATTTTATTTTTGGTATTTGAGCAAGAATATTAGGAACCTCATTAAGCTTATT
AATTCGAACAGAACTAGGAATTCCAGGTTCTTTAATTGGAGATGATCAAATTTATAATAC
TATTGTTACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGG
AGGATTTGGAAATTGATTAGTACCATTAATATTAGGGGCCCCTGATATAGCTTTTCCCCG
AATAAATAATATAAGATTCTGATTACTCCCCCCATCCTTAACTCTTTTAATTTCTAGTAT
AATTGTAGAAAATGGAGCTGGAACTGGTTGAACAGTCTATCCCCCTCTTTCATCTAATAT
TGCCCATGGAAGAAGATCTGTTGACTTAGTTATTTTCTCCCTCCATCTAGCAGGAATTTC
CTCAATTTTAGGAGCAATTAATTTTATTACAACAATTATTAATATAAAAATTAATAATAT
ATCATTTGATCAAATACCTTTATTTGTGTGAGCTGTAGGAATTACTGCTTTATTATTACT
TCTTTCCCTACCTGTTTTAGCTGGAGCTATTACTATATTATTAACAGATCGTAATTTAAA
TAC
```

Fig. 43 The partial sequence of mitochondrial COI gene of *P. polymnestor* CUJ5.

The conceptual translation of the consensus sequence of CUJ5 gave the following peptide sequence in Fig. 44.

>seq3 *Papilio polymnstor* CUJ5

```
TLYFIFGIWASMLGTSLSLLIRTELGIPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSMIVENGAGTGWTVYPPLSSNIAH
GSSSVDLVIFSLHLAGISSILGAINFITTIINMKINNMSFDQMPLFVWAVGITALLLLLSL
PVLAGAITMLLTDRNLNT
```

Fig. 44. The peptide sequence obtained from conceptual translation of consensus sequence of *P. polymnestor* CUJ5.

The nucleotide BLAST was conducted with the consensus sequence derived from *P. polymnestor* CUJ5, the table 10 below represent the BLAST hit table derived.

S. I. No.	**Subject Ids**	**% identity**	**Alignment length**	**Mismatches**	**Gap opens**	**Q. start**	**Q. end**	**S. start**	**S. end**	**Bit score**
1	*gi|584609671|gb|KJ195275.1|Papilio polymnestor isolate F415*	100.00	575	0	0	35	609	1	575	1062
2	*gi|658132123|dbj|AB969797.1|Papilio memnon , isolate: Pam_Ok01*	99.84	609	1	0	1	609	1	609	1120
3	*gi|545690939|gb|KF226564.1|Papilio memnon agenor voucher UMKL-JJW0038*	99.84	609	1	0	1	609	1	609	1120
4	*gi|42405483|gb|AY457578.1|Papilio memnon voucher UASM 9900091*	99.84	609	1	0	1	609	49	657	1120
5	*gi|197359532|gb|EU792477.1|Papilio polymnestor isolate SEC02BM05*	99.84	606	1	0	4	609	7	612	1114
6	*gi|584609651|gb|KJ195265.1|Papilio polymnstor F339*	99.83	575	1	0	35	609	1	575	1057
7	*gi|321121861|gb|HQ962218.1|Papilio memnon*	99.67	609	2	0	1	609	1	609	1114
8	*gi|584609693|gb|KJ195286.1|Papilio memnon*	99.48	575	3	0	35	609	1	575	1046
9	*gi|485475954|gb|KC810961.1|Papilio memnon*	99.16	596	5	0	14	609	1	596	1074
10	*gi|331271421|gb|JF681019.1|Papilio deiphobus voucher CBGPFLC_00198*	96.88	609	19	0	1	609	12	620	1020
11	*gi|42405495|gb|AY457582.1|Papilio rumanzovia voucher UASM 9900972*	96.72	609	20	0	1	609	49	657	1014
12	*gi|316994082|gb|GU372545.1|Papilio macilentus*	94.91	609	31	0	1	609	1	609	953

Table 10. The BLAST hit table of *P. polymnestor* CUJ5.

The consensus sequence of *P. polymnestor* CUJ5 after removing the primer sequence was BLAST against database showed 100% similarity to that of *P. polymnestor* KJ195275 from Pune, India, other 4 sequences were with 99.84% and the remaining 1 sequence has 99.83% similarity (table 10).

The sequences with top similarities were identified from the BLAST and the sequences were used with the sequences of *P. polymnestor* CUJ5 for phylogenetic analysis in MEGA6, the NJ- tree derived were represented below (Fig. 45).

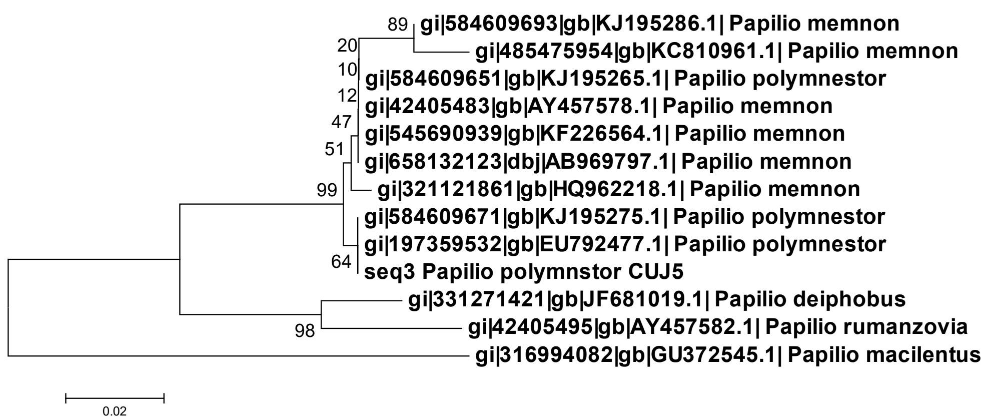

Fig. 45. The NJ-tree with Phylogenetic relationships of *P. polymnestor* CUJ5.

The phylogeny of CUJ5 was derived with NJ-tree developed from sequences of BLAST results along with CUJ5 in MEGA6 application (Fig. 45). The CUJ5 was found placed at the same clade with 2 samples of *P. polymnestor* KJ195275 from Pune and EU792477 from Bangalore India. The *P. memnon* with 99.67% identity found in the adjacent clade more close to the species.

Discussion

The *P. polymnestor* is endemic to Indian region among the 2 samples sequenced from India (EU792477 and EU792477) the 1 from Pune (EU792477) had 100% similarity to CUJ5, hence the sequence of CUJ5 can be useful as a molecular barcode. The sample from Bangalore has 99.84% similarity and it was a polymorphic variant of the species. The *P. memnon* with 99.67% similarity does not have the expected interspecies variability of 3% observed in most cases. The closely related *P. memnon* was isolated from Japan, Malaysia, Taiwan, Philippines and Australia were found more closely related to the species in the phylogenetic tree. This finding supports the common origin and distribution of the species in South East Asia, including, India (Condamine, 2013). In the Phylogenetic tree the more closely related sequence *P. polymnestor* (KJ195275) was from Pune belonging to Western Ghats with 100% similarity with CUJ5. The distance data of the tree revealed that the species was diverged from their closely related species *P. memnon* about 6,000 years ago and also from *P. deiphobus* about 60,000 years ago. This revealed a recent diversification of the genus in Western Ghats, about 60,000 years ago.

3.7. ***Hypolimnas bolina*** **Linnaeus, 1758 isolate CUJ6 (KT8806342)**

The specimen CUJ6 is identified as *Hypolimnas bolina* (Linnaeus, 1758) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio bolina* Linnaeus, 1758,

Nymphalis jacintha Drury (1773),

Hypolimnas parva Aurivillius, 1920

Hypolimnas bolina is a black-bodied butterfly common along India, South East Asia and Arabia. The species shows high degree of sexual dimorphism. The female is mimetic with many morphological forms.

The upper side of the wings; brownish black, fore wing; discal violet iridescence near costa, edges bear white markings, spots and lunules along outer margins of both wings

Larval food plants involve *Laportea interrupta, portulaca oleracea* and *sida rhombifolia* the larvae are bluish brown in colour and has two horns on the head, the body has six rows of orange yellow spines.

The PCR amplification of partial COI sequence of *H. bolina* isolate CUJ6 from 12012’56”N, 75016’54”E yielded a consensus with 609 bp. The chromatogram, DNA sequence obtained, its conceptual translation product, BLASTn hit table, and NJ-tree are presented in (Fig. 47 to 51).

Fig. 46. The upper side of the *H. bolina* CUJ6. It was a female resembling common crow, 1. The purple coloured iridescence at the anterior margin, 2. A series of white coloured circular markings in the interspaces along wing margins. 3. Hind wing has arrow shaped white outer markings, 4. The edges have white lace like markings.

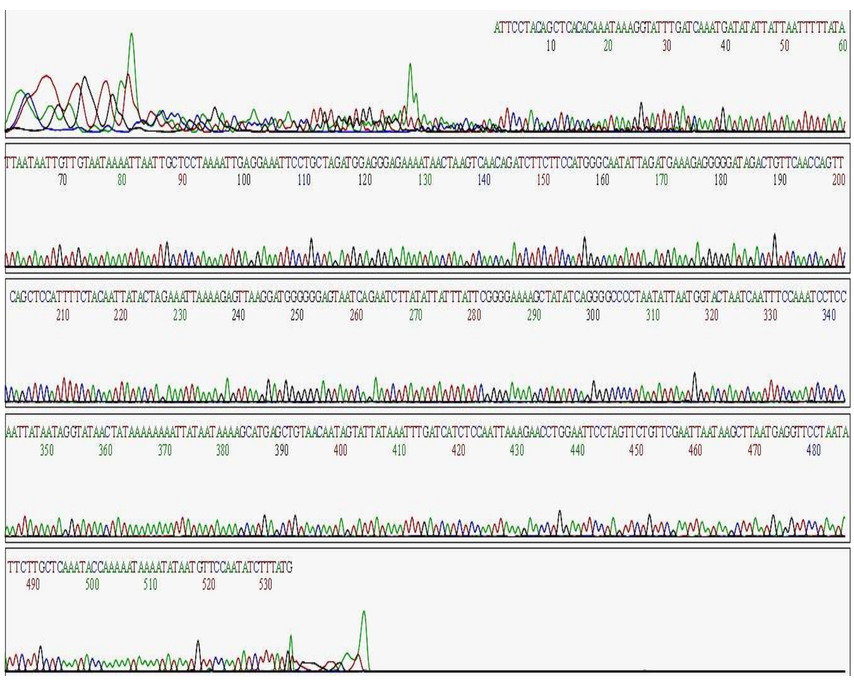

Fig. 47. The sequencing chromatogram of forward DNA strand of *H. bolina* CUJ6.

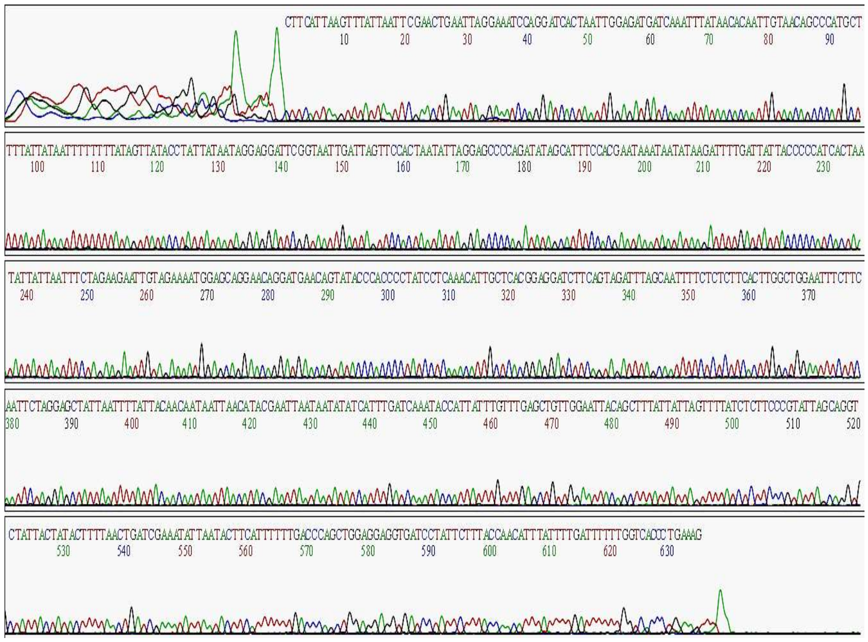

Fig. 48. The sequencing chromatogram of reverse DNA strand of *H. bolina* CUJ6.

```
>Hypolimnas bolina voucher CUJ6 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609bp)
TACTTTATATTTTATTTTTGGTATTTGAGCAGGAATAGTAGGAACTTCATTAAGTTTATT
AATTCGAACTGAATTAGGAAATCCAGGATCACTAATTGGAGATGATCAAATTTATAACAC
AATTGTAACAGCCCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATAGG
AGGATTCGGTAATTGATTAGTTCCACTAATATTAGGAGCCCCAGATATAGCATTTCCACG
AATAAATAATATAAGATTTTGATTATTACCCCCATCACTAATATTATTAATTTCTAGAAG
AATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTATACCCACCCCTATCCTCAAACAT
TGCTCACGGAGGATCTTCAGTAGATTTAGCAATTTTCTCTCTTCACTTGGCTGGAATTTC
TTCAATTCTAGGAGCTATTAATTTTATTACAACAATAATTAACATACGAATTAATAATAT
ATCATTTGATCAAATACCATTATTTGTTTGAGCTGTTGGAATTACAGCTTTATTATTAGT
TTTATCTCTTCCCGTATTAGCAGGTGCTATTACTATACTTTTAACTGATCGAAATATTAA
TACTTCATT
```

Fig. 49. The partial sequence of mitochondrial COI gene of *H. bolina* CUJ6.

The conceptual translation of the consensus sequence of CUJ6 gave the following peptide sequence in Fig. 50.

```
>seq4 Hypolimnas bolina CUJ6

TLYFIFGIWAGMVGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMMGG
FGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLMLLISSSIVENGAGTGWTVYPPLSSNIAH
GGSSVDLAIFSLHLAGISSILGAINFITTMINMRINNMSFDQMPLFVWAVGITALLLVLSL
PVLAGAITMLLTDRNINTS
```

Fig. 50. The peptide sequence obtained from the conceptual translation of consensus sequence *H. bolina* CUJ6.

The nucleotide BLAST was conducted with the consensus sequence derived from CUJ6, the (table 11) below represent the BLAST hit table derived from it.

S.I No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|589388708|gb|KF990127.1|Hypolimnas bolina*	100.00	658	0	0	1	658	1496	2153	1216
2	*gi|321121855|gb|HQ962215.1|Hypolimnas bolina jacintha voucher YB-KHC6551*	100.00	658	0	0	1	658	1	658	1216
3	*gi|302201964|gb|HM246468.1|Papilio clytia*	100.00	658	0	0	1	658	660	3	1216
4	*gi|301051529|gb|HM446465.1|Hypolimnas bolina isolate HZBJD-001*	100.00	658	0	0	1	658	1	658	1216
5	*gi|161332990|gb|EF683668.1|Hypolimnas bolina*	100.00	657	0	0	2	658	1	657	1214
6	*gi|545690759|gb|KF226474.1|Hypolimnas bolina voucher UMKL-JJW0213*	100.00	649	0	0	1	649	1	649	1199
7	*gi|8389249|gb|AF187775.1|Hypolimnas bolina voucher NW29-5*	100.00	627	0	0	26	652	1	627	1158
8	*gi|657172789|gb|KJ459843.1|Hypolimnas bolina isolate T73*	100.00	624	0	0	35	658	1	624	1153
9	*gi|330600279|gb|GU012527.1|Hypolimnas bolina voucher F72*	100.00	619	0	0	18	636	1	619	1144
10	*gi|330600281|gb|GU012528.1|Hypolimnas bolina voucher F73*	100.00	613	0	0	20	632	1	613	1133
11	*gi|330600247|gb|GU012509.1|Hypolimnas bolina voucher F15*	100.00	612	0	0	27	638	1	612	1131
12	*gi|6002806|gb|AF153916.1|Hypolimnas bolina voucher NW29-5*	100.00	600	0	0	26	625	1	600	1109
13	*gi|330600276|gb|GU012525.1|Hypolimnas bolina voucher F67*	99.68	619	0	2	26	642	1	619	1131
14	*gi|300432030|gb|GU091495.1|Hypolimnas bolina strain B11*	99.20	627	5	0	20	646	1	627	1131
15	*gi|197359551|gb|EU792493.1|Hypolimnas bolina isolate SEC18GE05*	98.63	657	6	2	5	658	7	663	1160
16	*gi|255927077|gb|GQ240284.1|Hypolimnas diomea voucher NW80-5*	95.74	633	27	0	26	658	1	633	1020
17	*gi|313581234|gb|HM906265.1|Hypolimnas deois voucher USNM:00704486*	95.59	658	29	0	1	658	1	658	1055
18	*gi|529386293|gb|KF404781.1|Hypolimnas antilope voucher 11ANIC-07485*	94.99	658	33	0	1	658	1	658	1033
19	*gi|58614895|gb|AY788636.1|Hypolimnas pandarus voucher NW80-11*	94.63	633	34	0	26	658	1	633	981
20	*gi|545690753|gb|KF226471.1|Hypolimnas anomala anomala voucher UMKL-JJW0209*	94.07	658	39	0	1	658	1	658	1000

21	*gi|58614889|gb|AY788633.1|Hypolimnas alimena voucher NW81-1*	94.00	633	38	0	26	658	1	633	959
22	*gi|169672607|gb|EU368161.1|Hypolimnas misippus isolate jinbanjiadie3*	93.46	657	43	0	2	658	1	657	976
23	*gi|667668748|gb|KJ752718.1|Junonia almana voucher JAS2*	91.49	658	56	0	1	658	6	663	905
24	*gi|321121851|gb|HQ962213.1|Junonia lemonias lemonias voucher YB-KHC6544*	91.05	659	57	2	1	658	1	658	889

Table 11. The BLAST hit table of *H. bolina* CUJ6.

. The consensus sequence of CUJ6 was BLAST against database showed 100% similarity to that of 11 samples among the 14 *H. bolina* sequences deposited in GenBank, they involve KF990127, HM246468, HM446465 and EF683668) from P. R. China, HQ962215 from Thailand, KF226474 from Malaysia, AF187775 from Finland, KJ459843, GU012527, GU012528, GU012528, GU012509 from Pune, and AF153916 from Finland. The three sequences of *H. bolina* GU012525 and GU091495 from Pune, and EU792493 from Bangalore were with 99.68%, 99.20% and 98.63% similarities (table 11).

The phylogeny of CUJ6 was derived with NJ tree developed from the top similar sequences of BLAST hit results along with CUJ6 was developed (Fig. 51) in which the CUJ6 was found placed in the same clade with11samples of *H. bolina*. The *Hypolimnas antilope* voucher (KF404781) and *Hypolimnas anomala* (KF226471) were with 94.99% and 94.07% identity respectively in the adjacent clade more close to the species.

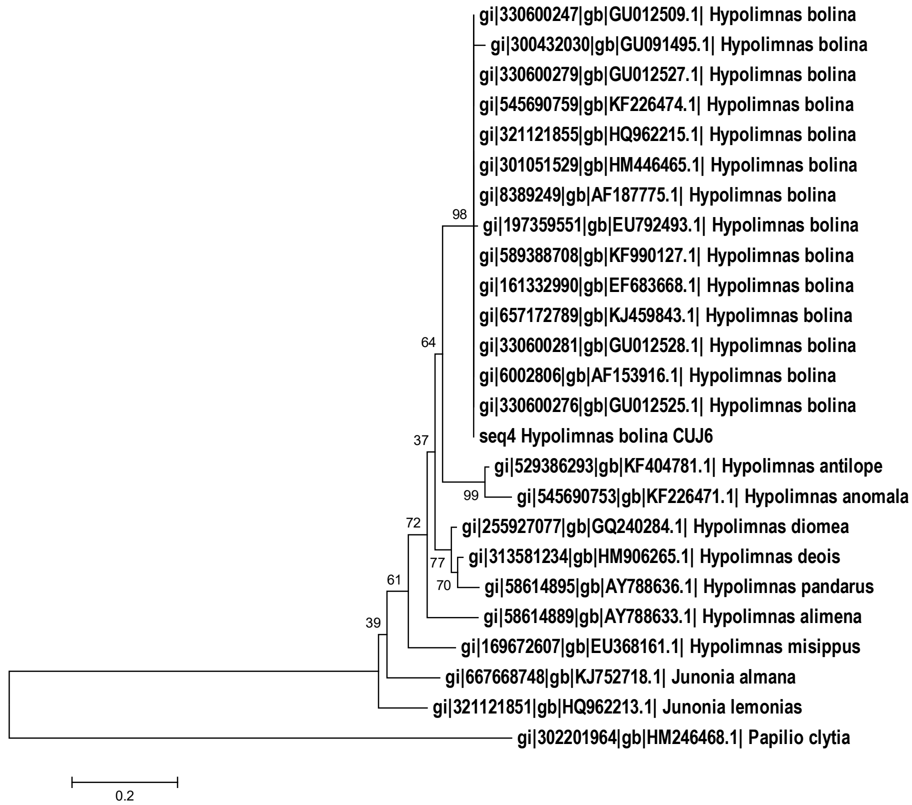

Fig. 51. The NJ-tree with Phylogenetic relationships of *H. bolina* CUJ6

Discussion

The *Hypolimnas bolina* CUJ6 was BLAST against database showed 100% similarity to that of 11 sequences deposited in GenBank. Therefor the COI sequence of CUJ6 will be useful as a molecular barcode for identification of the species. The CUJ6 was placed in the same clade with 14 samples of *H. bolina*. The *H. antelope* (KF404781) and *H. anomala* (KF226471) were more close to *H. bolina* and found in the same clade, they were monophylectic and sharing common ancestor. The NJ-tree distance data revealed that the species was diverged from their closely related species about one, 00, 000 years ago. They diverged from *Junonia almana* about two, 40, 000 years ago. The sequences isolated from samples of P. R. China, Japan, Malaysia, Thailand and India were found closer in the genus. This findings supports common origin of the species in Asia (Condamine, 2013). In the Phylogenetic tree the sequences of *H. bolina* isolated from Western Ghats were more closely related with 100% similarity except some samples (GU012525.1), (GU091495.1) from Pune and (EU792493.1) from Bangalore were with 99.68%, 99.20% and 98.63% similarities (table. 11) with CUJ6 they were polymorphic to the loci. The distance data of the tree also revealed a recent diversification of the genus in South East Asia about 1, 00, 000 years ago.

3.8. *Papilio clytia* (Linnaeus, 1758) isolateCUJ7 (KT880643)

The specimen CUJ7 is identified as *Papilio clytia* (Linnaeus, 1758) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonym: *Papilio similis* (Grote, 1899)

It is the Common Mime butterfly frequently observed in garden plants with long, narrow corolla tubes like *Ixora coccinia*. It is a polymorphic butterfly having two morphs, the clytia form and dissimilis form, the clytia form resembles *Papilio polytes* and *Papilio dravidaram*, while the dissimilis form resemble *Thirumala limniace*. This is also an example for Batesian mimicry.

In dissimilis form Upper side, fore wing: cell with four streaks coalescent at base and four spots beyond at apex, a long streak in interspace 1a, two streaks with two spots beyond which more or less coalescent with them in interspace 1, a broad streak with an outwardly emarginated spot in interspace 2, similar spots, one at base and one beyond, in 3, a single similar spot in 4, elongate streaks in 5 and 6, and much smaller elongate spots in interspaces 8 and 9 all these streaks and spots cream-white with diffuse edges (Fig. 52); sub terminal and terminal truncate white spots; Antennae, head, thorax and abdomen black, the thorax anteriorly and beneath and the abdomen on the sides spotted with white (Fig. 52).

Hind wing; markings; discoidal cell entirely white, discal white streaks longer, reach quite up to the outer margin of the cell and are continued anteriorly to the costa by elongate streaks in interspaces 6 and 7, two spots in interspace 8 and a slender streak along the costa (Fig. 52).

The larval food plants involve *Cinnamomum zeylanicum*, *Cinnamomum camphora* and *Litsea chinensis* the larvae are characterized with light yellow colour with red spots.

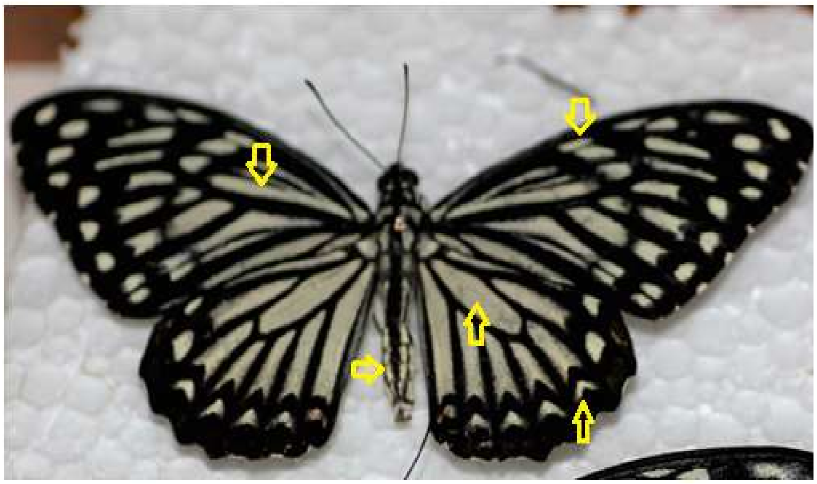

Fig. 52. The upper side of *P. clytia* CUJ7, 1. Four streaks coalescent at base and four spots beyond at apex, 2. Sub terminal and terminal truncate white spots, 3 Body black the abdomen on the sides spotted with white.

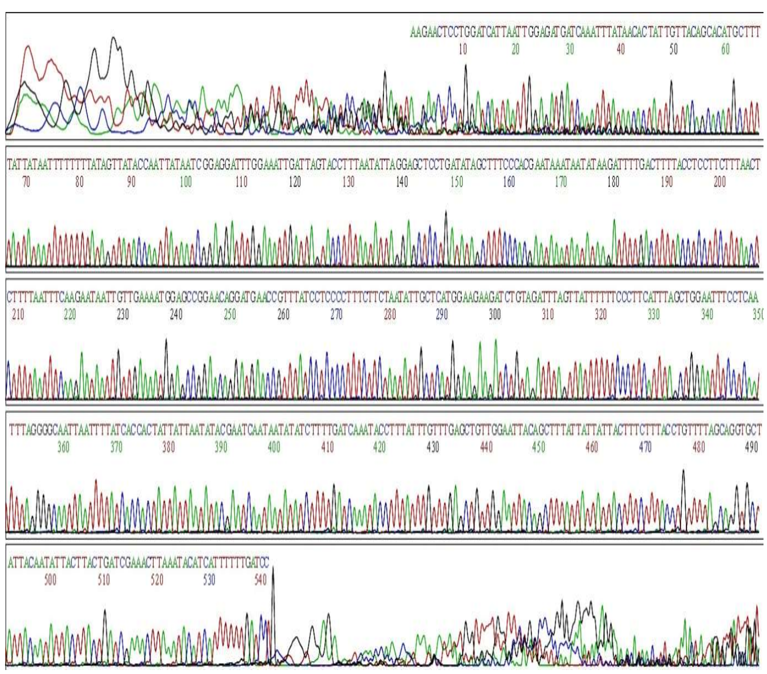

Fig. 53. The sequencing chromatogram of forward DNA strand of *P. clytia* CUJ7.

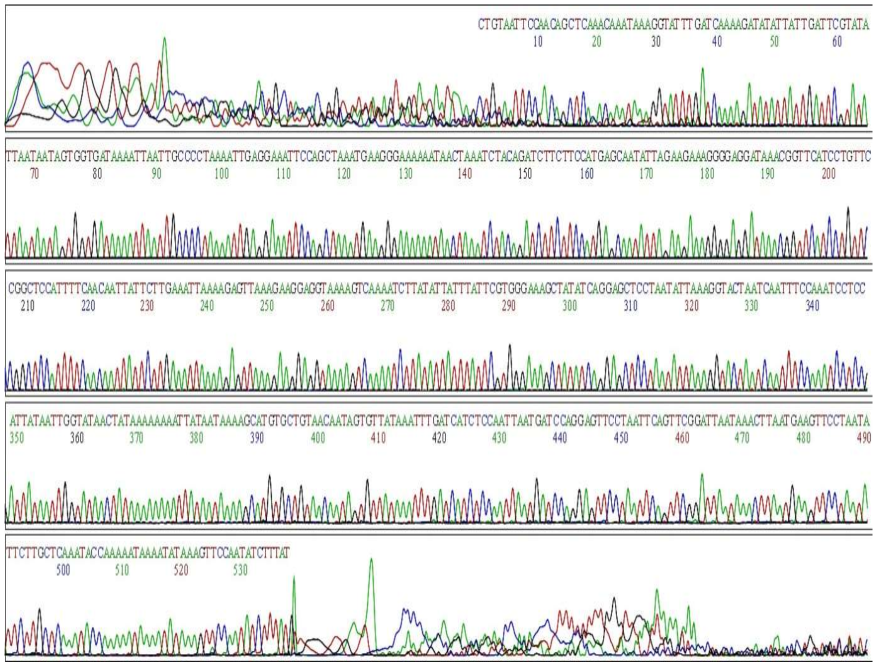

Fig. 54. The sequencing chromatogram of reverse DNA strand of *P. clytia* CUJ7.

The PCR amplification of partial COI sequence of *P. clytia* CUJ7 from Thalassery (11046’37”N, 75028’12”E) yielded a consensus with 609 bp The chromatogram, DNA sequence obtained, its conceptual translation product, BLASTn hit table, and NJ-tree are presented in (Fig. 53-57).

```
>Papilio clytia voucher CUJ7 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
AACTTTATATTTTATTTTTGGTATTTGAGCAAGAATATTAGGAACTTCATTAAGTTTATT
AATCCGAACTGAATTAGGAACTCCTGGATCATTAATTGGAGATGATCAAATTTATAACAC
TATTGTTACAGCACATGCTTTTATTATAATTTTTTTTATAGTTATACCAATTATAATCGG
AGGATTTGGAAATTGATTAGTACCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCACG
AATAAATAATATAAGATTTTGACTTTTACCTCCTTCTTTAACTCTTTTAATTTCAAGAAT
AATTGTTGAAAATGGAGCCGGAACAGGATGAACCGTTTATCCTCCCCTTTCTTCTAATAT
TGCTCATGGAAGAAGATCTGTAGATTTAGTTATTTTTTCCCTTCATTTAGCTGGAATTTC
CTCAATTTTAGGGGCAATTAATTTTATCACCACTATTATTAATATACGAATCAATAATAT
ATCTTTTGATCAAATACCTTTATTTGTTTGAGCTGTTGGAATTACAGCTTTATTATTATT
ACTTTCTTTACCTGTTTTAGCAGGTGCTATTACAATATTACTTACTGATCGAAACTTAAA
TACATCATT
```

Fig. 55. The partial sequence of mitochondrial COI gene of *P. clytia* CUJ7.

The conceptual translation of the consensus sequence of CUJ7 gave the following peptide sequence.

```
>seq5 Papilio clytia CUJ7
TLYFIFGIWASMLGTSLSLLIRTELGTPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSMIVENGAGTGWTV
YPPLSSNIAHGSSSVDLVIFSLHLAGISSILGAINFITTIINMRINNMSFDQMPLF
VWAVGITALLLLLSLPVLAGAITMLLTDRNLNTS
```

Fig. 56. The peptide sequence obtained from conceptual translation of consensus sequence *P. clytia* CUJ7

The nucleotide BLAST was conducted with the consensus sequence derived from *P. clytia* CUJ7, the Table 12 below represent the BLAST hit table derived from it. The nucleotide BLAST showed 99.51% identity to 1 sample of *P. clytia* AY457594 sequence from Malaysia (table 12).

S.I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|42405531|gb|AY457594.1|Papilio clytia voucher UASM 9900073*	99.51	609	3	0	1	609	49	657	1109
2	*gi|374900991|gb|JQ548606.1|Papilio torquatus tolmides*	93.70	571	36	0	39	609	3	573	856
3	*gi|284020382|gb|GU163878.1|Papilio torquatus tolmides*	93.50	600	39	0	10	609	1	600	893
4	*gi|669633617|gb|KJ828915.1|Papilio torquatus voucher P54_2*	93.44	564	37	0	40	603	1	564	837
5	*gi|379769552|gb|JQ606290.1|Papilio torquatus tolmides*	93.43	609	40	0	1	609	1	609	904
6	*gi|284020334|gb|GU163854.1|Papilio torquatus tolmides*	93.43	609	40	0	1	609	1	609	904
7	*gi|284020332|gb|GU163853.1|Papilio torquatus tolmides*	93.43	609	40	0	1	609	1	609	904
8	*gi|284020330|gb|GU163852.1|Papilio torquatus tolmides*	93.43	609	40	0	1	609	1	609	904
9	*gi|545690955|gb|KF226572.1|Papilio paradoxa aenigma voucher UMKL-JJW0116*	92.79	610	42	2	1	609	1	609	881
10	*gi|119655457|gb|EF126457.1|Papilio glaucus voucher PgFY03*	92.78	609	44	0	1	609	18	626	881

Table12. The BLAST hit table of *P. clytia* CUJ7.

The sequences with top similarities were identified from the BLAST and the sequences were used with the sequences of *P. clytia* CUJ7 for phylogenetic analysis in MEGA6, the NJ- trees derived were represented below (Fig. 57).

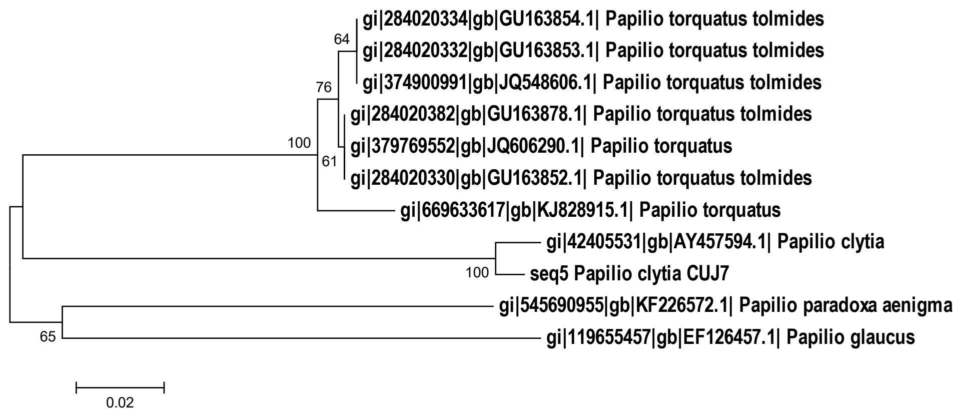

Fig. 57. The NJ-tree with Phylogenetic relationships of *P. clytia* CUJ7.

The CUJ7 was placed at the same clade with 1 sample of *P. clytia* AY457594 from Malaysia. The *P. torquatus* JQ548606 from Costa Rica with 93.70% identity was in the adjacent clade more close to the species.

Discussion

The CUJ7 BLAST results showed 99.51% identity to *P. clytia* AY457594 from Malaysia. Therefore the COI sequence of CUJ7 isolated in this study will be useful as a barcode to identify the species *P. clytia*. The COI sequence of CUJ7 showed considerable variation from related species of the genus *Papilio*, hence it is a novel sequence. The phylogeny of CUJ7 with NJ-tree (Fig. 57) showed the CUJ7 in the same clade with *P. clytia* (AY457594) from Malaysia. Whereas the *P. torquatus tolmide* (JQ548606) from Costa Rica with 93.70% identity was in the adjacent clade more close to the species, their ancestors were monophyletic. The

NJ-tree distance data revealed that the species was diverged from their closely related species about 1,36,000 years ago. It was a widely distributed butterfly of South East Asia.

3.9. ***Pachliopta hector*** **(Linnaeus, 1758) isolate CUJ8 (KT880644)**

The specimen CUJ8 is identified as *Pachliopta hector* (Linnaeus, 1758) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Atrophaneura hector* (Linnaeus, 1758)

Papilio hector Linnaeus, 1758

This is a red-bodied butterfly common in south India, North-East India, Sri Lanka, and Myanmar. They are common in Northern Kerala during Monsoon. They are common in flowers during sunny days for nectar feeding, the basking during morning and roosting in groups on tree branches during night were also observed.

Upper side; black, Fore wing; broad white interrupted band from the subcostal nervure opposite the origin of veins 10 and 11, extended obliquely to the tornus, and a second short pre-apical similar band; both bands composed of detached irregularly indented broad streaks in the interspaces (Fig. 58). Hind wing; with a discal posteriorly strongly curved series of seven crimson spots followed by a sub terminal series of crimson limulus (Fig. 58). Cilia black alternated with white Under side; fore wing; dull brownish black, hind wing; black markings as on the upper side, but the crimson spots and crescentic markings on the hind wing larger (Fig. 63). Antennae, thorax and abdomen above at base, black; head and rest of the abdomen bright crimson; beneath; palpi, the sides of the thorax and abdomen crimson. The larval food plants involve *Aristolochia indica* and *Thottea siliquosa*, their larvae are similar to that of *Atrophaneura aristolochiae* and *Troides minos*.

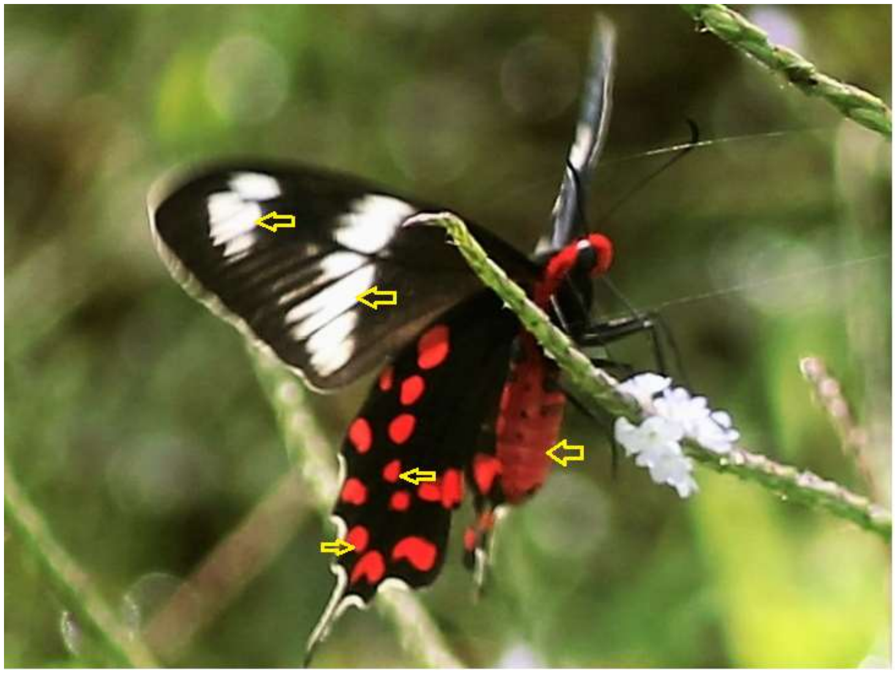

Fig. 58. *P. hector* CUJ8 black fore wing with broad white interrupted band from the subcostal nervure opposite the origin of veins 10 and 11, extended obliquely to the tornus, and a second short pre-apical similar band; both bands composed of detached irregularly indented broad streaks in the interspaces, 2. Hind wing; with a discal posteriorly strongly curved series of seven crimson spots followed by a sub terminal series of crimson limulus, 3. Red coloured body.

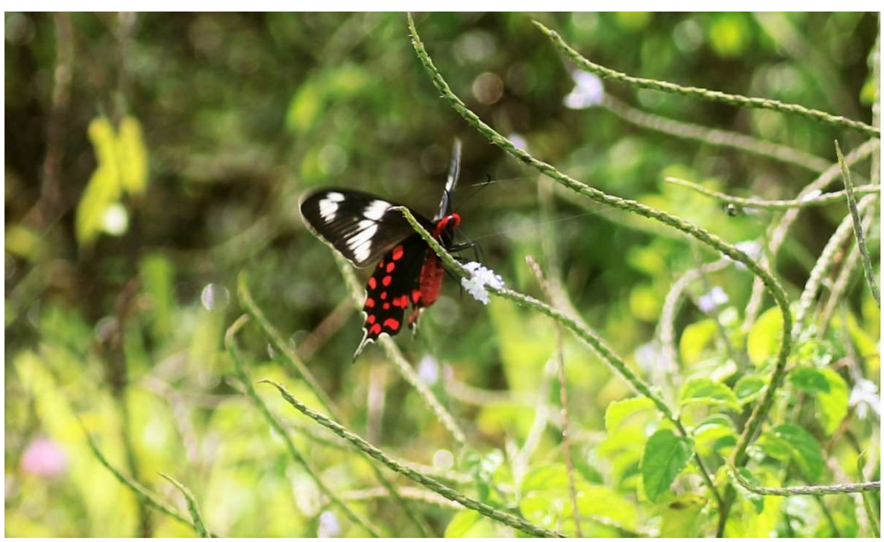

Fig. 59. The habit *P. hector* CUJ8.

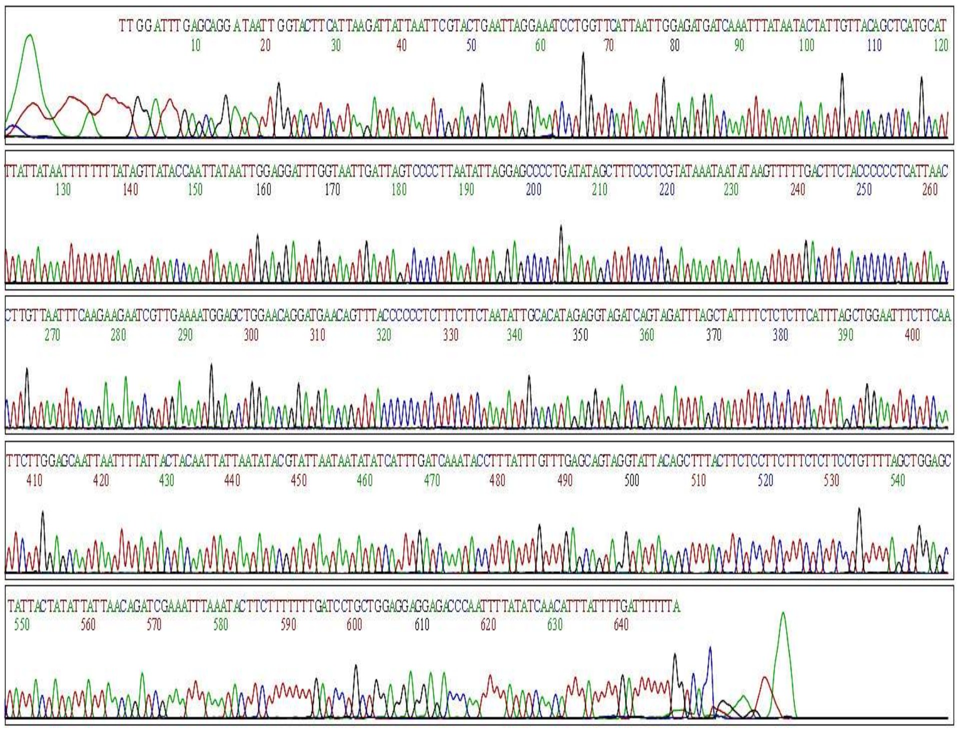

Fig. 60 The sequencing chromatogram of forward DNA strand of *P. hector* CUJ8

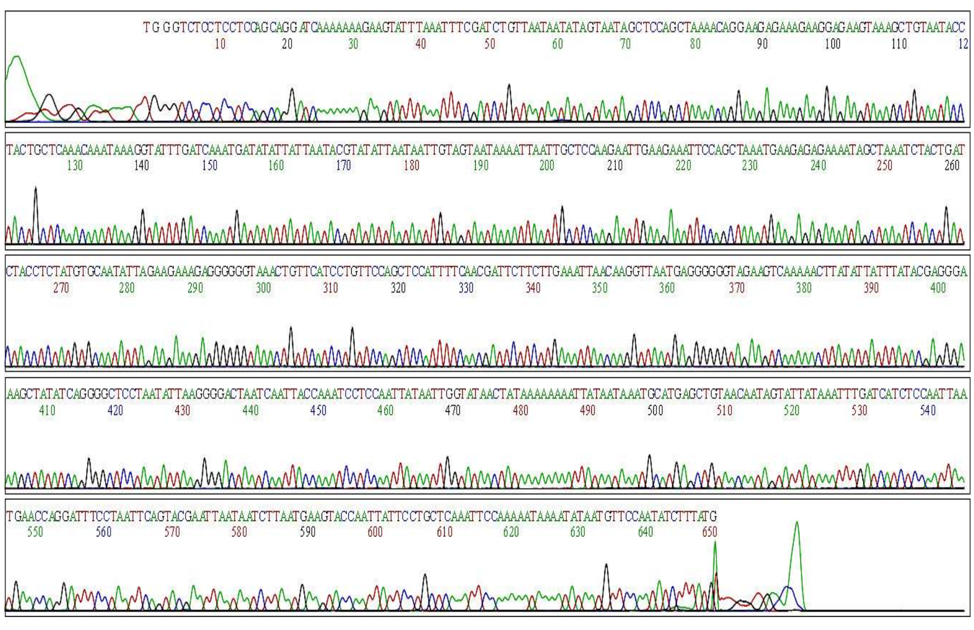

Fig. 61. The sequencing chromatogram of reverse DNA strand of *P.hector* CUJ8.

The PCR amplification of partial COI sequence of *P. hector* (Linnaeus, 1758) CUJ8 from Aravanchal (12012’56”N, 75016’54”E) yielded a consensus with 622 bp. The chromatogram, DNA sequence obtained, its conceptual translation product, BLASTn hit table, and NJ-tree are presented in (Fig. 60 to 64).

```
>Pachliopta hector voucher CUJ8 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (622)
ATAAAGATATTGGAACATTATATTTTATTTTTGGAATTTGAGCAGGAATAATTGGTACTT
CATTAAGATTATTAATTCGTACTGAATTAGGAAATCCTGGTTCATTAATTGGAGATGATC
AAATTTATAATACTATTGTTACAGCTCATGCATTTATTATAATTTTTTTTATAGTTATAC
CAATTATAATTGGAGGATTTGGTAATTGATTAGTCCCCTTAATATTAGGAGCCCCTGATA
TAGCTTTCCCTCGTATAAATAATATAAGTTTTTGACTTCTACCCCCCTCATTAACCTTGT
TAATTTCAAGAAGAATCGTTGAAAATGGAGCTGGAACAGGATGAACAGTTTACCCCCCTC
TTTCTTCTAATATTGCACATAGAGGTAGATCAGTAGATTTAGCTATTTTCTCTCTTCATT
TAGCTGGAATTTCTTCAATTCTTGGAGCAATTAATTTTATTACTACAATTATTAATATAC
GTATTAATAATATATCATTTGATCAAATACCTTTATTTGTTTGAGCAGTAGGTATTACAG
CTTTACTTCTCCTTCTTTCTCTTCCTGTTTTAGCTGGAGCTATTACTATATTATTAACAG
ATCGAAATTTAAATACTTCTTT
```

Fig. 62. The partial sequence of mitochondrial COI gene of *P. hector* CUJ8.

The conceptual translation of the consensus sequence of CUJ8 gave the following peptide sequence.

>seq6 *pachiliopta hector* CUJ8

```
TLYFIFGIWAGMIGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSSIVENGAGTGWTVYPPLSSNIAH
SGSSVDLAIFSLHLAGISSILGAINFITTIINMRINNMSFDQMPLFVWAVGITALLLLLSL
PVLAGAITMLLTDRNLNTS
```

Fig. 63. The peptide sequence obtained from conceptual translation of consensus sequence of *P. hector* CUJ8.

The nucleotide BLAST was conducted with the consensus sequence derived from *P. hector* CUJ8, the Table 13 below represent the BLAST hit table derived from it.

S. I. No .	Subject Ids	% Identi ty	Alig nme nt leng th	Mi sm atc hes	Ga p op ens	Q. sta rt	Q. end	S. sta rt	S. end	Bit score
1	*gi|197359545|gb|EU792488.1|Pachliopta hector isolate SEC13CRR05*	100	662	0	0	32	693	7	668	1223
2	*gi|572169453|gb|KF733790.1|Graphium nomius*	100	658	0	0	28	685	1	658	1216
3	*gi|396576492|gb|JX261944.1|Pachliopta hector voucher BUZOOUGC-Ph*	100	623	0	1	38	660	7	628	1144
4	*gi|545690921|gb|KF226555.1|Pachliopta aristolochiae asteris voucher UMKL-JJW0033*	97	658	23	0	28	685	1	658	1088
5	*gi|321121995|gb|HQ962285.1|Pachliopta aristolochiae asteris voucher YB-KHC6901*	97	658	23	0	28	685	1	658	1088
6	*gi|545690919|gb|KF226554.1|Pachliopta aristolochiae asteris voucher UMKL-JJW0035*	96	658	24	0	28	685	1	658	1083
7	*gi|312451706|gb|HQ424202.1|Atrophaneura aristolochiae voucher CUJ2*	96	658	24	0	28	685	1	658	1083

Table 13 The BLAST hit table of *P. hector* CUJ8.

The consensus sequence of CUJ8 was BLAST against database showed 100% identity with two sample of *P. hector* sequences deposited in GenBank (table 13). *P. hector* KT879883 and EV92488 both from Bangalore, India. The *Graphium nomius* KF733790.1 from Madurai, India also with 100% similarity to the *P. hector* CUJ8

The phylogeny of CUJ8 was derived with NJ-tree developed from the similar sequences of BLAST results (Fig. 64). The CUJ8 was placed at the same clade with 2 samples of *P. hector* (KT879883) and *P. hector* (EV92488) from Bangalore, India. The *G. nomius* (KF733790) from Madurai, India also found in the same clade.

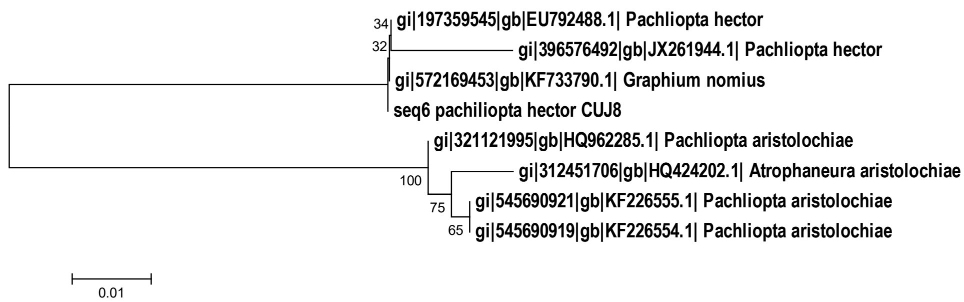

Fig. 64. The NJ-tree with Phylogenetic relationships of *P. hector* CUJ8.

Discussion

The COI sequence from CUJ8 BLAST results showed 100% identity with the two sample of *P. hector* deposited in GenBank the KT879883 and EV92488 both from Bangalore, India (table 13). Therefore, the COI sequence from CUJ8 will be useful as molecular barcode for identification. The *Graphium nomius* (KF733790) from Madurai, India also has 100% similarity to the *P. hector* CUJ8. This result challenges the barcoding gap and the ability of COI sequence in species identification. Even though the COI sequence from CUJ8 can help the species identification from its 100% similarity with two other samples of *P. hector* KT879883 and EV92488 deposited in GenBank and clarifies the species recognition power of sequence. The COI sequences from other samples of *G. nomius* are there in the database (KJ195277, AB377395) but they have no similarity with CUJ8 like the *Graphium nomius* (KF733790). Therefore, the case of *G. nomius* (KF733790) needs further clarification. The NJ-tree developed from the top similar sequences of BLAST hit results (Fig. 64), the CUJ8 was placed in the same clade with 2 samples of *P. hector* KT879883 and EV92488 from Bangalore, India. The *G. nomius* (KF733790) from Madurai, India also found in the same clade. The

P. hector is endemic to South East Asian region among the 2 samples sequenced from India the KT879883 and EV92488 from Bangalore has 100% similarity to CUJ8. However, the sample from Coimbatore JX261944 has 99% similarity and this was a polymorphic novel variant of the sequence. The distance data of the tree revealed that the species was diverged from their closely related species *Atrophaneura aristolochiae* about 59,000 years ago. The data supports the recent diversification of *Pachliopta hector* having limited distribution from their close relative *Atrophaneura aristolochiae* enjoying a wide distribution.

3.10. *Papilio liomedon* Moore, 1874 isolate CUJ10 (KT880646)

The specimen CUJ10 was identified as *Papilio liomedon* Moore, 1874 referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio demolion liomedon* Rothschild, 1895

The *Papilio liomedon* is commonly called as Malabar Banded swallowtail butterfly, which is an endemic butterfly of Western Ghats.

Upper side; brownish black, Fore and hind wings; crossed by a broad prominent oblique pale greenish or yellowish-white band that commences from the middle of the dorsal margin of the hind wing, crosses over on to the fore wing and is continued as a series of separate spots that diminish in size in the upper interspaces to the apex of that wing (Fig. 65). On the hind wing this is followed by a sub terminal series of similarly-coloured limulus (Fig. 65). Under side; sooty black, the transverse band that crosses the wings as on the upper side, Fore wing; cell with a series of four slender longitudinal pale lines from base; the veins also picked out with pale lines (Fig. 66), on the veins that run to the terminal margin these lines are conspicuous only at the apices; there are besides short similar lines between the veins that extend to the terminal margin. Hind wing; the interspaces beyond the transverse medial greenish-white band marked with broad jet-black streaks up to the sub terminal line of greenish-white lunules; these streaks medially interrupted by a transverse line of blue scales and succeeded in interspaces 1 and 7 by preapical ochraceous-yellow spots (Fig. 66); terminal margin beyond the line of lunules black. Antennae, head, thorax and abdomen fuliginous black; beneath, the palpi and abdomen greenish white, the thorax dark grey (Fig. 65 and 66).

The larval food plants involve *Evodia roxburghiana* and *Acronychia pedunculata* the eggs are yellowish colored, laid one over the other as stalks glued on small twigs.

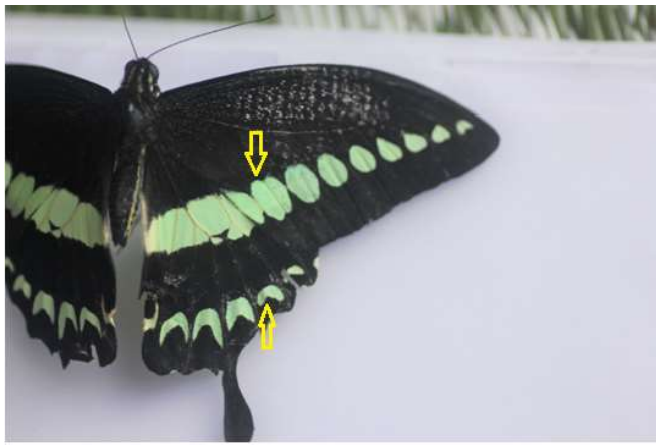

Fig. 65. The upper side of the *Papilio liomedon* CUJ10, 1. Fore and hind wings; crossed by a broad prominent oblique pale greenish or yellowish-white band that commences from the middle of the dorsal margin of the hind wing, crosses over on to the fore wing and is continued as a series of separate spots that diminish in size in the upper interspaces to the apex. 2. Hind wing has a sub terminal series of similarly-coloured limulus.

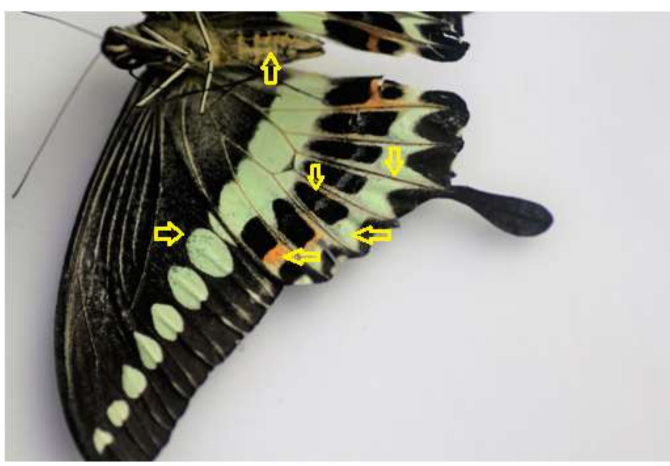

Fig. 66. The under side of *P. liomedon* CUJ10, 1. Fore wing; cell with a series of four slender longitudinal pale lines, the veins also picked out with pale lines. 2. Hind wing; the interspaces beyond the transverse medial greenish-white band

marked with broad jet-black streaks up to the sub terminal line of greenish-white limulus. 3. The linules are with broad jet-black streaks up to the sub terminal line of greenish-white lunules; 4. In interspaces 1 and 7 by preapical ochraceous-yellow spots. 5. Antennae, head, thorax and abdomen fuliginous black; beneath, the palpi and abdomen greenish white, the thorax dark grey.

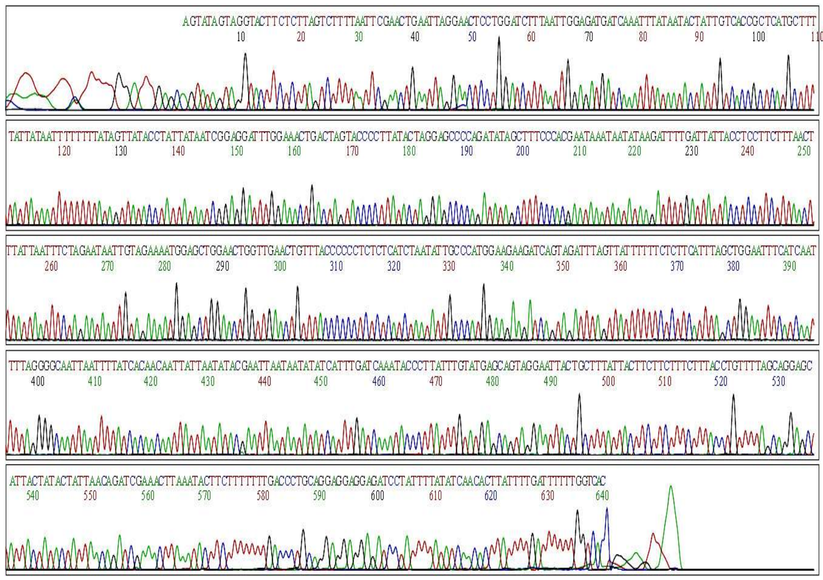

Fig. 67. The sequencing chromatogram of forward DNA strand of *P. liomedon* CUJ10.

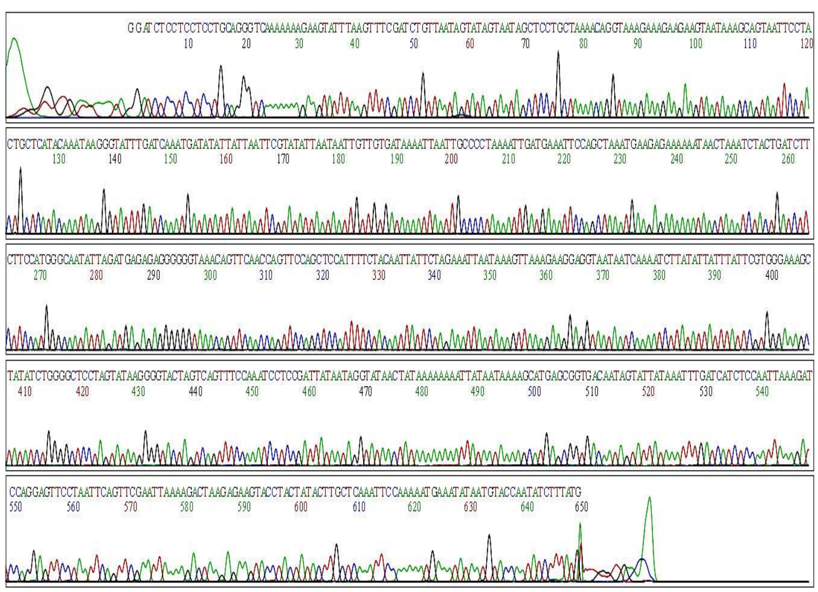

Fig. 68. The sequencing chromatogram of reverse DNA strand of *P.liomedon* CUJ10.

The PCR amplification of partial COI sequence of *P. liomedon* CUJ10 from Chapparapadav (12007’40”N, 75024’59”E) yielded a consensus with 609 bp. The chromatogram, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 67 to 71).

The sequencing of PCR product from template DNA isolated from CUJ10 gave chromatograms of two complimentary DNA strands each from both sides of the mitochondrial COI gene. The chromatograms are with clear signals for most of the base positions.

```
>Papilio liomedon voucher CUJ10 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
TACATTATATTTCATTTTTGGAATTTGAGCAAGTATAGTAGGTACTTCTCTTAGTCTTTT
AATTCGAACTGAATTAGGAACTCCTGGATCTTTAATTGGAGATGATCAAATTTATAATAC
TATTGTCACCGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATCGG
AGGATTTGGAAACTGACTAGTACCCCTTATACTAGGAGCCCCAGATATAGCTTTCCCACG
AATAAATAATATAAGATTTTGATTATTACCTCCTTCTTTAACTTTATTAATTTCTAGAAT
AATTGTAGAAAATGGAGCTGGAACTGGTTGAACTGTTTACCCCCCTCTCTCATCTAATAT
TGCCCATGGAAGAAGATCAGTAGATTTAGTTATTTTTTCTCTTCATTTAGCTGGAATTTC
ATCAATTTTAGGGGCAATTAATTTTATCACAACAATTATTAATATACGAATTAATAATAT
ATCATTTGATCAAATACCCTTATTTGTATGAGCAGTAGGAATTACTGCTTTATTACTTCT
TCTTTCTTTACCTGTTTTAGCAGGAGCTATTACTATACTATTAACAGATCGAAACTTAAA
TACTTCTTT
```

Fig. 69. . The partial sequence of mitochondrial cytochrome oxidase subunit I gene of *P. liomedon* CUJ10.

The conceptual translation of the consensus sequence of CUJ10 gave the following peptide sequence.

```
>seq8 Papilio liomedon CUJ10

TLYFIFGIWASMVGTSLSLLIRTELGTPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSMIVENGAGTGWTV
YPPLSSNIAHGSSSVDLVIFSLHLAGISSILGAINFITTIINMRINNMSFDQMPLF
VWAVGITALLLLLSLPVLAGAITMLLTDRNLNTS
```

Fig. 70. The peptide sequence obtained from conceptual translation of consensus sequence of *P. liomedon* CUJ10.

The nucleotide BLAST was conducted with the consensus sequence derived from *P. liomedon* CUJ10, the table14 below represent the BLAST hit results derived from it.

S. I. No.	Subject Ids	% Identity	Align ment length	Mis mat ches	Gap opens	Q. start	Q. en d	S. start	S. end	Bit score
1	*gi|331271423|gb|JF681020.1| Papilio demolion voucher CBGPFLC_00200*	97.26	621	17	0	1	621	12	632	1053
2	*gi|564657727|gb|JF747532.1| Papilio noblei voucher FD22*	97.26	656	15	3	2	657	2	654	1109
3	*gi|545690927|gb|KF226558.1| Papilio demoleus malayanus voucher UMKL-JJW0037*	96.81	658	21	0	1	658	1	658	1099
4	*gi|331271431|gb|JF681024.1| Papilio gigon voucher CBGPFLC_00211*	94.06	657	39	0	2	658	10	666	998
5	*gi|545690937|gb|KF226563.1| Papilio iswara iswara voucher UMKL-JJW0029*	92.11	659	48	4	2	658	2	658	926

Table 14. The BLAST hit table of *P. liomedon* CUJ10.

The consensus sequence of CUJ10 was BLAST against database showed 97.26% identity with the *Papilio demolion* (JF681020) from Thailand, and *Papilio noblei* (JF747532) from P. R. China. While it has 96.81% similarity with *Papilio demoleus malayanus* (KF226558) from Malaysia (table 14).

The phylogeny of *P. liomedon* CUJ10 was derived with NJ tree developed from the top similar sequences of database with BLAST results along with the sequence of COI derived from *P. liomedon* CUJ10 (Fig. 71). The *Papilio liomedon* CUJ10 was placed between *P. demoleus malayanus* (KF226558) from Malaysia and *P. iswara iswara* (KF226563) from Malaysia.

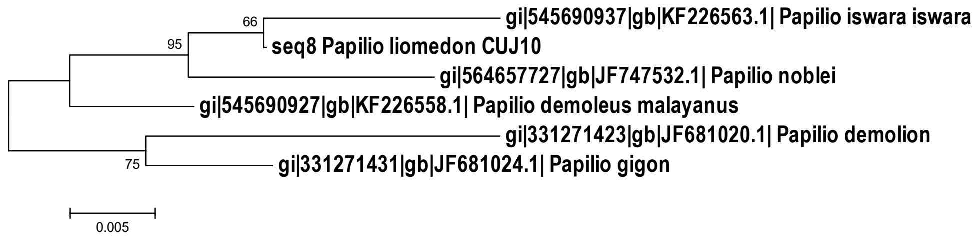

Fig. 71. The NJ-tree with Phylogenetic relationships of *P. liomedon* CUJ10.

Discussion

The *P. liomedon* CUJ10 is endemic to Western Ghats, this is the first time reporting of mitochondrial COI gene sequence of *P. liomedon.* The COI sequence of CUJ10 showed 97.26% identity with the *P. demolion* (JF681020) from Thailand, and *P. noblei* (JF747532) from P. R. China. While it has 96.81% similarity with *P. demoleus malayanus* (KF226558) from Malaysia. In the NJ-tree, the *P. liomedon* CUJ10 was found to be placed between *P. demoleus malayanus* (KF226558) from Malaysia and *P. iswara iswara* (KF226563) from Malaysia. The distance data of the tree revealed that the species was diverged from their closely related species *P. demoleus malayanus* (KF226558) about 15,500 years ago. The *P. iswara iswara* (KF226563) was diverged from *P. liomedon* CUJ10 about 19,000 years after its evolution. The data supports the relation with *P. demoleus* of South East Asia and their recent diversification after its isolation in Western Ghats with the loss of continuity of their distribution. This isolation was associated with the vicariance and climatic changes in Deccan plateau.

3. 11. ***Delias eucharis*** **(Drury, 1773) isolate CUJ11 (KT880647)**

The specimen CUJ11 was identified as *Delias eucharis* (Drury, 1773) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio eucharis* Drury, 1773

Pieris epicharis Godart, 1819

The *Delias eucharis* is the Common Jezebel butterfly, which is a medium sized pierid butterfly, found in many areas of South Asia and Southeast Asia, especially in the non-arid regions of India, Sri Lanka, Indonesia, Myanmar and Thailand. The Common Jezebel is one of the most common butterfly of the genus *Delias*.

Upper side; white, the fore wings; veins broadly black, broadened triangularly at the termination of the veins, costal margin narrowly black (Fig 72); a broad black post discal transverse band from costa to dorsum sloped obliquely outwards from costa to vein 4, thence parallel to termen. Hind wing; veins similar but for three-fourths of their length much more narrowly black, a post discal transverse black band as on the fore wing but curved, narrower, and extended only between veins 2 and 6; beyond this the veins are more broadly black and this colour as on the fore wing, broadens out triangularly at the termination of the veins, the interspaces beyond the post discal black band pink, due to the vermilion coloration of the underside showing through (Fig. 72).

Under side of the fore wings; similar to upper side but the black edging to the veins much broader, two interspaces beyond the post discal transverse band tinged with yellow. Hind wing; ground colour bright yellow, veins and transverse post discal band, much more broadly black, the latter extended from the costa to vein 2; the interspaces between the veins beyond the post discal fascia with a series of broadly lanceolate vermilion-red spots, each spot very narrowly edged with white (Fig. 72). The basal portion of interspace 6 white, in contrast to the bright yellow of the ground-colour. Antenna; black, head, thorax and abdomen; white, the apical joint of the palpi black, the head and thorax; with a mixture of black hairs that give these parts a grey-blue appearance (Fig. 72).

The larval food plants involve different species of the parasitic plant *Loranthus* involving *Dendrophthoe falcate, Helianthes elastic, Scurrula parasitica*. The predators usually avoid this butterflies because the toxicants sequestered in their body from these larval host plants.

The PCR amplification of partial COI sequence of *Delias eucharis* isolate CUJ11 from Peringome (12013’35”N, 75018’41”E) yielded a consensus with 609 bp. The chromatogram, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 73 to 77).

The sequencing of PCR product from template DNA isolated from CUJ11 gave chromatograms of two complimentary DNA strands each from both sides of the mitochondrial COI gene. The chromatograms are with clear base calls for most of the base positions without any ambiguous peaks (Fig. 73 and 74).

Fig. 72. The under side of *D. eucharis* CUJ11, 1. Fore wings; veins broadly black, broadened triangularly at the termination of the veins, 2. The interspaces beyond the post discal black band pink, due to the vermilion coloration of the under side, 3. Under side of hind wing; ground colour bright yellow, veins and transverse post discal band, much more broadly black, 4. Under side of hind wing the interspaces between the veins beyond the post discal fascia with a series of broadly lanceolate vermilion-red spots. 5. The head and thorax; with a mixture of black hairs that give these parts a grey-blue appearance.

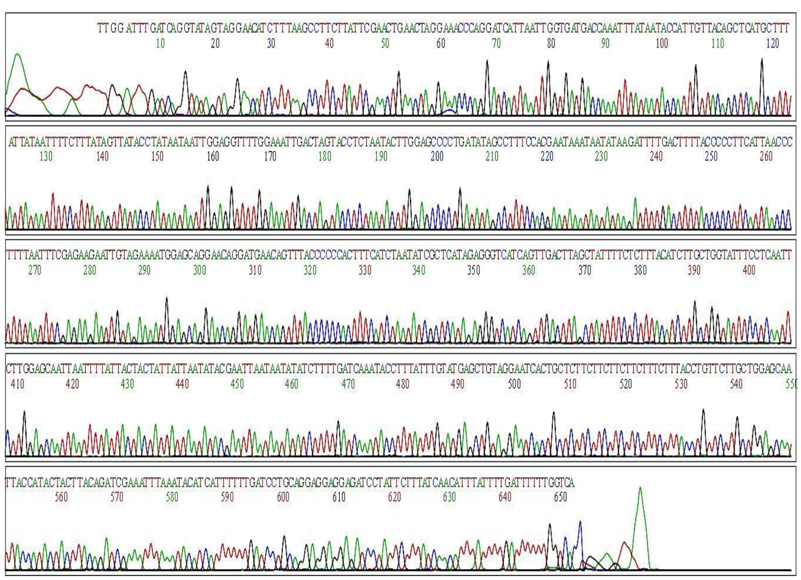

Fig. 73. The sequencing chromatogram of forward DNA strand of *D. eucharis* CUJ11.

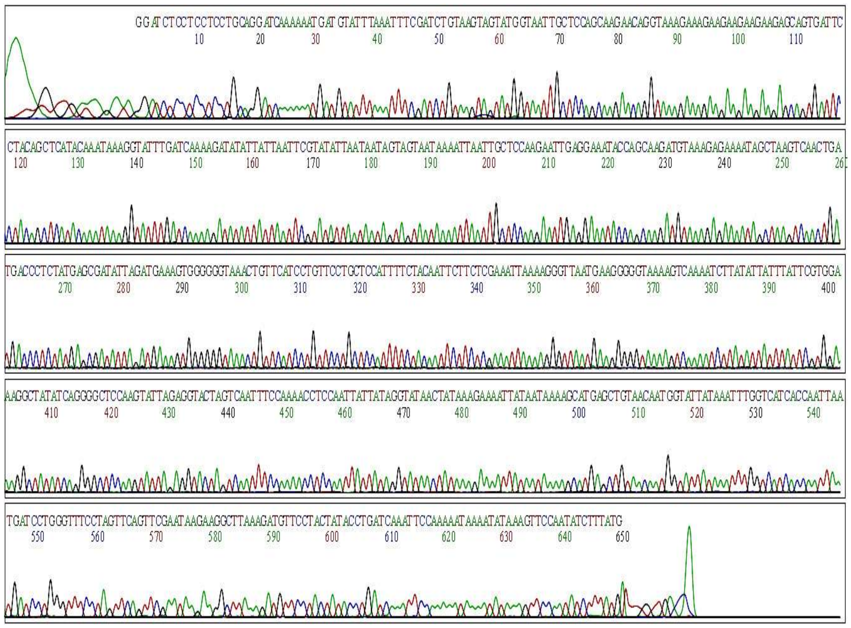

Fig. 74. The sequencing chromatogram of reverse DNA strand of *D. eucharis* CUJ11

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>Delias eucharis voucher CUJ11 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
AACTTTATATTTTATTTTTGGAATTTGATCAGGTATAGTAGGAACATCTTTAAGCCTTCT
TATTCGAACTGAACTAGGAAACCCAGGATCATTAATTGGTGATGACCAAATTTATAATAC
CATTGTTACAGCTCATGCTTTTATTATAATTTTCTTTATAGTTATACCTATAATAATTGG
AGGTTTTGGAAATTGACTAGTACCTCTAATACTTGGAGCCCCTGATATAGCCTTTCCACG
AATAAATAATATAAGATTTTGACTTTTACCCCCTTCATTAACCCTTTTAATTTCGAGAAG
AATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTTTACCCCCCACTTTCATCTAATAT
CGCTCATAGAGGGTCATCAGTTGACTTAGCTATTTTCTCTTTACATCTTGCTGGTATTTC
CTCAATTCTTGGAGCAATTAATTTTATTACTACTATTATTAATATACGAATTAATAATAT
ATCTTTTGATCAAATACCTTTATTTGTATGAGCTGTAGGAATCACTGCTCTTCTTCTTCT
TCTTTCTTTACCTGTTCTTGCTGGAGCAATTACCATACTACTTACAGATCGAAATTTAAA
TACATCATT
```

Fig. 75. The partial sequence of mitochondrial cytochrome oxidase subunit I gene of *D. eucharis* CUJ1.

The conceptual translation of the consensus sequence of CUJ11 gave a peptide sequence.

>seq9 *Delias eucharis* CUJ11

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TLYFIFGIWSGMVGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
MMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSSIVENGAGTGWTV
YPPLSSNIAHSGSSVDLAIFSLHLAGISSILGAINFITTIINMRINNMSFDQMPLF
VWAVGITALLLLLSLPVLAGAITMLLTDRNLNTS
```

Fig. 76. The peptide sequence obtained from conceptual translation of consensus sequence of *D. eucharis* CUJ11.

Through the nucleotide BLAST conducted with consensus sequences obtained from *D. eucharis* CUJ11 is provided here under.

S. I. N o.	Subject Ids	% Ident ity	Alig nme nt lengt h	Mis mat ches	Gap open s	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|675401521|gb|KJ422911.1| Delias eucharis isolate F356*	100	611	0	0	35	645	1	611	1129
2	*gi|443611377|gb|JX978938.1| Delias eucharis isolate CMPM18_04*	99.83	597	1	0	62	658	4	600	1099
3	*gi|529386663|gb|KF404966.1| Delias mysis voucher 11ANIC-06886*	94.53	658	36	0	1	658	1	658	1016
4	*gi|545690595|gb|KF226392.1| Delias hyparete metarete voucher UMKL-JJW0064*	94.38	658	37	0	1	658	1	658	1011
5	*gi|393190343|gb|JX094279.1| Delias hyparete*	94.23	658	38	0	1	658	1480	213 7	1005
6	*gi|529370387|gb|KF396828.1| Delias argenthona voucher 11ANIC-06882*	93.62	658	42	0	1	658	1	658	983
7	*gi|443611363|gb|JX978931.1| Delias doylei isolate CJM-226-001*	94.00	617	37	0	42	658	1	617	937
8	*gi|443611369|gb|JX978934.1| Delias eileenae isolate CJM-116-001*	94.00	617	37	0	42	658	1	617	935
9	*gi|443611311|gb|JX978905.1| Delias argenthona isolate CMPM18_03*	93.52	617	40	0	42	658	1	617	922
10	*gi|529375337|gb|KF399303.1| Delias ennia voucher 11ANIC-06888*	91.64	658	55	0	1	658	1	658	911
11	*gi|443611319|gb|JX978909.1| Delias bagoe isolate CJM-089-001*	93.34	616	41	0	42	657	1	616	911
12	*gi|545690593|gb|KF226391.1| Delias agostina voucher UMKL-SJ008*	91.34	658	57	0	1	658	1	658	900

13	*gi|443611523|gb|JX979011.1| Delias timorensis isolate CMPM18_13*	93.50	600	39	0	59	658	1	600	893
14	*gi|443611497|gb|JX978998.1| Delias salvini isolate CJM-082-001*	93.90	590	36	0	42	631	1	590	893
15	*gi|302487945|gb|GU813976.1| Delias pasithoe voucher BX1*	91.98	636	51	0	23	658	1	636	893
16	*gi|443611335|gb|JX978917.1| Delias ceneus isolate CJM-096-001*	93.72	589	37	0	42	630	1	589	887
17	*gi|443611405|gb|JX978952.1| Delias hyparete isolate CJM-042-001*	93.56	590	38	0	42	631	1	590	883
18	*gi|443611307|gb|JX978903.1| Delias apatela isolate CJM-098-001*	92.32	612	47	0	47	658	6	617	870
19	*gi|443611469|gb|JX978984.1| Delias narses isolate CJM-057-002*	92.16	612	48	0	47	658	6	617	865

Table 15. The BLAST hit table of *D. eucharis* CUJ11.

The consensus sequence of CUJ11 was BLAST against database showed 100% identity with the *D. eucharis* isolate F356 (KJ422911) from Pune, 99.83% similarity with *D. eucharis* isolate CMPM18_04 (JX978938) from Australia and 94.53% with *D. mysis* voucher 11ANIC-06886 (KF404966) from Australia (table 15).

The sequences with highest similarities identified from the BLAST and compared with the sequences of *D. eucharis* CUJ11 for phylogenetic analysis with NJ-tree (Fig. 77). The CUJ11 was found placed along with *D. eucharis* (KJ422911) from Pune, India, and JX978938 from Australia in the same clade.

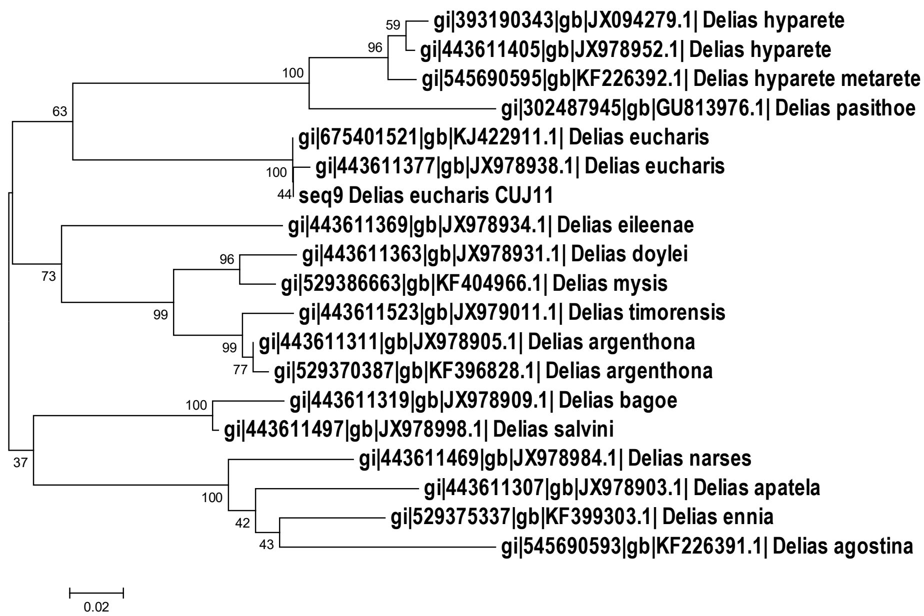

Fig. 77. NJ-tree with Phylogenetic relationship of *D. eucharis* CUJ11.

Discussion

The nucleotide BLAST analysis of COI sequence from *D. eucharis* CUJ11 against database showed 100% identity with the *D. eucharis* (KJ422911) from Pune hence it will be useful as a molecular barcode helping species identification. However, it has 99.83% similarity with *D. eucharis* (JX978938) from Australia this was a polymorphic novel variant of the gene. The phylogenetic tree developed with the sequence from CUJ11 was showed the CUJ11 along with *D. eucharis* (KJ422911) from Pune, India, and *D. eucharis* (JX978938) from Australia in the same clade. The distance data of the tree revealed that the species diverged from their closely related species *D. hyparete* about 82,000 years ago. The data supports the relation with South East Asia and the recent diversification of them associated with the vicariance and climatic changes in Deccan plateau.

3.12. *Eurema hecabe* (Linnaeus, 1758) isolates CUJ12 (KT880648)

The specimen CUJ12 was identified as *Eurema hecabe* (Linnaeus, 1758) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio hecabe* Linnaeus, 1758

Terias solifera Butler, 1875

Terias bisinuata Butler, 1876

Terias chalcomiaeta Butler, 1879

Terias dentilimbata Butler, 1879

Terias bewsheri Butler, 1879

Terias orientis Butler, 1888

Terias hecabe Staudinger, 1889

Terias aethiopica Trimen & Bowker, 1889

Terias butleri Trimen & Bowker, 1889

The *Eurema hecabe* is the Common Grass Yellow butterfly which is seen common in India it is very common in Kerala.

Upper side; yellow, rich lemon-yellow, fore wing; apex and termen brownish black, this colour continued narrowly along the costal margin to base of wing, becomes diffuse; the inner margin of the black area from costa to vein 4 oblique and irregular, between veins 2 and 4 excavate on the inner side, this excavation outwardly rounded between the veins and inwardly toothed on vein 3; below vein 2 the black area is suddenly dilated into a square spot which occupies the whole of the tornal angle (Fig. 78) the inner margin of this dilation is slightly

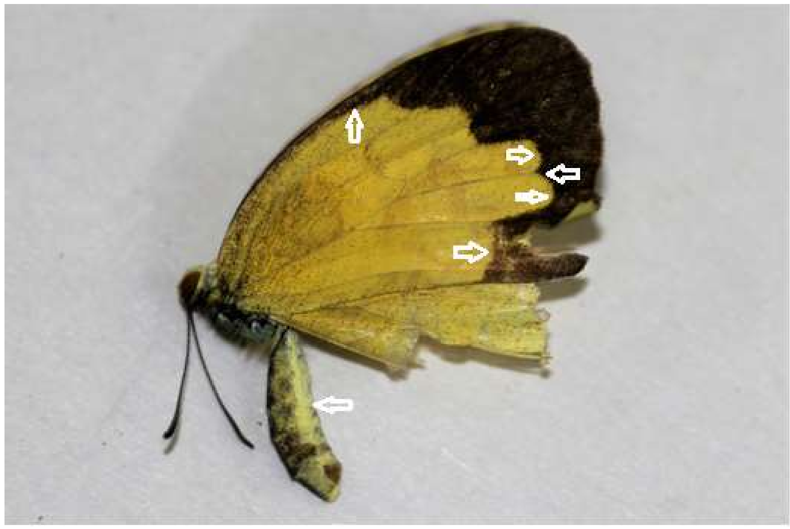

Fig 78 The under side of the *E. hecabe* CUJ12, Upper side; lemon-yellow, Fore wing; apex and termen brownish black, narrowly along the costal margin to base, between veins 2 and 4 excavate on the inner side, this excavation outwardly rounded between the veins and inwardly toothed on vein 3; below vein 2 the black area is suddenly dilated into a square spot which occupies the whole of the tornal angle, Antenna; greyish yellow, the club black; head, thorax and abdomen yellow, shaded with fuscous scales; beneath; palpi, thorax and abdomen yellowish white.

Fig. 79. The Habit of *E. hecabe* CUJ12

concave. Hind wing; terminal margin with a narrow black band attenuated anteriorly and posteriorly, dorsal margin; broadly paler than the ground-colour.

Under side; yellow, slightly paler than that of the upper side, with following reddish-brown markings, fore wing; two small specks in basal half of cell and a ring on the disco cellulars Hind wing; slightly curved sub basal series of three small spots, an irregular slender ring on the disco cellulars, followed by a highly irregular, curved, transverse, discal series of specks, all of which are often obsolescent. On both fore and hind wings the veins that attain the costal and terminal margins end in minute reddish-brown specks. Antenna; greyish yellow, the club black; head, thorax and abdomen yellow, shaded with fuscous scales; beneath; palpi, thorax and abdomen yellowish white (Fig. 78). The larval food plants involve *Cassia fistula, Cassia tora, Cassia alata, Cassia sophera, Cassia mimosoides* and *Moulluva spicata.* The larvae are dark green with white parallel lines.

The PCR amplification of partial COI sequence of *E. hecabe* CUJ12 from Aravanchal ($12^0$12'56"N, $75^0$16'55"E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 80 to 84).

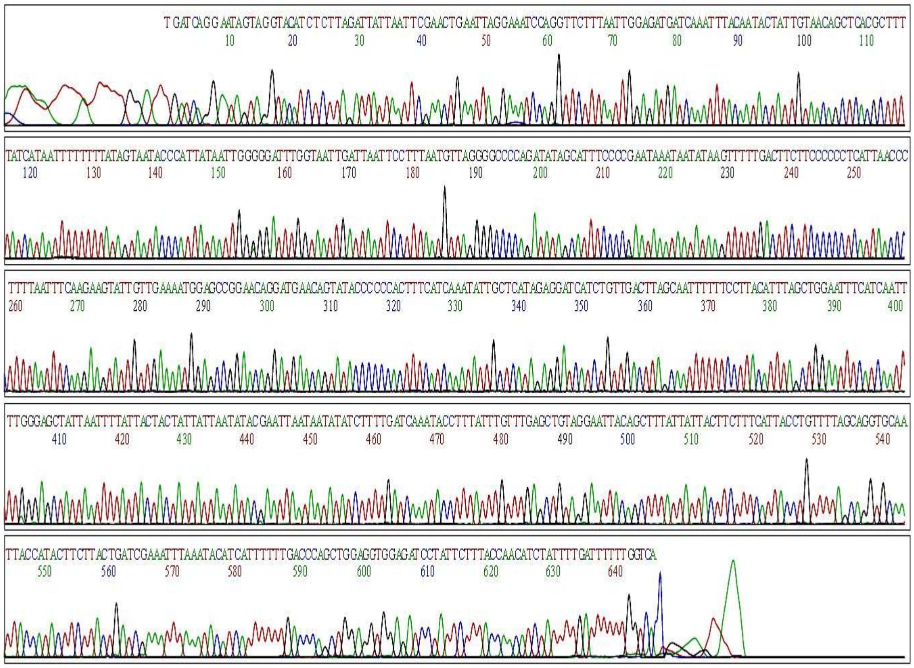

Fig. 80. The sequencing chromatogram of forward DNA strand of *E. hecabe* CUJ12.

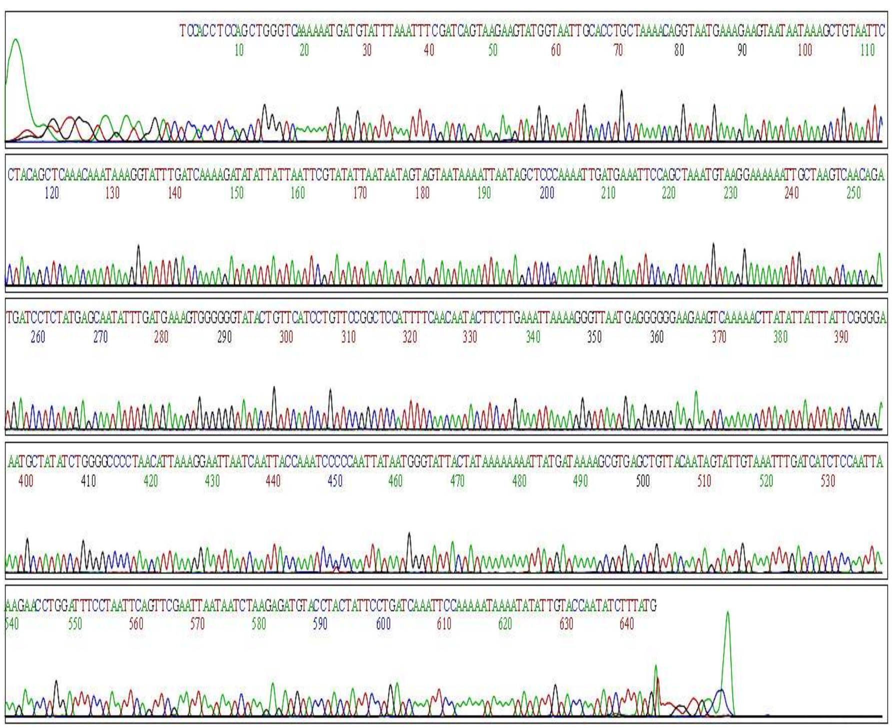

Fig. 81. The sequencing chromatogram of reverse DNA strand of *E. hecabe* CUJ12

```
>Eurema hecabe voucher CUJ12 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
TACAATATATTTTATTTTTGGAATTTGATCAGGAATAGTAGGTACATCTCTTAGATTATT
AATTCGAACTGAATTAGGAAATCCAGGTTCTTTAATTGGAGATGATCAAATTTACAATAC
TATTGTAACAGCTCACGCTTTTATCATAATTTTTTTTATAGTAATACCCATTATAATTGG
GGGATTTGGTAATTGATTAATTCCTTTAATGTTAGGGGCCCCAGATATAGCATTTCCCCG
AATAAATAATATAAGTTTTTGACTTCTTCCCCCCTCATTAACCCTTTTAATTTCAAGAAG
TATTGTTGAAAATGGAGCCGGAACAGGATGAACAGTATACCCCCCACTTTCATCAAATAT
TGCTCATAGAGGATCATCTGTTGACTTAGCAATTTTTTCCTTACATTTAGCTGGAATTTC
ATCAATTTTGGGAGCTATTAATTTTATTACTACTATTATTAATATACGAATTAATAATAT
ATCTTTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACAGCTTTATTATTACT
TCTTTCATTACCTGTTTTAGCAGGTGCAATTACCATACTTCTTACTGATCGAAATTTAAA
TACATCATT
```

Fig. 82. The partial sequence of mitochondrial COI gene of *E. hecabe* CUJ12

The conceptual translation of the consensus sequences of *E. hecabe* isolate CUJ12 gave following sequence (Fig. 83).

```
>seq10 Eurema hecabe CUJ12
TMYFIFGIWSGMVGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLIPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSSIVENGAGTGWTVYPPLSSNIAH
SGSSVDLAIFSLHLAGISSILGAINFITTIINMRINNMSFDQMPLFVWAVGITALLLLLSL
PVLAGAITMLLTDRNLNTS
```

Fig. 83. The peptide sequence obtained from conceptual translation of consensus sequence of *E. hecabe* CUJ12.

The nucleotide BLAST was conducted with the consensus sequence derived from *E. hecabe* CUJ12. The details about similarities of sequences obtained and alignment details were represented below.

S.I. No.	**Subject ids**	**% Identity**	**Alignment length**	**Mismatches**	**Gap opens**	**Q. start**	**Q. end**	**S. start**	**S. end**	**Bit score**
1	*gi|321485695|gb|HQ990344.1|Eurema hecabe voucher NIBGE BUT-00026*	100.00	658	0	0	1	658	1	658	1216

2	*gi|731772987|gb|KP216719.1|Eurema hecabe voucher A-11980*	100.00	624	0	0	35	658	1	624	1153
3	*gi|731772983|gb|KP216717.1|Eurema hecabe voucher A-11978*	100.00	617	0	0	42	658	1	617	1140
4	*gi|731772985|gb|KP216718.1|Eurema hecabe voucher A-11979*	100.00	612	0	0	47	658	1	612	1131
5	*gi|731772981|gb|KP216716.1|Eurema hecabe voucher A-11976*	100.00	612	0	0	47	658	1	612	1131
6	*gi|675401423|gb|KJ422862.1|Eurema hecabe isolate F618*	100.00	611	0	0	35	645	1	611	1129
7	*gi|675401659|gb|KJ422980.1|Eurema hecabe isolate F434*	100.00	609	0	0	35	643	1	609	1125
8	*gi|675401645|gb|KJ422973.1|Eurema hecabe isolate F459*	100.00	606	0	0	35	640	1	606	1120
9	*gi|675401599|gb|KJ422950.1|Eurema hecabe isolate F43*	100.00	603	0	0	35	637	1	603	1114
10	*gi|675401585|gb|KJ422943.1|Eurema hecabe isolate F47*	100.00	598	0	0	35	632	1	598	1105
11	*gi|675401491|gb|KJ422896.1|Eurema hecabe isolate F31*	100.00	591	0	0	35	625	1	591	1092
12	*gi|545690697|gb|KF226443.1|Eurema hecabe contubernalis voucher UMKL-JJW0086*	99.85	658	1	0	1	658	1	658	1210
13	*gi|316994110|gb|GU372559.1|Eurema hecabe*	99.85	658	1	0	1	658	1	658	1210
14	*gi|147724814|gb|EF584862.1|Eurema brigitta*	99.84	629	1	0	30	658	4	632	1157
15	*gi|675401757|gb|KJ423029.1|Eurema hecabe isolate EUUR7*	99.84	611	1	0	35	645	1	611	1123
16	*gi|675401667|gb|KJ422984.1|Eurema hecabe isolate F475*	99.84	611	1	0	35	645	1	611	1123
17	*gi|545690699|gb|KF226444.1|Eurema hecabe contubernalis voucher UMKL-JJW0077*	99.70	658	2	0	1	658	1	658	1205
18	*gi|545690685|gb|KF226437.1|Eurema ada iona voucher UMKL-JJW0069*	99.54	658	3	0	1	658	1	658	1199
19	*gi|529387481|gb|KF405375.1|Eurema hecabe voucher 11ANIC-06844*	99.39	657	4	0	1	657	1	657	1192
20	*gi|147724812|gb|EF584861.1|Eurema blanda*	99.36	628	4	0	31	658	3	630	1138
21	*gi|317467885|gb|HQ689635.1|Eurema hecabe voucher CNCLEP00056719*	98.78	658	8	0	1	658	1	658	1186
22	*gi|658132137|dbj|AB969804.1|Eurema blanda, isolate: Eub_Is02*	94.49	653	36	0	6	658	6	658	1007
23	*gi|321121947|gb|HQ962261.1|Eurema simulatrix voucher YB-KHC6764*	94.33	653	37	0	6	658	6	658	1002

Table 16. The BLAST hit table of *Eurema hecabe* isolate CUJ12

The consensus sequence of *E.hecabe* CUJ12 after removing the primer sequences BLAST against database (table 16) and showed 100% similarity to that of *E. hecabe* HQ990344 from Punjab of Pakistan, KP422980, KP2216717 and KP2216716 from Dehradun, KJ422980, KJ422973, KJ422950, KJ 422943, KJ422896 and KJ 422862 from Pune, India. Whereas 99% similarity with KF226443 from Malaysia, KJ422949 from Pune, GU372559 from Republic of Korea and EF584862 from P. R. of China. The pairwise BLAST analysis of *E. hecabe* CUJ12 collected from Aravanchal of Kannur District, Kerala and *E. hecabe* CUJ13 from Thalassery of Kannur District, Kerala showed only 99% identity. The sequences with top similarities with the sequences of *E. hecabe* CUJ12 and CUJ13 were identified from the BLAST results and were used for phylogenetic analysis, the NJ- trees derived were represented below (Fig. 84).

The phylogeny of *E. hecabe* CUJ12 was derived with NJ-tree developed from BLAST results along with CUJ12 (Fig. 84). The *E. hecabe* CUJ12 was found placed at a different position from CUJ13 in the tree. The CUJ12 found placed in a clade with 13 samples of *E. hecabe,* involving of HQ990344 from Punjab of Pakistan, KP422980, KP2216717 and KP2216716 from Dehradun, India, KJ422980, KJ422973, KJ422950, KJ 422943, KJ422896 and KJ 422862 from Pune, India. The CUJ13 with KF226443 from Malaysia, KJ422949 from Pune, GU372559 from Republic of Korea and EF584862 from P. R. of China.

The variation in nucleotides of COI gene of *E. hecabe* CUJ12 was analysed with multiple sequence alignments using ClustalW.

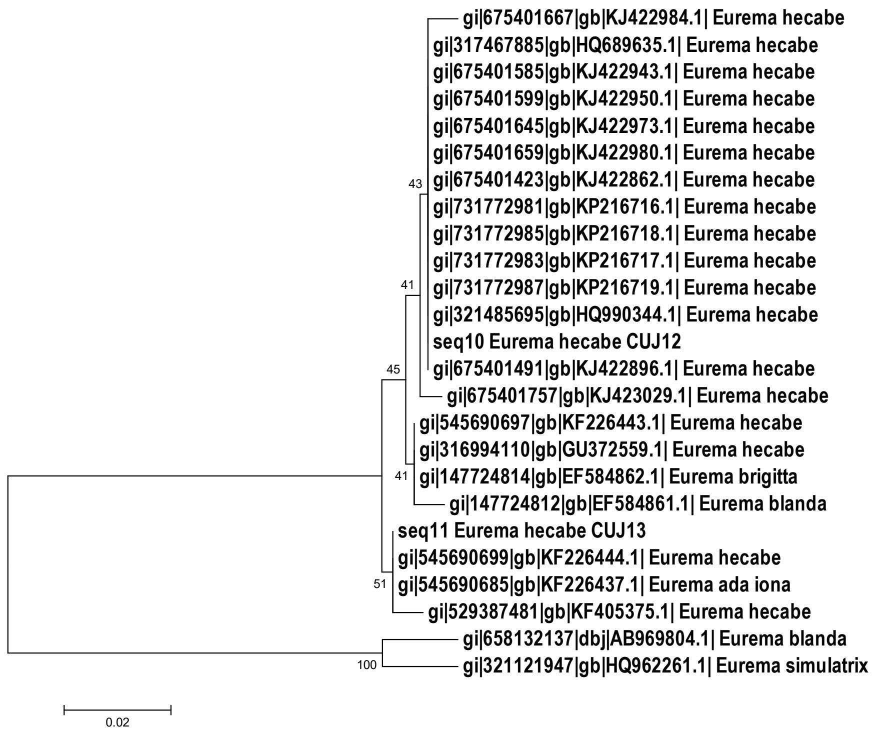

Fig. 84. NJ-tree with Phylogenetic relationship of *E. hecabe* CUJ12. The diagram revealed the dissimilarity in mitochondrial cytochrome oxidase subunit I gene of *Eurema hecabe* isolate CUJ12nucleotide sequences with CUJ13 they belongs to different positions in the diagram.

CUJ12	152	T	T	T	T	T	T	T	A	T	A	G	T	A	A	T	A	C	C	C	A	T	T	A	T	A	A	T	T	G	G	G	G	G	A
CUJ13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
HQ990344		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216719		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216717		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216718		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KJ422943		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KF226443		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
GU372559		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
KJ422984		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KF226444		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
CUJ12	405	C	A	T	T	T	A	G	C	T	G	G	A	A	T	T	T	C	A	T	C	A	A	T	T	T	T	G	G	G	A	G	C	T	A
CUJ13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
HQ990344		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216719		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216717		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216718		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KJ422943		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KF226443		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gGU372559		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KJ422984		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KF226444		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.

Fig. 85. The multiple sequence alignments showing nucleotide polymorphism *E. hecabe* CUJ12 and CUJ13. The variations involve two G to A transitions in different positions.

```
Query  1    TACAATATATTTTATTTTTGGAATTTGATCAGGAATAGTAGGTACATCTCTTAGATTATT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TACAATATATTTTATTTTTGGAATTTGATCAGGAATAGTAGGTACATCTCTTAGATTATT  60

Query  61   AATTCGAACTGAATTAGGAAATCCAGGTTCTTTAATTGGAGATGATCAAATTTACAATAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AATTCGAACTGAATTAGGAAATCCAGGTTCTTTAATTGGAGATGATCAAATTTACAATAC  120

Query  121  TATTGTAACAGCTCACGCTTTTATCATAAttttttttATAGTAATACCCATTATAATTGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TATTGTAACAGCTCACGCTTTTATCATAATTTTTTTTATAGTAATACCCATTATAATTGG  180

Query  181  GGGATTTGGTAATTGATTAATTCCTTTAATGTTAGGGGCCCCAGATATAGCATTTCCCCG  240
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AGGATTTGGTAATTGATTAATTCCTTTAATGTTAGGGGCCCCAGATATAGCATTTCCCCG  240

Query  241  AATAAATAATATAAGTTTTTGACTTCTTCCCCCCTCATTAACCCTTTTAATTTCAAGAAG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AATAAATAATATAAGTTTTTGACTTCTTCCCCCCTCATTAACCCTTTTAATTTCAAGAAG  300

Query  301  TATTGTTGAAAATGGAGCCGGAACAGGATGAACAGTATACCCCCCACTTTCATCAAATAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TATTGTTGAAAATGGAGCCGGAACAGGATGAACAGTATACCCCCCACTTTCATCAAATAT  360

Query  361  TGCTCATAGAGGATCATCTGTTGACTTAGCAATTTTTTCCTTACATTTAGCTGGAATTTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TGCTCATAGAGGATCATCTGTTGACTTAGCAATTTTTTCCTTACATTTAGCTGGAATTTC  420

Query  421  ATCAATTTTGGGAGCTATTAATTTTATTACTACTATTATTAATATACGAATTAATAATAT  480
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  ATCAATTTTAGGAGCTATTAATTTTATTACTACTATTATTAATATACGAATTAATAATAT  480

Query  481  ATCTTTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACAGCTTTATTATTACT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  ATCTTTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACAGCTTTATTATTACT  540

Query  541  TCTTTCATTACCTGTTTTAGCAGGTGCAATTACCATACTTCTTACTGATCGAAATTTAAA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  TCTTTCATTACCTGTTTTAGCAGGTGCAATTACCATACTTCTTACTGATCGAAATTTAAA  600

Query  601  TACATCATT  609
            |||||||||
Sbjct  601  TACATCATT  609
```

Fig. 86. *E. hecabe* CUJ12 and CUJ13, CO I gene partial cds; mitochondrial (609) pair wise BLAST alignment showing 99% similarity.

Discussion

The COI sequence of *E. hecabe* CUJ12, BLAST results showed 100% similarity to that of 10 isolates of *E. hecabe* deposited in databases. Therefore, the sequence isolated from CUJ12 can be useful as a molecular barcode helping identification of the species. Whereas 99% similarity with another four sequences of

E. hecabe involving KF226443 from Malaysia, KJ422949 from Pune, GU372559 from Republic of Korea, EF584862 from P. R. of China and the CUJ13 isolate from Thalassery of Kannur District. They were polymorphic novel sequences of the species. The pairwise BLAST analysis of *E .hecabe* CUJ12 collected from Aravanchal of Kannur District ($12^0$12'56"N, $75^0$16'55"E) and CUJ13 from Thalassery of Kannur District ($11^0$47'22"N, $75^0$28'22"E) Kerala, showed only 99% identity. This was an instance of nucleotide polymorphism the mutations involved were studied with multiple sequence alignments using ClustalW, (Fig. 85) the two different single nucleotide substitutions were involved, both with G to A transition. The involved mutations were silent and no peptide changes were noticed. The phylogeny of *E. hecabe* CUJ12 was derived with NJ-tree (Fig. 84). The *E. hecabe* CUJ12 was found placed at a different position from *E. hecabe* CUJ13 in the tree. The CUJ12 found in a clade with 13 samples of *E. hecabe.* The NJ-tree distance data revealed that the species was diverged from their closely related species *Eurema blanda* about 1,28,000 years ago. The CUJ12 was further diverged more than 10,000 years from CUJ13. This findings supported the distribution of ancestors of *E. hecabe* of India and Pakistan from South East Asia. The findings also supports the view of distribution of the species from North Pole to South East Asia (Condamine, 2013). In the Phylogenetic tree the more closely related sequence of *E. hecabe* was from Pune belonging to Western Ghats, Pakistan and Dehradun with 100% similarity at the same time the populations confirmed the presence of polymorphism of COI sequences with 99% identity in the same geographical location. The distance data of the tree also revealed a recent diversification of the genus in Western Ghats about 80,000 years ago.

3.13. *Eurema hecabe* (Linnaeus, 1758) isolates CUJ13, (KT880649)

The specimen CUJ12 was identified as *Eurema hecabe* (Linnaeus, 1758) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio hecabe* Linnaeus, 1758
Terias hecabe
Terias solifera Butler, 1875
Terias bisinuata Butler, 1876
Terias chalcomiaeta Butler, 1879
Terias dentilimbata Butler, 1879
Terias bewsheri Butler, 1879
Terias orientis Butler, 1888
Terias aethiopica Trimen & Bowker, 1889
Terias butleri Trimen & Bowker, 1889

The *Eurema hecabe* is the Common Grass Yellow butterfly which is seen common in India it is very common in Kerala.

Upper side; yellow, rich lemon-yellow, Fore wing; apex and termen brownish black, this colour continued narrowly along the costal margin to base of wing, becomes diffuse; the inner margin of the black area from costa to vein 4 oblique and irregular, between veins 2 and 4 excavate on the inner side, this excavation outwardly rounded between the veins and inwardly toothed on vein 3; below vein 2 the black area is suddenly dilated into a square spot which occupies the whole of the tornal angle (Fig. 87); the inner margin of this dilatation is slightly concave. Hind wing; terminal margin with a narrow black band attenuated anteriorly and posteriorly, dorsal margin; broadly paler than the ground-colour.

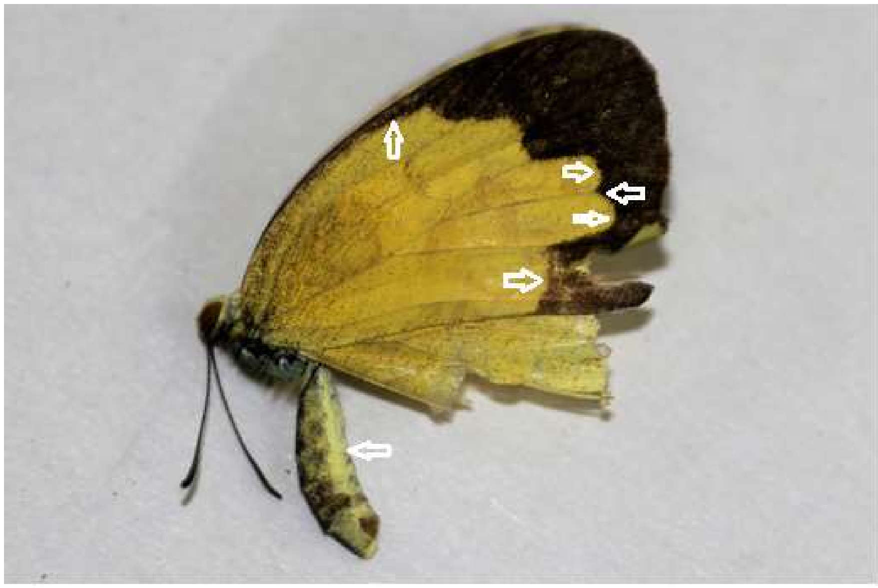

Fig 87 The under side of the *E. hecabe* CUJ13 Upper side; lemon-yellow, Fore wing; apex and termen brownish black, narrowly along the costal margin to base, between veins 2 and 4 excavate on the inner side, this excavation outwardly rounded between the veins and inwardly toothed on vein 3; below vein 2 the black area is suddenly dilated into a square spot which occupies the whole of the tornal angle, Antenna; greyish yellow, the club black; head, thorax and abdomen yellow, shaded with fuscous scales; beneath; palpi, thorax and abdomen yellowish white.

Fig. 88. The Habit of *E. hecabe* CUJ13

Under side; yellow, slightly paler than that of the upper side, with following reddish-brown markings, fore wing; two small specks in basal half of cell and a ring on the disco cellulars Hind wing; slightly curved sub basal series of three small spots, an irregular slender ring on the disco cellulars, followed by a highly irregular, curved, transverse, discal series of specks, all of which are often obsolescent On both fore and hind wings the veins that attain the costal and terminal margins end in minute reddish-brown specks

Antenna; greyish yellow, the club black; head, thorax and abdomen yellow, shaded with fuscous scales; beneath; palpi, thorax and abdomen yellowish white (Fig. 87) The larval food plants involve *Cassia fistula, Cassia tora, Cassia alata, Cassia sophera, Cassia mimosoides* and *Moulluva spicata*. The larvae are dark green with white parallel lines.

The PCR amplification of partial COI sequence of *E. hecabe* CUJ13 from Thalassery (11^{0}47'22"N, 75^{0}28'22"E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 89-93).

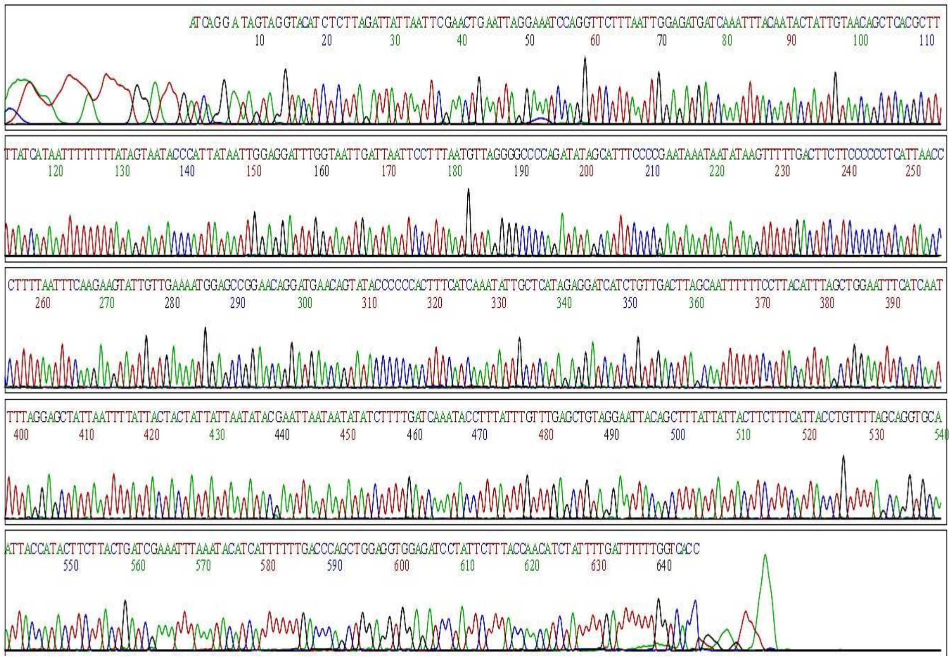

Fig. 89. The sequencing chromatogram of forward DNA strand of *E. hecabe* CUJ13.

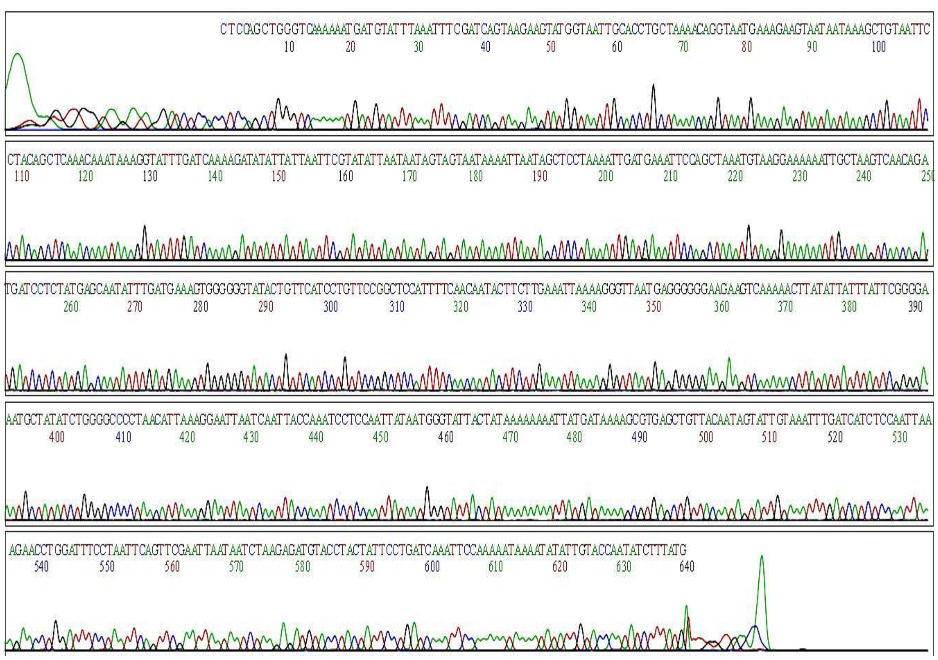

Fig. 90. The sequencing chromatogram of reverse direction DNA strand of *E. hecabe* CUJ13.

```
>Eurema hecabe voucher CUJ13 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
TACAATATATTTTATTTTTGGAATTTGATCAGGAATAGTAGGTACATCTCTTAGATTATT
AATTCGAACTGAATTAGGAAATCCAGGTTCTTTAATTGGAGATGATCAAATTTACAATAC
TATTGTAACAGCTCACGCTTTTATCATAATTTTTTTATAGTAATACCCATTATAATTGG
AGGATTTGGTAATTGATTAATTCCTTTAATGTTAGGGGCCCCAGATATAGCATTTCCCCG
AATAAATAATATAAGTTTTTGACTTCTTCCCCCCTCATTAACCCTTTTAATTTCAAGAAG
TATTGTTGAAAATGGAGCCGGAACAGGATGAACAGTATACCCCCCACTTTCATCAAATAT
TGCTCATAGAGGATCATCTGTTGACTTAGCAATTTTTTCCTTACATTTAGCTGGAATTTC
ATCAATTTTAGGAGCTATTAATTTTATTACTACTATTATTAATATACGAATTAATAATAT
ATCTTTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACAGCTTTATTATTACT
TCTTTCATTACCTGTTTTAGCAGGTGCAATTACCATACTTCTTACTGATCGAAATTTAAA
TACATCATT
```

Fig. 91. The partial sequence of mitochondrial cytochrome oxidase subunit I gene of *E. hecabe* CUJ13.

The conceptual translation of the consensus sequences of *E. hecabe* CUJ13 gave a peptide sequence.

```
>seq11 Eurema hecabe CUJ13
TMYFIFGIWSGMVGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLIPLMLGAPDMAFPRMNNMSFWLLPPSLTLLISSSIVENGAGTGWTVYPPLSSNIAH
SGSSVDLAIFSLHLAGISSILGAINFITTIINMRINNMSFDQMPLFVWAVGITALLLLLSL
PVLAGAITMLLTDRNLNTS
```

Fig. 92. The peptide sequence obtained from conceptual translation of consensus sequence of *E. hecabe* CUJ13.

The nucleotide BLAST was conducted with the consensus sequence derived from *E. hecabe* CUJ13. The details about similarities of sequences obtained and alignment details were represented below.

SI No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|545690699|gb|KF226444.1|Eurema hecabe contubernalis voucher UMKL-JJW0077*	100.00	609	0	0	1	609	1	609	1125
2	*gi|675401699|gb|KJ423000.1|Eurema hecabe isolate F201*	100.00	575	0	0	35	609	1	575	1062
3	*gi|675401647|gb|KJ422974.1|Eurema*	100.00	575	0	0	35	609	1	575	1062

	hecabe isolate F345									
4	*gi|675401499|gb|KJ422900.1|Eurema hecabe isolate F347*	100.00	575	0	0	35	609	1	575	1062
5	*gi|675401439|gb|KJ422870.1|Eurema hecabe isolate A17*	100.00	575	0	0	35	609	1	575	1062
6	*gi|545690701|gb|KF226445.1|Eurema hecabe contubernalis voucher UMKL-JJW0078*	100.00	564	0	0	1	564	1	564	1042
7	*gi|545690697|gb|KF226443.1|Eurema hecabe contubernalis voucher UMKL-JJW0086*	99.84	609	1	0	1	609	1	609	1120
8	*gi|545690685|gb|KF226437.1|Eurema ada iona voucher UMKL-JJW0069*	99.84	609	1	0	1	609	1	609	1120
9	*gi|316994110|gb|GU372559.1|Eurema hecabe*	99.84	609	1	0	1	609	1	609	1120

Table 17. The BLAST hit tableof *E. hecabe* CUJ13.

The COI sequence of *E. hecabe* CUJ13, BLAST against database and showed 100% similarity to that of *E. hecabe* KJ422974, KJ422900 and KJ422870 from Pune, India. Whereas 99% similarity with KF226445, KF226443, KF226437 from Malaysia and GU372559 from Republic of Korea.

The phylogeny of *E. hecabe.* Isolate CUJ13 was derived with NJ-tree developed from the similar sequences identified from BLAST results along with *E. hecabe* isolate CUJ13 (Fig. 93). The CUJ13 was found at a different position from that of CUJ12 in the tree. The CUJ13 was found placed in a clade with *E. hecabe* KJ422974, KJ422900 and KJ422870 from Pune, India.

The variation in nucleotides of cytochrome oxidase subunit I gene of *E. hecabe* CUJ12 was analysed with multiple sequence alignments using ClustalW.

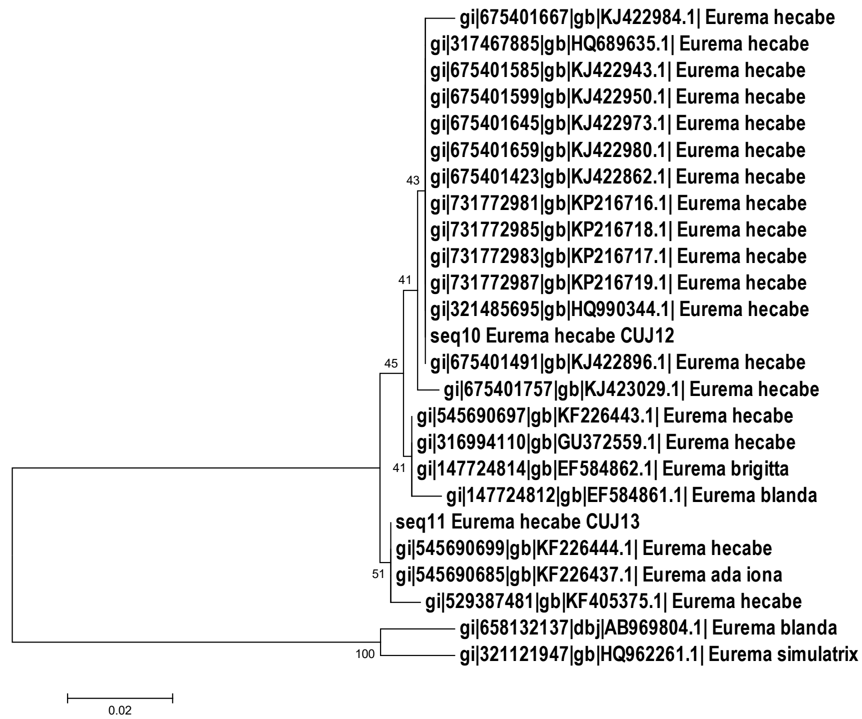

Fig.93 NJ-tree with Phylogenetic relationship of *E. hecabe* CUJ12. The diagram revealed the dissimilarity in mitochondrial cytochrome oxidase subunit I gene of *E. hecabe* CUJ12 and CUJ13 they belongs to different positions in the diagram.

CUJ12	152	T	T	T	T	T	T	T	A	T	A	G	T	A	A	T	A	C	C	C	A	T	T	A	T	A	A	T	T	G	G	G	G	G	A
CUJ13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
HQ990344		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216719		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216717		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216718		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KJ422943		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KF226443		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
GU372559		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
KJ422984		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KF226444		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
CUJ12	405	C	A	T	T	T	A	G	C	T	G	G	A	A	T	T	T	C	A	T	C	A	A	T	T	T	T	G	G	G	A	G	C	T	A
CUJ13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
HQ990344		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216719		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216717		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP216718		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KJ422943		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KF226443		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
gGU372559		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KJ422984		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KF226444		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.

Fig. 94. The multiple sequence alignments showing nucleotide polymorphism *Eurema hecabe* isolate CUJ12 and CUJ13. The variation involved two G to A transitions in different positions.

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Query  1    TACAATATATTTTATTTTTGGAATTTGATCAGGAATAGTAGGTACATCTCTTAGATTATT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TACAATATATTTTATTTTTGGAATTTGATCAGGAATAGTAGGTACATCTCTTAGATTATT  60

Query  61   AATTCGAACTGAATTAGGAAATCCAGGTTCTTTAATTGGAGATGATCAAATTTACAATAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AATTCGAACTGAATTAGGAAATCCAGGTTCTTTAATTGGAGATGATCAAATTTACAATAC  120

Query  121  TATTGTAACAGCTCACGCTTTTATCATAAttttttttATAGTAATACCCATTATAATTGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TATTGTAACAGCTCACGCTTTTATCATAATTTTTTTTATAGTAATACCCATTATAATTGG  180

Query  181  GGGATTTGGTAATTGATTAATTCCTTTAATGTTAGGGGCCCCAGATATAGCATTTCCCCG  240
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AGGATTTGGTAATTGATTAATTCCTTTAATGTTAGGGGCCCCAGATATAGCATTTCCCCG  240

Query  241  AATAAATAATATAAGTTTTTGACTTCTTCCCCCCTCATTAACCCTTTTAATTTCAAGAAG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AATAAATAATATAAGTTTTTGACTTCTTCCCCCCTCATTAACCCTTTTAATTTCAAGAAG  300

Query  301  TATTGTTGAAAATGGAGCCGGAACAGGATGAACAGTATACCCCCCACTTTCATCAAATAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TATTGTTGAAAATGGAGCCGGAACAGGATGAACAGTATACCCCCCACTTTCATCAAATAT  360

Query  361  TGCTCATAGAGGATCATCTGTTGACTTAGCAATTTTTTCCTTACATTTAGCTGGAATTTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TGCTCATAGAGGATCATCTGTTGACTTAGCAATTTTTTCCTTACATTTAGCTGGAATTTC  420

Query  421  ATCAATTTTGGGAGCTATTAATTTTATTACTACTATTATTAATATACGAATTAATAATAT  480
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  ATCAATTTTAGGAGCTATTAATTTTATTACTACTATTATTAATATACGAATTAATAATAT  480

Query  481  ATCTTTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACAGCTTTATTATTACT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  ATCTTTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACAGCTTTATTATTACT  540

Query  541  TCTTTCATTACCTGTTTTAGCAGGTGCAATTACCATACTTCTTACTGATCGAAATTTAAA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  TCTTTCATTACCTGTTTTAGCAGGTGCAATTACCATACTTCTTACTGATCGAAATTTAAA  600

Query  601  TACATCATT  609
            |||||||||
Sbjct  601  TACATCATT  609
```

Fig. 95 *E. hecabe* CUJ12 and CUJ13 cytochrome oxidase subunit I gene, partial cds; mitochondrial (609) pair wise BLAST alignment showing 99% similarity.

Discussion

The pairwise alignment of CUJ12 collected from Aravanchal of Kannur District, Kerala and CUJ13 from Thalassery of Kannur District, Kerala (Table No.17) showed only 99% identity. This was an instance of nucleotide polymorphism the mutations involved were studied with multiple sequence alignments using ClustalW, (Fig. 94) the two different single nucleotide substitutions were involved, both with G to A transition. The involved mutations were silent and no peptide changes were noticed. The BLAST results of CUJ12 and CUJ13 were identified as two variable forms among the populations irrespective of their geographical locations both forms were found in Western Ghats even in a district. The COI sequnces isolated from CUJ12 and CUJ13 can be useful as a molecular barcode to segregate both varients from other populations. The phylogeny of *E. hecabe* CUJ13 was derived with NJ-tree along with *E. hecabe* CUJ12 (Fig. 93). The CUJ12 was found at a different position from that of CUJ13 in the tree. The NJ-tree distance data revealed that the species was diverged from their closely related species *Eurema blanda* about 1,28,000 years ago. The CUJ13 further diverged more than 10,000 years from CUJ12. These findings suggest the distribution of ancestors of *E. hecabe* of India and Pakistan was South East Asia. The finding also supports the view of distribution of the species from North Pole to South East Asia. In the Phylogenetic tree the more closely related sequence of *E. hecabe* was from Pune belonging to Western Ghats, Pakistan and Dehradun, India with 100% similarity at the same time the populations confirmed the presence of polymorphism of COI sequences with 99% identity in the same geographical location. The distance data of the tree also revealed a recent diversification of the genus in Western Ghats about 80,000 years ago.

3.14. *Orsotriaena medus* (Fabricius, 1775) isolate CUJ14 (KT880650)

The specimen CUJ14 was identified as *Orsotriaena medus* (Fabricius, 1775) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio medus* Fabricius, 1775

Mycalesis gamaliba Butler, 1867

Mycalesis mandosa Butler, 1868

The *Orsotriaena medus* is dark grass-brown butterfly which is commonly found in South Asia, Southeast Asia, and Australia. The butterfly has historically been called the Nigger but it has been renamed as Smooth-eyed Bush brown.

Upper side; dark brown with a thin marginal pale border, upper hind wing having a thin sub marginal line no ocellus on the upper side of the wings.

Underside; a white discal band which runs across both wings, five ocelli marginaly on the under sides of both wings. In the fore wing; has two ocelli, anterior ocellus slightly smaller. In hind wing; two ocelli, one on apical region, another ocellus in the tornal region. The uppermost ocellus in the hind wing is greatly smaller, while the remaining ocelli are of equal sizes (Fig. 96)

The larvae feed on grasses, including rice plants (*Oryza sativa*), sugarcanes (*Saccharum officinarum*), and para grass (*Brachiaria mutica*). The eggs are spherical and yellowish, laid on the leaf blades and stems of grasses. The larvae are spindle-shaped, wrinkled, and covered with small tubercles, giving it a rough appearance. The colour above is rosy-red with a blue dorsal and a white lateral line, below which, the underparts are green , two long brown spines on the head pointed forwards, while a pair of pinkish prongs project from the anal segment.

Fig. 96. The underside of the *O. medus* CUJ14. A white discal band which runs across both wings, five ocelli marginally on the under sides of both wings, dark brawn colour, thin pale marginal boarder.

Fig. 97. The habit of the *O. medus* CUJ14.

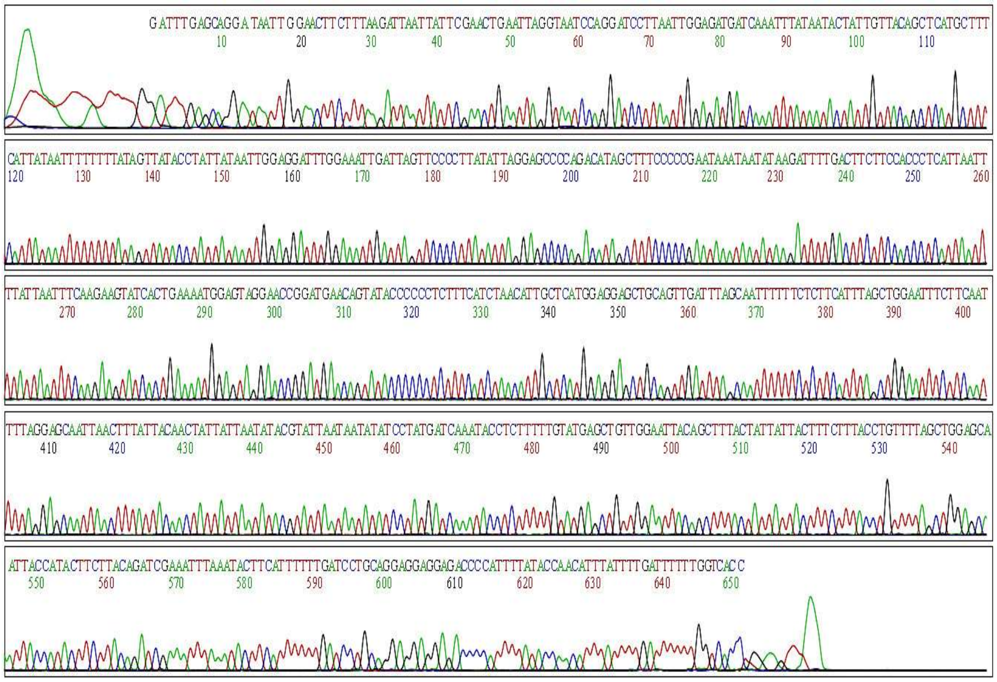

Fig. 98 The sequencing chromatogram of forward DNA strand of *O. medus* CUJ14.

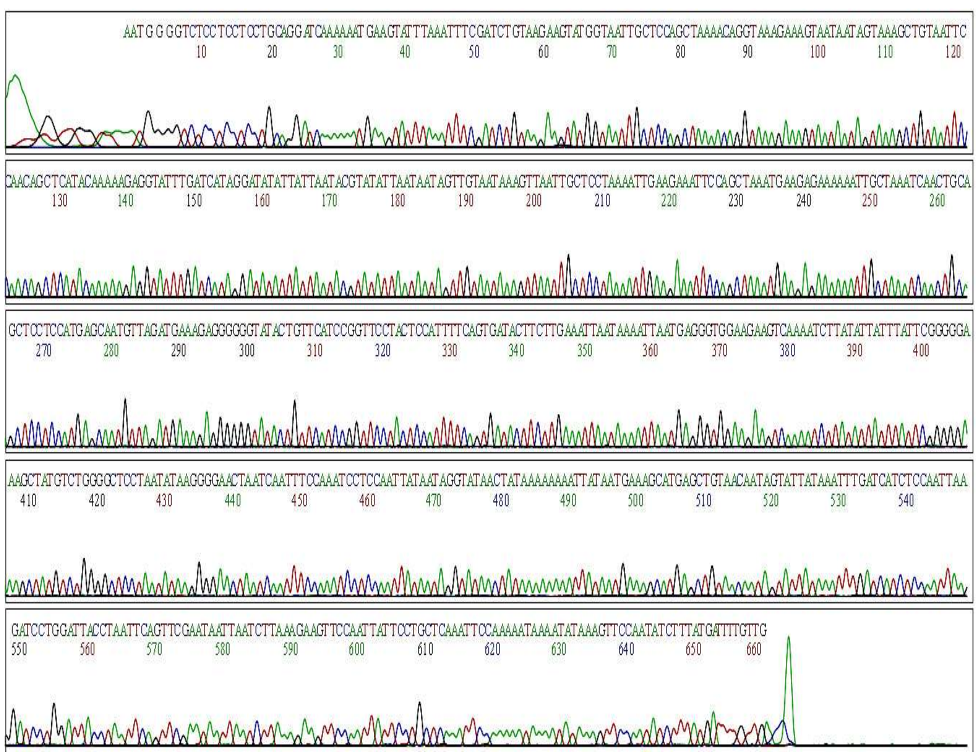

Fig. 99. The sequencing chromatogram of reverse DNA strand of *O. medus* CUJ14.

The PCR amplification of partial COI sequence of *O. medus* CUJ14 from Aravanchal (12^{0}12'57"N, 75^{0}16'51"E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 98-102).

>Orsotriaena medus voucher CUJ14 cytochrome oxidase subunit I gene, partial cds; mitochondrial(609)
AACTTTATATTTTATTTTTGGAATTTGAGCAGGAATAATTGGAACTTCTTTAAGATTAAT
TATTCGAACTGAATTAGGTAATCCAGGATCCTTAATTGGAGATGATCAAATTTATAATAC
TATTGTTACAGCTCATGCTTTCATTATAATTTTTTTTATAGTTATACCTATTATAATTGG
AGGATTTGGAAATTGATTAGTTCCCCTTATATTAGGAGCCCCAGACATAGCTTTCCCCCG
AATAAATAATATAAGATTTTGACTTCTTCCACCCTCATTAATTTTATTAATTTCAAGAAG
TATCACTGAAAATGGAGTAGGAACCGGATGAACAGTATACCCCCCTCTTTCATCTAACAT
TGCTCATGGAGGAGCTGCAGTTGATTTAGCAATTTTTTCTCTTCATTTAGCTGGAATTTC
TTCAATTTTAGGAGCAATTAACTTTATTACAACTATTATTAATATACGTATTAATAATAT
ATCCTATGATCAAATACCTCTTTTTGTATGAGCTGTTGGAATTACAGCTTTACTATTATT
ACTTTCTTTACCTGTTTTAGCTGGAGCAATTACCATACTTCTTACAGATCGAAATTTAAA
TACTTCATT

Fig. 100. The partial sequence of mitochondrial COI gene of *O. medus* CUJ14.

The conceptual translation of the consensus sequence of *O. medus* CUJ14 gave the following peptide sequence.

>seq12 *Orsotriaena medus* CUJ14

TLYFIFGIWAGMIGTSLSLIIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLILLISSSITENGVGTGWTV
YPPLSSNIAHGGAAVDLAIFSLHLAGISSILGAINFITTIINMRINNMSYDQMPLF
VWAVGITALLLLLSLPVLAGAITMLLTDRNLNTS

Fig. 101. The peptide sequence obtained from conceptual translation of consensus sequence of *O. medus* CUJ14.

The nucleotide BLAST was conducted with the consensus sequence derived from *O. medus* CUJ14. The details of sequence similarities obtained and their alignment details represented below (table 18).

S. I. N o.	Subject Ids	% identi ty	Align ment length	Mi sm atc hes	Ga p op ens	Q. sta rt	Q. end	S. sta rt	S. end	Bit scor e
1	*gi|330600380|gb|GU012586.1|Orsotriaena medus voucher F372*	100.00	644	0	0	1	644	6	649	1190
2	*gi|657172811|gb|KJ459854.1|Orsotriaena medus isolate OR45*	100.00	624	0	0	35	658	1	624	1153
3	*gi|85013872|gb|DQ338766.1|Orsotriaena medus*	99.84	630	1	0	29	658	4	633	1158
4	*gi|545690917|gb|KF226553.1|Orsotriaena medus cinerea voucher UMKL-JJW0258*	99.70	658	2	0	1	658	1	658	1205
5	*gi|657172751|gb|KJ459824.1|Orsotriaena medus isolate T4*	99.68	624	2	0	35	658	1	624	1142
6	*gi|558413324|dbj|AB863289.1|Orsotriaena medus medus*	99.54	647	2	1	12	658	1	646	1177
7	*gi|330600403|gb|GU012598.1|Orsotriaena medus voucher F377*	99.38	647	0	4	7	649	1	647	1170
8	*gi|317185814|gb|HM240615.1|Orsotriaena jopas voucher UK5-24Orsotriaena jopas voucher UK5-24*	96.05	658	26	0	1	658	13	670	1072
9	*gi|747021398|gb|KM572807.1|Aphantopus hyperantus voucher TLMF Lep 05821*	90.59	659	60	2	1	658	1	658	872

Table 18. The BLAST hit table of *O. medus* CUJ14.

The sequence of *O. medus* CUJ14 BLAST against database and showed 100% similarity to GU012586 and KJ459854 from Pune, India. It has 99% similarity with DQ338766 from Sweden, KF226553 from Malaysia and KJ459824 from Pune, India.

The phylogeny of *O. medus* CUJ14 was derived with NJ-tree using related sequences from database (Fig. 102). The CUJ14 was found placed in a clade with 5 samples involving GU012586, KJ459854 from Pune, DQ338766 from Sweden and KF226553 from Malaysia.

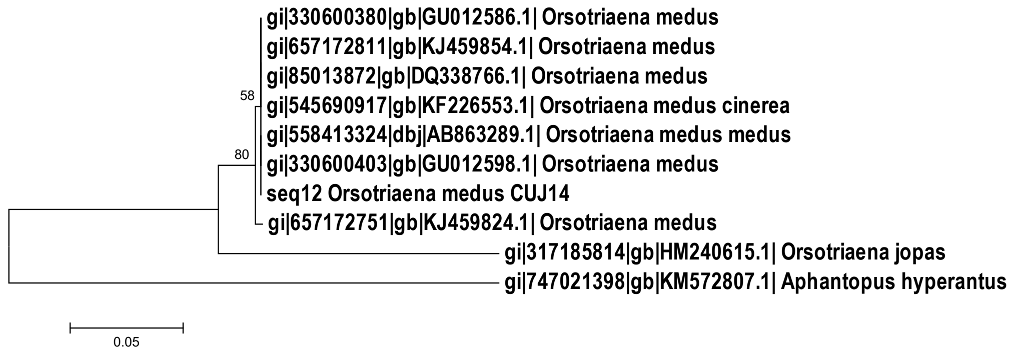

Fig. 102. NJ-tree with Phylogenetic relationship of *O. medus* CUJ14.

Discussion

The BLAST results of *O. medus* CUJ14 against database showed 100% similarity to that of GU012586 and KJ459854 from Pune, India. Therefore, the COI sequence of CUJ14 will be a molecular barcode helping identification of the species. It has 99% similarity with DQ338766 from Sweden, KF226553 from Malaysia and KJ459824 from Pune, India. They were polymorphic novel sequences. The CUJ14 was found in a clade with five samples involving GU012586, KJ459854 from Pune, DQ338766 from Sweden KF226553 from Malaysia and KJ459824 from Pune in the adjacent clade. The NJ-tree distance data revealed that the CUJ14 diverged from their closely related species *Orsotriaena jopas* HM240615 about 35,000 years ago. The presence of *O. medus* KJ459824 from Pune, India, with 99.68% similarity suggested the presence of variability among the samples from Western Ghats. *O. medus* is a widely distributed butterfly species. The close relationship in COI sequence of *O. medus* CUJ14 with that of KF226553 from Malaysia, suggesting the association of Western Ghat population with South East Asia.

3.15. ***Cynitia lepidea* (Butler, 1868), isolate CUJ15 (KT880651)**

The specimen CUJ15 was identified as *Cynitia lepidea* (Butler 1868) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonym: *Tanaecia lepidea* (Butler, 1868)

Euthalia lepidea, (Butler, 1868)

Cynitia lepidea, the Grey Count butterfly which is a Nymphalidae butterfly found in South and South East Asia.

Upper side; dark brown, with very vague black markings of transverse lines across the cells of both fore and hind wings, an oblique discal fascia on the fore wing. An ash-grey continuous band along the termen of both fore and hind wings, gradually broadening from the apex of the fore wing, where it is very narrow, to the tornus of the hind wing, where it covers about one-third of the wing, white cilia present. Antennae, head, thorax and abdomen; dark brown above, beneath. Antennae; ochraceous, the rest of the body dusky white washed with ochraceous (Fig. 103).

Under side; ochraceous brown, the colours paler on hind wing; the fore wing somewhat narrow, hind wing much more broadly suffused with lilacine-grey on the terminal margins and along the dorsal margin of the hind wing. Cells of both wings; with dark brown sinuous transverse lines and loop like markings. Both fore and hind wings; crossed by somewhat diffuse broad discal and narrower postdiscal dark bands, prominent on the fore wing, obscure on the hind wing, a patch of specialized dark scales above vein 4 on the upper side of the hind wing.

Fig. 103 The upper side of the *C. lepidea* CUJ15, Upper side dark brown, with vague black markings of transverse lines across the cells of both fore and hind wings, an ash-grey continuous band along the termen of both fore and hind wings which gradually broadening from the apex of the fore wing to the tornus of the hind wing.

Fig.104 The habit of the *C. lepidea* CUJ15.

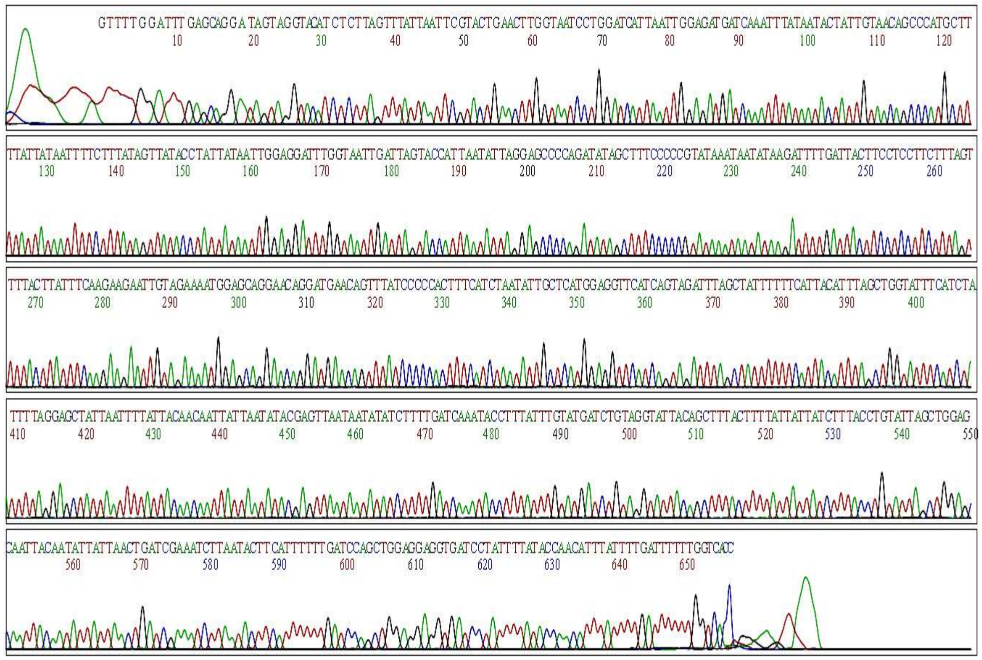

Fig. 105 The sequencing chromatogram of forward DNA strand of *C. lepidea* CUJ15.

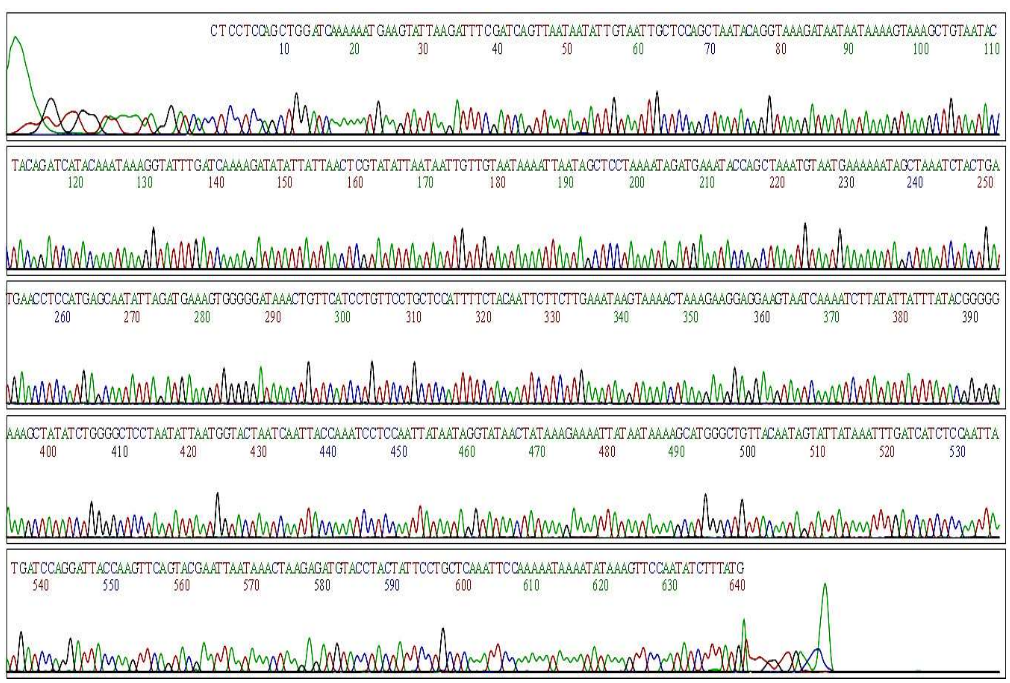

Fig. 106. The sequencing chromatogram of reverse DNA strand of *C. lepidea* CUJ15.

The larval food plants involve *Careya arborea,* and *Melastoma malabathricum* the larvae are bluish green in colour, the pupae is green coloured with golden spots.

The PCR amplification of partial COI sequence of *C. lepidea* CUJ15 from Aravanchal (12^{0}12'57"N, 75^{0}16'53"E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 105-109).

```
> Cynitia lepidea voucher CUJ15 cytochrome oxidase subunit I gene,
partial cds; mitochondrial(609)
AACTTTATATTTTATTTTTGGAATTTGAGCAGGAATAGTAGGTACATCTCTTAGTTTATT
AATTCGTACTGAACTTGGTAATCCTGGATCATTAATTGGAGATGATCAAATTTATAATAC
TATTGTAACAGCCCATGCTTTTATTATAATTTTCTTTATAGTTATACCTATTATAATTGG
AGGATTTGGTAATTGATTAGTACCATTAATATTAGGAGCCCCAGATATAGCTTTCCCCCG
TATAAATAATATAAGATTTTGATTACTTCCTCCTTCTTTAGTTTTACTTATTTCAAGAAG
AATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTTTATCCCCCACTTTCATCTAATAT
TGCTCATGGAGGTTCATCAGTAGATTTAGCTATTTTTTCATTACATTTAGCTGGTATTTC
ATCTATTTTAGGAGCTATTAATTTTATTACAACAATTATTAATATACGAGTTAATAATAT
ATCTTTTGATCAAATACCTTTATTTGTATGATCTGTAGGTATTACAGCTTTACTTTTATT
ATTATCTTTACCTGTATTAGCTGGAGCAATTACAATATTATTAACTGATCGAAATCTTAA
TACTTCATT
```

Fig. 107. The partial sequence of mitochondrial COI gene of *C. lepidea* CUJ15.

The conceptual translation of the consensus sequence of *C. lepidea* CUJ15 gave the following peptide sequence.

```
>seq13 Cynitia lepidea CUJ15
TLYFIFGIWAGMVGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLVLLISSSIVENGAGTGWTVYPPLSSNIAH
GGSSVDLAIFSLHLAGISSILGAINFITTIINMRVNNMSFDQMPLFVWSVGITALLLLLSL
PVLAGAITMLLTDRNLNTS
```

Fig. 108. The peptied sequence obtained from conceptual translation of consensus sequence of *C. lepidea* isolate CUJ15.

The nucleotide BLAST was conducted with the consensus sequence derived from *C. lepidea* CUJ15. The details about similarities of sequences obtained and alignment details are represented below.

S.I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|330600314|gb|GU012547.1|Cynitia lepidea voucher D255*	100.00	635	0	0	1	635	14	648	1173
2	*gi|657172665|gb|KJ459781.1|Cynitia lepidea isolate F379*	100.00	624	0	0	35	658	1	624	1153
3	*gi|321121661|gb|HQ962118.1|Tanaecia iapis voucher YB-KHC3351*	99.85	658	1	0	1	658	1	658	1210
4	*gi|545690559|gb|KF226374.1|Cynitia lepidea voucher UMKL-JJW0327*	99.53	633	3	0	1	633	1	633	1153
5	*gi|545690563|gb|KF226376.1|Cynitia lepidea voucher UMKL-JJW0324*	99.52	629	3	0	1	629	1	629	1146
6	*gi|545690561|gb|KF226375.1|Cynitia lepidea voucher UMKL-JJW0326*	99.52	628	3	0	1	628	1	628	1144
7	*gi|330600307|gb|GU012543.1|Cynitia lepidea voucher D238*	99.52	625	0	3	11	632	1	625	1134
8	*gi|545690557|gb|KF226373.1|Cynitia lepidea voucher UMKL-JJW0311*	99.39	658	4	0	1	658	1	658	1194
9	*gi|321122076|gb|HQ962324.1|Tanaecia iapis voucher YB-KHC6667*	99.39	658	4	0	1	658	1	658	1194
10	*gi|374857580|dbj|AB511420.1|Cynitia lepidea cognata; isolate: MY-Ny21*	97.87	658	14	0	1	658	5	662	1138
11	*gi|269117152|gb|GQ864809.1|Tanaecia julii voucher NW100-15*	97.31	633	17	0	26	658	1	633	1075
12	*gi|545691079|gb|KF226634.1|Tanaecia munda waterstradti voucher UMKL-JJW0399*	97.26	658	18	0	1	658	1	658	1116
13	*gi|374857582|dbj|AB511421.1|Tanaecia pelea lutala isolate: MY-Ny24*	97.26	658	18	0	1	658	5	662	1116
14	*gi|545691067|gb|KF226628.1|Tanaecia aruna aruna voucher UMKL-JJW0315*	96.81	658	21	0	1	658	1	658	1099
15	*gi|321121917|gb|HQ962246.1|Tanaecia godartii voucher YB-KHC6694*	96.81	658	21	0	1	658	1	658	1099
16	*gi|545690723|gb|KF226456.1|Euthalia monina voucher UMKL-JJW0200*	95.75	658	28	0	1	658	1	658	1061

Table 19. The BLAST hit table of *C. lepidea* CUJ15.

The 609bp COI sequence of *C. lepidea* CUJ15 was BLAST against database and showed 100% similarity to that of *C. lepidea* (GU012547 and KJ459781) from Pune, India, 99.85% similarity with *Tanaecia iapis* (HQ962118) from Thailand and *C. lepidea* (GU012543) from Pune, the *C. lepidea* (KF226376, KF226375, KF226374 and KF226373) from Malaysia, with 99.53%.

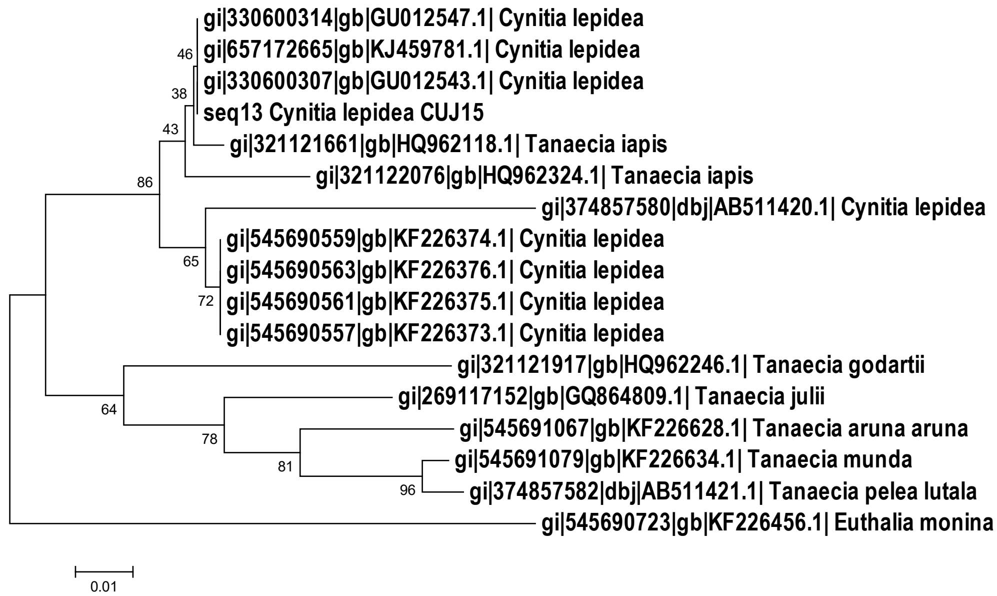

Fig. 109. NJ-tree with Phylogenetic relationship of *C. lepidea* CUJ15.

The phylogenetic analysis of *C. lepidea* CUJ15 was derived with NJ-tree developed from the database sequences using MEGA6 application (Fig. 109) revealed that the CUJ15 was placed in a clade with 3 samples of *C. lepidea* (GU012547, GU012543 and KJ459781) from Pune, India. *Tanaecia iapis* (HQ962118) from Thailand in an adjacent clade with 99.585% similarities. The *C. lepidea* (KF226376, KF226375, KF226374, KF226373) from Malaysia was placed in a separate clade with 99.53% similarity.

Discussion

The PCR amplification of genomic DNA from *C. lepidea* CUJ15 yielded a consensus of 609 bp. The BLAST results showed 100% similarity to *C. lepidea* (GU012547, KJ459781) from Pune, hence it is a molecular barcode for species identification. However, CUJ15 showed 99.85% similarity with *Tanaecia iapis* (HQ962118) from Thailand, here the barcoding gap is challenged and not existing between this two species. Even though the species can be identified while considering the differences with other database sequences. Whereas *C. lepidea* (GU012543) from Pune with 99.85% similarity and KF226376, KF226375, KF226374 and KF226373 from Malaysia, with 99.53% they were polymorphic novel variants of CUJ15. The NJ-tree revealed that the *C. lepidea* CUJ15 was placed in a clade with 3 samples of GU012547, GU012543 and KJ459781 from Pune and *Tanaecia iapis* (HQ962118) from Thailand in an adjacent clade with 99.585% similarities. The *C. lepidea* (KF226376, KF226375, KF226374 and KF226373) from Malaysia was placed in a separate clade with 99.53% similarity. Each clade represent samples either from India or Thailand or Malaysia here the difference in geographical location was clearly reflected in sequence variations. The NJ-tree distance data revealed that the species evolveded from their closely related species *Tanaecia julii* (GQ864809) about 23,500 years ago. The data revealed that *C. lepidea* CUJ15 also has recent origin and there close relatives were from South East Asia. The COI gene partial coding sequence can recognise the species, is found to be suitable as a Molecular barcode for identifying species, and is an effective tool in phylogenetic analysis.

3.16. *Junonia iphita* (Cramer, 1779) isolate CUJ16 (KT880652)

The specimen CUJ16 was identified as *Junonia iphita* (Cramer, 1779) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonym: *Precis iphita* Moore, 1878

Junonia iphita, is the chocolate pansy, which is a common butterfly found in forests and areas with rich vegetation.

Upper side; brown of varying shades of colour, fore wing; cell with one pair of sub basal and one pair of apical transverse sinuous fasciae, the outermost defining the disco-cellulars; a short, broad, dark, oblique fascia beyond to vein 4, its inner margin diffuse, its outer sinuous but sharply defined. Below vein 4 a sinuous, transverse, more faint fascia, followed by a discal blackish fascia, very broad and diffuse, below costa; bordered by a row of faint ocelli, and a post discal and a sub terminal similar fascia following the outline of the termen. Hind wing; a slender blackish loop near apex of cellular area, a broad inwardly diffuse, outwardly well-defined short discal fascia in continuation of the one on the fore wing, a series of post discal somewhat ochraceous ocelli with black pupils minutely centred with white; post discal and sub terminal broad lines as on the fore wing. Under side; brown, with very broad darker brown transverse fasciae, interspaces between the markings irrorated with purplish silvery scales. Fore wing with two sinuous fasciae on basal half succeeded by a discal fascia, very broad at the costal margin and decreasing in width to the dorsum, on its outer border a row of obscure ocelli. This is succeeded by a zig zag dark line, and sinuous sub terminal and terminal lines; apex and tornal area suffused with purplish silvery scales.

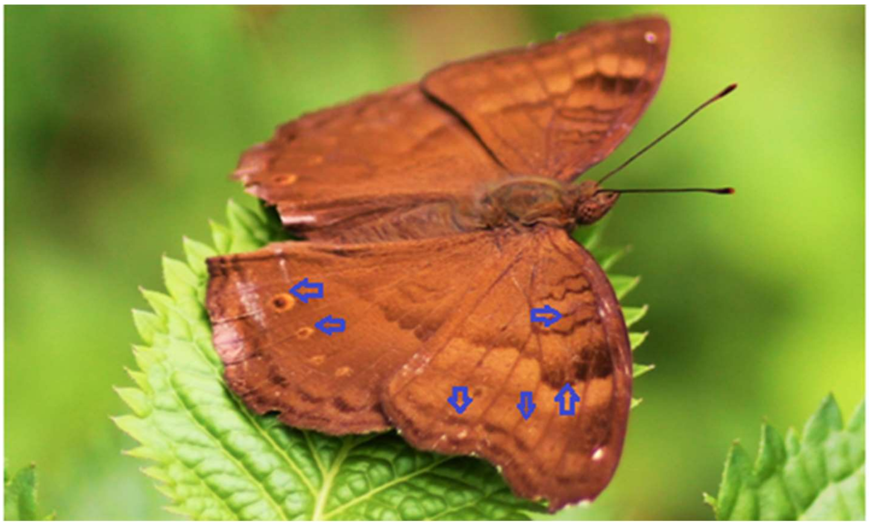

Fig. 110. The upper side of the *J. iphita* CUJ16, Upper side; brown with varying shades of colour. Fore wing; cell with one pair of sub basal and one pair of apical transverse sinuous fasciae, base of costa; bordered by a row of faint ocelli, and a post discal and a sub terminal similar fascia following the outline of the termen, hind wing with a series of post discal somewhat ochraceous ocelli with black pupils minutely centred with white.

Fig. 111. The habit of the *J. iphita* CUJ16.

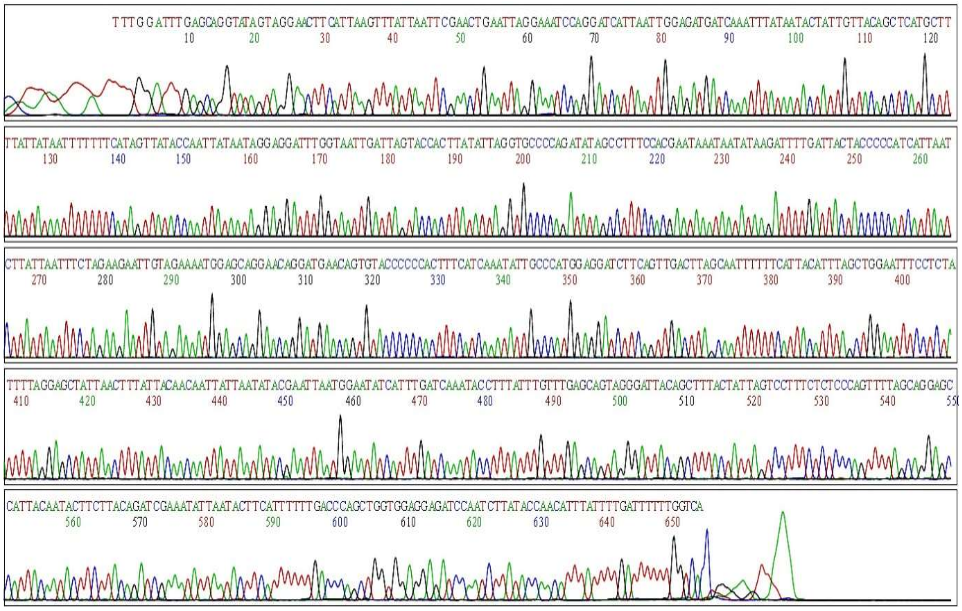

Fig. 112. The sequencing chromatogram of forward DNA strand of *J. iphita* CUJ16.

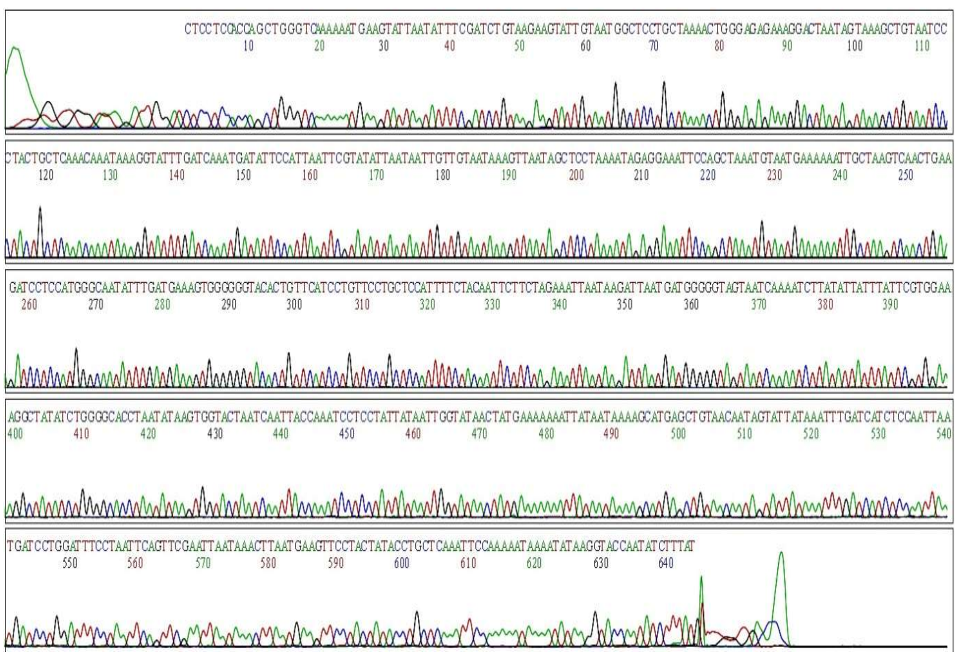

Fig. 113. The sequencing chromatogram of reverse DNA strand of *J. iphita* CUJ16.

Hind wing; two irregular, dark brown, very broad, curved short fasciae near base; a straight, narrow, transverse, prominent, ochreous-brown discal band defined outwardly by a black line. A transverse post discal dark brown fascia, widest in the middle and bearing outwardly a curved row of ochreous-brown white-centred ocelli, followed by a zig zag dark line in continuation of the one on the fore wing. A sub terminal somewhat diffuse dark fascia and a terminal dark line. Antennae, head, thorax and abdomen, dark brown (Fig. 110). The larval food plants involve *Hygrophila auriculata, Carvia callosa* and *Justicia neesii*, the larvae are reddish brown in colour with brawn branched spines.

The PCR amplification of partial COI sequence of *J. iphita* CUJ16 from Thalassery (11^{0}46’40”N, 75^{0}28’14”E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 112 to 116). The sequencing of PCR product from template DNA isolated from *Junoniphita* CUJ16 gave chromatograms of two complimentary DNA strands each from both sides of the mitochondrial COI gene. The chromatograms are with clear signals for most of the base positions (Fig. 112 and Fig. 113).

```
>Junonia iphita voucher CUJ16 cytochrome oxidase subunit I
gene, partial cds; mitochondrial(609)
TACCTTATATTTTATTTTTGGAATTTGAGCAGGTATAGTAGGAACTTCATTAAGTTTATT
AATTCGAACTGAATTAGGAAATCCAGGATCATTAATTGGAGATGATCAAATTTATAATAC
TATTGTTACAGCTCATGCTTTTATTATAATTTTTTTCATAGTTATACCAATTATAATAGG
AGGATTTGGTAATTGATTAGTACCACTTATATTAGGTGCCCCAGATATAGCCTTTCCACG
AATAAATAATATAAGATTTTGATTACTACCCCCATCATTAATCTTATTAATTTCTAGAAG
AATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTGTACCCCCCACTTTCATCAAATAT
TGCCCATGGAGGATCTTCAGTTGACTTAGCAATTTTTTCATTACATTTAGCTGGAATTTC
CTCTATTTTAGGAGCTATTAACTTTATTACAACAATTATTAATATACGAATTAATGGAAT
ATCATTTGATCAAATACCTTTATTTGTTTGAGCAGTAGGGATTACAGCTTTACTATTAGT
CCTTTCTCTCCCAGTTTTAGCAGGAGCCATTACAATACTTCTTACAGATCGAAATATTAA
TACTTCATT
```

Fig. 114. The partial sequence of mitochondrial CO I gene of *J. iphita* CUJ16.

The conceptual translation of the consensus sequence of *J. iphita* CUJ16 gave the following peptide sequence.

>seq14 *Junonia iphita* CUJ16

TLYFIFGIWAGMVGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMMGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLILLISSSIVENGAGTGWTV
YPPLSSNIAHGGSSVDLAIFSLHLAGISSILGAINFITTIINMRINGMSFDQMPLF
VWAVGITALLLVLSLPVLAGAITMLLTDRNINTS

Fig. 115. The peptide sequence obtained from conceptual translation of consensus sequence of *J. iphita* CUJ16.

The nucleotide BLAST was conducted with the consensus sequence derived from of *J. iphita* CUJ16. The details about similarities of sequences obtained and alignment details were represented below.

S.I. No.	Subject Ids	% identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S start	S. end	Bit score
1	*gi|330600420|gb|GU012609.1| Junonia iphita voucher F334*	100.00	656	0	0	3	658	1	656	1212
2	*gi|330600356|gb|GU012574.1| Junonia iphita voucher F365*	100.00	645	0	0	1	645	6	650	1192
3	*gi|330600411|gb|GU012604.1| Junonia iphita voucher F413*	100.00	633	0	0	4	636	1	633	1170
4	*gi|330600447|gb|GU012623.1| Junonia iphita voucher F422*	100.00	631	0	0	5	635	4	634	1166
5	*gi|330600426|gb|GU012612.1| Junonia iphita voucher F357*	100.00	627	0	0	1	627	3	629	1158
6	*gi|330600265|gb|GU012519.1| Junonia iphita voucher F57*	100.00	625	0	0	8	632	1	625	1155
7	*gi|657172715|gb|KJ459806.1|Junonia iphita isolate F493*	99.84	624	1	0	35	658	1	624	1147
8	*gi|330600256|gb|GU012514.1| Junonia iphita voucher F38*	99.84	623	1	0	20	642	1	623	1146
9	*gi|657172743|gb|KJ459820.1|Junonia iphita isolate F702*	99.68	618	2	0	35	652	1	618	1131
10	*gi|197359536|gb|EU792480.1|Junonia iphita isolate SEC05CP05*	99.39	658	2	2	2	658	6	662	1192
11	*gi|330600332|gb|GU012558.1| Junonia iphita voucher F399*	98.92	645	0	7	1	638	13	657	1146

12	*gi|442535848|gb|KC158394.1|J unonia iphita voucher NIBGE BUT-00212*	97.42	658	17	0	1	658	1	658	1122
13	*gi|302201966|gb|HM246469.1| Papilio slateri*	96.51	658	23	0	1	658	660	3	1088
14	*gi|169672599|gb|EU368157.1|J unonia iphita isolate gouchiyan2*	96.50	657	23	0	2	658	1	657	1086
15	*gi|321121871|gb|HQ962223.1| Junonia iphita voucher YB-KHC6584*	94.83	658	34	0	1	658	1	658	1027
16	*gi|545690809|gb|KF226499.1|J unonia hedonia voucher UMKL-JJW0294*	94.53	658	36	0	1	658	1	658	1016
17	*gi|330600418|gb|GU012608.1| Junonia atlites voucher F330*	94.42	645	36	0	5	649	4	648	992
18	*gi|699975928|gb|KM115626.1| Junonia almana isolate JA_N2*	93.47	658	43	0	1	658	6	663	977
19	*gi|893640295|gb|KP941756.1|J unonia lemonias*	93.03	660	42	4	1	658	1494	2151	961

Table 20 The BLAST hit table of *J. iphita* CUJ16.

The nucleotide BLAST of COI sequence of *J. iphita* CUJ16 against database and showed 100% similarity to that GU012612, GU012574, GU012609, GU012604, GU012623, GU012519 and KJ459806 from Pune, India, 99.85% similarity with EU792480 from Bangalore and GU012514, KJ459820 and GU012558 from Pune, the KC158394 from Pakistan, EU368157 from Shanxi, P. R. China were with 97% similarity. The KM115628 from Mizoram with 96% identity. The *J. Iatlites* (GU12608) from Pune and *Junonia hedonia* (KF226499) from Malaysia, with 95% similarity and was different species of the genus remain close to *J. Iphita*.

The NJ-tree developed from the similar sequences from BLAST results (Fig. 116). The *J. iphita* CUJ16 was placed in a clade with 12 samples involving GU012612, GU012574, GU012609, GU012604, GU012623, GU012519, KJ459806, GU012514, KJ459820 and GU012558 from Pune and another

EU792480 from Bangalore, they were monophyletic sharing same ancestor. The *J. iphita* (KC158394) from Pakistan, *J. iphita* (EU368157) from Shanxi, P. R. China were with 97% similarity. The *J. iphita* (KM115628) from Mizoram with 96% identity were found in the adjacent clade. The *J. atlites* (GU12608) from Pune and *J. hedonia* (KF226499) from Malaysia were with 95% similarity and was different species of the genus remain close to *J. Iphita*, in a separate clade.

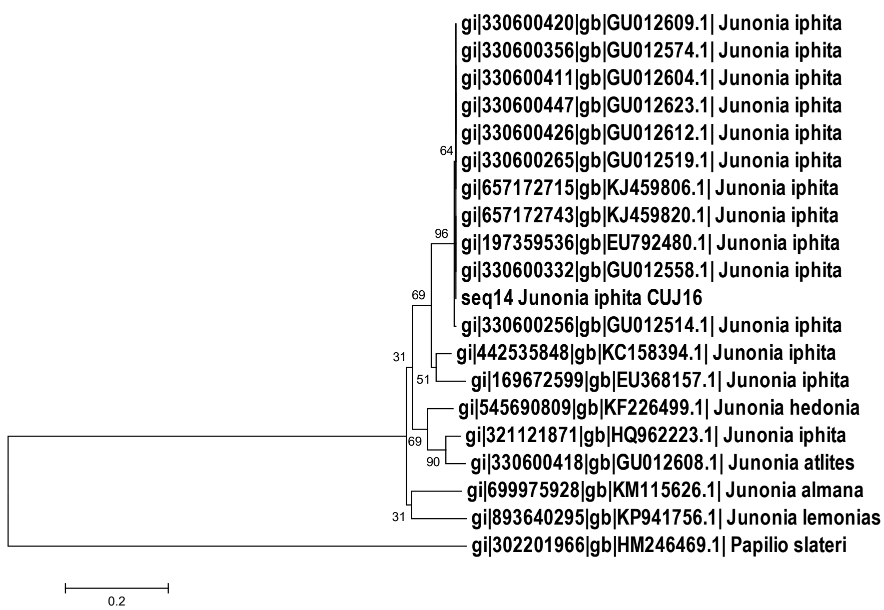

Fig. 116. NJ-tree with Phylogenetic relationship of *J. iphita* CUJ16.

Discussion

The consensus sequence of *J. iphita* CUJ16, BLAST results showed 100% similarity to that *J. iphita* GU012612, GU012574, GU012609, GU012604, GU012623, GU012519 and KJ459806 from Pune, India, therefore it will be a molecular barcode for species identification. The EU792480 from Bangalore has 99.85% similarity. While GU012514, KJ459820 and GU012558 from Pune and the

KC158394 from Pakistan, EU368157 from Shanxi, P. R. China were with 97% similarity. Another sample KM115628 from Mizoram has 96% identity are the polymorphic novel isolates from different geographical regions. In the present study the intra species variability in most cases was observed below 3% but the sample KM115628 from Mizoram, the variability is about 4% and has only 96% similarity with others. The *J. atlites* GU12608 from Pune and *J. hedonia* KF226499 from Malaysia were with 95% similarity and remain close to *J. Iphita*. The phylogeny of *J. iphita* CUJ16 was derived with NJ-tree developed from BLAST hit results (Fig. 116). The *J. iphita* CUJ16 was found in a clade with 12 samples involving *J. iphita* GU012612, GU012574, GU012609, GU012604, GU012623, GU012519, KJ459806, GU012514, KJ459820 and GU012558 from Pune and another EU792480 from Bangalore, were monophyletic sharing same ancestor. The KC158394 from Pakistan, EU368157 from Shanxi, P. R. China and The KM115628 from Mizoram were found in the adjacent clade. The *J. atlites* (GU12608) from Pune and *J. hedonia* (KF226499) from Malaysia were with 95% similarity and the species remain close to *J. Iphita*, in a separate clade. The NJ-tree distance data revealed that the species was diverged from their closely related species *J. hedonia* about 2,00,000 years ago. The data also revealed that *J. iphita* CUJ16 also has recent origin and were diverged from South East Asia.

3.17. *Junonia almana* (Linnaeus, 1758) isolate CUJ17 (KT880653)

The specimen CUJ17 was identified as *Junonia almana* (Linnaeus, 1758) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Alcyoneis almane* Hubner, [1819]

Junonia asterie; Moore, [1881]

Junonia almana, is the peacock pansy, which is a Nymphalidae butterfly found in South Asia. It exhibits seasonal polymorphic forms, this butterfly is very common in Kerala.

Upper side; rich orange-yellow, fore wing; pale dusky, darker short transverse bar with lateral jet-black marginal lines across cell, somewhat similar bar defining the discocellulars. Costal margin; an inner and an outer sub terminal line, and a terminal line dusky black. A large minutely white-centred ocellus with an inner slender and outer black ring on disc in interspace 2; two similar but smaller geminate sub apical ocelli with an obscure pale spot above them and a short oblique bar connecting them to the black on the costa. Hind wing; a small minutely white-centred and very slenderly black-ringed discal ocellus in interspace 2, with a very much larger pale yellow and black-ringed ocellus above it spreading over interspaces 4, 5 and 6, the centre of this ocellus inwardly brownish orange, outwardly bluish black, with two minute white spots in vertical order between the two colours. Finally, post discal sub terminal and terminal black sinuous lines. Antennae dark brown; head, thorax and abdomen slightly darker (Fig. 117).

Fig 117. The upper side of the *J. almana* CUJ17 rich orange – yellow colour, fore wing; pale dusky, darker short transverse bar. Costal margin; an inner and an outer sub terminal line, and a terminal line dusky black. a large minutely white-centred ocellus with an inner slender and outer black ring on disc in interspace 2., two similar but smaller geminate sub apical ocelli with an obscure pale spot above them and a short oblique bar connecting them to the black on the costa, smaller geminate sub apical ocelli with an obscure pale spot above them and a short oblique bar connecting them to the black on the costa. Hind wing; a small minutely white-centred and very slenderly black-ringed discal ocellus in interspace 2, a very much larger pale yellow and black-ringed ocellus above it spreading over interspaces 4, 5 and 6, the centre of this ocellus inwardly brownish orange, outwardly bluish black, with two minute white spots.

Fig. 118. The habit of the *J. almana* CUJ17.

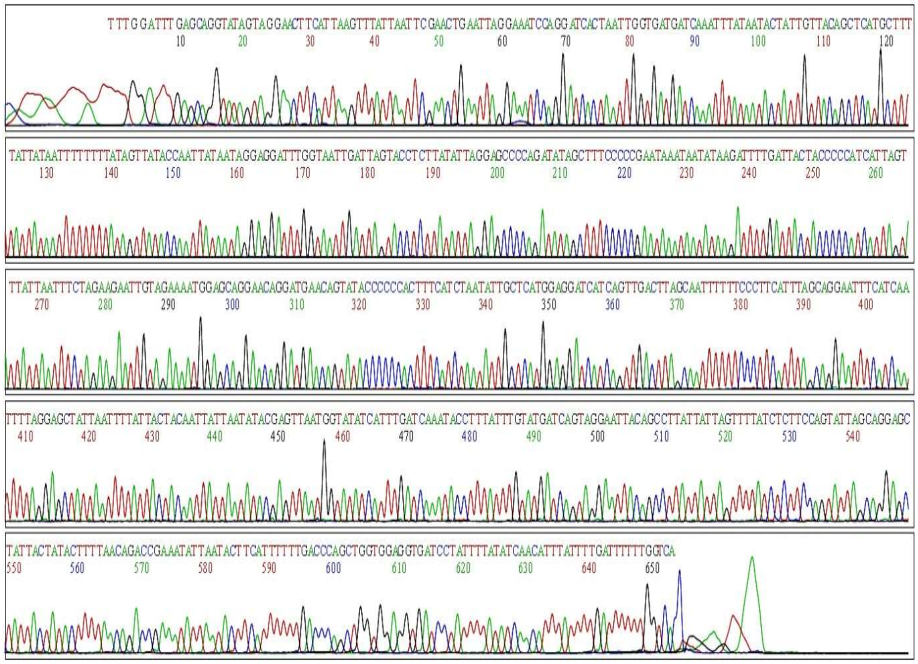

Fig. 119. The sequencing chromatogram of forward DNA strand of *J. almanac* CUJ17.

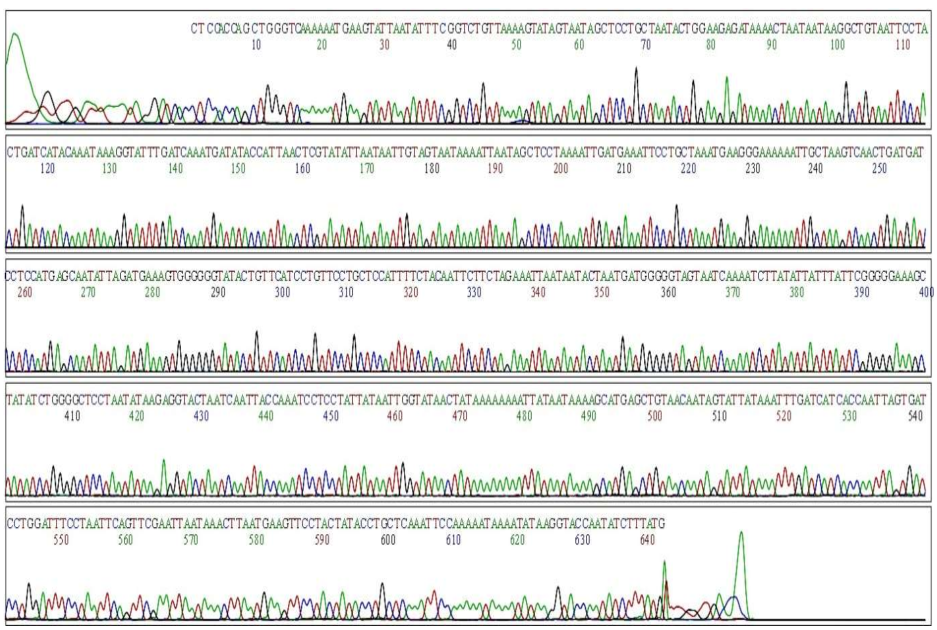

Fig. 120. The sequencing chromatogram of reverse DNA strand of *J. almana* CUJ17.

The larval food plants involve *Hygrophila auriculata* and *Phyla nodiflora*, the foggy orange coloured neck and spines with pale yellow are characterise their larvae.

The PCR amplification of partial COI sequence of *J. almana* CUJ17 from Aravanchal ($12^0$12'58"N, $75^0$16'52"E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 119 to 121).

```
>Junonia almana voucher CUJ17 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
TACCTTATATTTTATTTTTGGAATTTGAGCAGGTATAGTAGGAACTTCATTAAGTTTATT
AATTCGAACTGAATTAGGAAATCCAGGATCACTAATTGGTGATGATCAAATTTATAATAC
TATTGTTACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCAATTATAATAGG
AGGATTTGGTAATTGATTAGTACCTCTTATATTAGGAGCCCCAGATATAGCTTTCCCCCG
AATAAATAATATAAGATTTTGATTACTACCCCCATCATTAGTATTATTAATTTCTAGAAG
AATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTATACCCCCCACTTTCATCTAATAT
TGCTCATGGAGGATCATCAGTTGACTTAGCAATTTTTTCCCTTCATTTAGCAGGAATTTC
ATCAATTTTAGGAGCTATTAATTTTATTACTACAATTATTAATATACGAGTTAATGGTAT
ATCATTTGATCAAATACCTTTATTTGTATGATCAGTAGGAATTACAGCCTTATTATTAGT
TTTATCTCTTCCAGTATTAGCAGGAGCTATTACTATACTTTTAACAGACCGAAATATTAA
TACTTCATT
```

Fig. 121. The partial sequence of mitochondrial COI gene of *J. almana* CUJ17.

The conceptual translation of the consensus sequence of *J. almana* CUJ17 gave the following peptide sequence.

```
>seq15 Junonia almana CUJ17

TLYFIFGIWAGMVGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMMGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLVLLISSSIVENGAGTGWTV
YPPLSSNIAHGGSSVDLAIFSLHLAGISSILGAINFITTIINMRVNGMSFDQMPLF
VWSVGITALLLVLSLPVLAGAITMLLTDRNINTS
```

Fig. 122. The peptide sequence obtained from conceptual translation of consensus sequence of COI gene, DNA of *J. almana* CUJ17.

The nucleotide BLAST was conducted with the consensus sequence derived from of *J. almana* CUJ17. The details about similarities of sequences obtained and alignment details are represented below.

S.I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|699975928|gb|KM115626.1|Junonia almana isolate JA_N2*	100.00	658	0	0	1	658	6	663	1216
2	*gi|667668748|gb|KJ752718.1|Junonia almana voucher JAS2*	100.00	658	0	0	1	658	6	663	1216
3	*gi|667668736|gb|KJ752712.1|Junonia almana voucher JAN2*	100.00	658	0	0	1	658	6	663	1216
4	*gi|557469442|gb|KF590539.1|Junonia almana isolate N877*	100.00	658	0	0	1	658	1493	2150	1216
5	*gi|555929157|dbj|AB855871.1|Junonia almana almana , isolate: t030*	100.00	658	0	0	1	658	18	675	1216
6	*gi|321485691|gb|HQ990342.1|Junonia almana voucher NIBGE BUT-00024*	100.00	658	0	0	1	658	1	658	1216
7	*gi|301051531|gb|HM446466.1|Junonia almana almana isolate MYJD-001*	100.00	658	0	0	1	658	1	658	1216
8	*gi|330600360|gb|GU012576.1|Junonia almana voucher D243*	100.00	657	0	0	1	657	10	666	1214
9	*gi|169672605|gb|EU368160.1|Junonia almana isolate meiyan1*	100.00	657	0	0	2	658	1	657	1214
10	*gi|330600399|gb|GU012596.1|Junonia almana voucher N283*	100.00	639	0	0	4	642	1	639	1181
11	*gi|330600358|gb|GU012575.1|Junonia almana voucher F84*	100.00	636	0	0	1	636	13	648	1175
12	*gi|330600312|gb|GU012546.1|Junonia almana voucher D246*	100.00	635	0	0	1	635	9	643	1173
13	*gi|155968143|gb|EU053288.1|Junonia almana voucher NW131-8*	100.00	633	0	0	26	658	1	633	1170
14	*gi|155968141|gb|EU053287.1|Junonia almana voucher NW131-7*	100.00	633	0	0	26	658	1	633	1170
15	*gi|699975914|gb|KM115619.1|Junonia almana isolate JA_N3*	100.00	624	0	0	35	658	1	624	1153
16	*gi|330600362|gb|GU012577.1|Junonia almana voucher F131*	100.00	619	0	0	6	624	1	619	1144
17	*gi|330600319|gb|GU012550.1|Junonia almana voucher F282*	100.00	618	0	0	24	641	1	618	1142
18	*gi|657172619|gb|KJ459758.1|Junonia almana isolate N292*	100.00	617	0	0	35	651	1	617	1140
19	*gi|330600309|gb|GU012544.1|Junonia almana voucher D242*	100.00	610	0	0	36	645	1	610	1127
20	*gi|925175959|gb|KP997222.1|Junonia almana almana voucher MMJ-09*	100.00	604	0	0	24	627	1	604	1116
21	*gi|699975950|gb|KM115637.1|Junonia almana isolate JA_S2*	100.00	601	0	0	11	611	4	604	1110

22	*gi|545690803|gb|KF226496.1|Junonia almana javana voucher UMKL-JJW0231*	99.85	658	1	0	1	658	1	658	1210
23	*gi|505490278|gb|KC755864.1|Junonia almana voucher NIBGE BUT-00332*	99.85	658	1	0	1	658	1	658	1210
24	*gi|321485689|gb|HQ990341.1|Junonia almana voucher NIBGE BUT-00023*	99.85	658	1	0	1	658	1	658	1210
25	*gi|699975942|gb|KM115633.1|Junonia almana isolate JA_S1*	99.84	636	1	0	23	658	1	636	1170
26	*gi|657172787|gb|KJ459842.1|Junonia almana isolate T71*	99.84	624	1	0	35	658	1	624	1147
27	*gi|305695040|gb|GU681859.1|Junonia almana voucher NIBGE IMB-00166*	99.83	602	1	0	23	624	1	602	1107
28	*gi|321485687|gb|HQ990340.1|Junonia almana voucher NIBGE BUT-00021*	99.83	587	1	0	38	624	1	587	1079
29	*gi|699975962|gb|KM115643.1|Junonia almana isolate JA_N1*	99.53	641	3	0	14	654	1	641	1168
30	*gi|657172795|gb|KJ459846.1|Junonia almana isolate T81*	99.52	624	3	0	35	658	1	624	1136
31	*gi|330600311|gb|GU012545.1|Junonia almana voucher D245*	99.38	641	1	3	10	647	1	641	1158
32	*gi|155968145|gb|EU053289.1|Junonia almana voucher NW29-3*	99.37	633	4	0	26	658	1	633	1147
33	*gi|348162089|gb|JN609221.1|Junonia almana isolate MZUbp102*	99.04	626	6	0	33	658	1	626	1123
34	*gi|699975936|gb|KM115630.1|Junonia almana isolate JA_S3*	99.04	624	6	0	35	658	1	624	1120
35	*gi|657635988|gb|KJ469090.1|Junonia genoveva isolate LCB192*	95.75	658	28	0	1	658	1	658	1061
36	*gi|657635936|gb|KJ469064.1|Junonia zonalis isolate LCB162*	95.60	659	27	2	1	658	1	658	1057
37	*gi|529812362|gb|KF292251.1|Junonia wahlbergi voucher ARG20*	95.59	658	29	0	1	658	24	681	1055
38	*gi|851776938|gb|KP849041.1|Junonia evarete voucher YB-BCI55882*	95.45	659	28	2	1	658	1	658	1050

Table 21. The BLAST hit Table of *J.almana* CUJ17.

The consensus sequence of *J. almana* CUJ17 sequences BLAST results showed 100% similarity to that of *J. almana* GU012575, GU012576, GU012546 from Pune, KM115626, KJ757718, KJ757712 from Mizoram, KF590539 from Taiwan, AB855871 from Japan, HQ990342 from Pakistan and HM446466 from P. R. China. However 99.85% similarity with KF226496 from Malaysia KC755864 and HQ990341 from Pakistan. The KM115633 from Mizoram was with 99.83% similarity. The JN609221 and KM115630 from Mizoram were with 99.04%

similarity.

The phylogeny of *J. almana* CUJ17 was derived with NJ-tree developed from the similar sequences obtained from BLAST hit results (Fig.123). The *J. almana* CUJ17 was found to be placed in a clade with 31 samples involving *J. almana* GU012575, GU012576, GU012546 from Pune, KM115626, KJ757718, KJ757712 from Mizoram, India, KF590539) from Taiwan, AB855871 from Japan*,* and HQ990342 from Pakistan and HM446466 from P. R. China. The KF226496 from Malaysia, KC755864, HQ990341 from Pakistan. The *J. almana* JN609221 and KM115630 from Mizoram were found in the adjacent clade. The *J. genoveva* KJ469090 from French Guiana a different species of the genus remain close to *J. almana*, formed a separate clade.

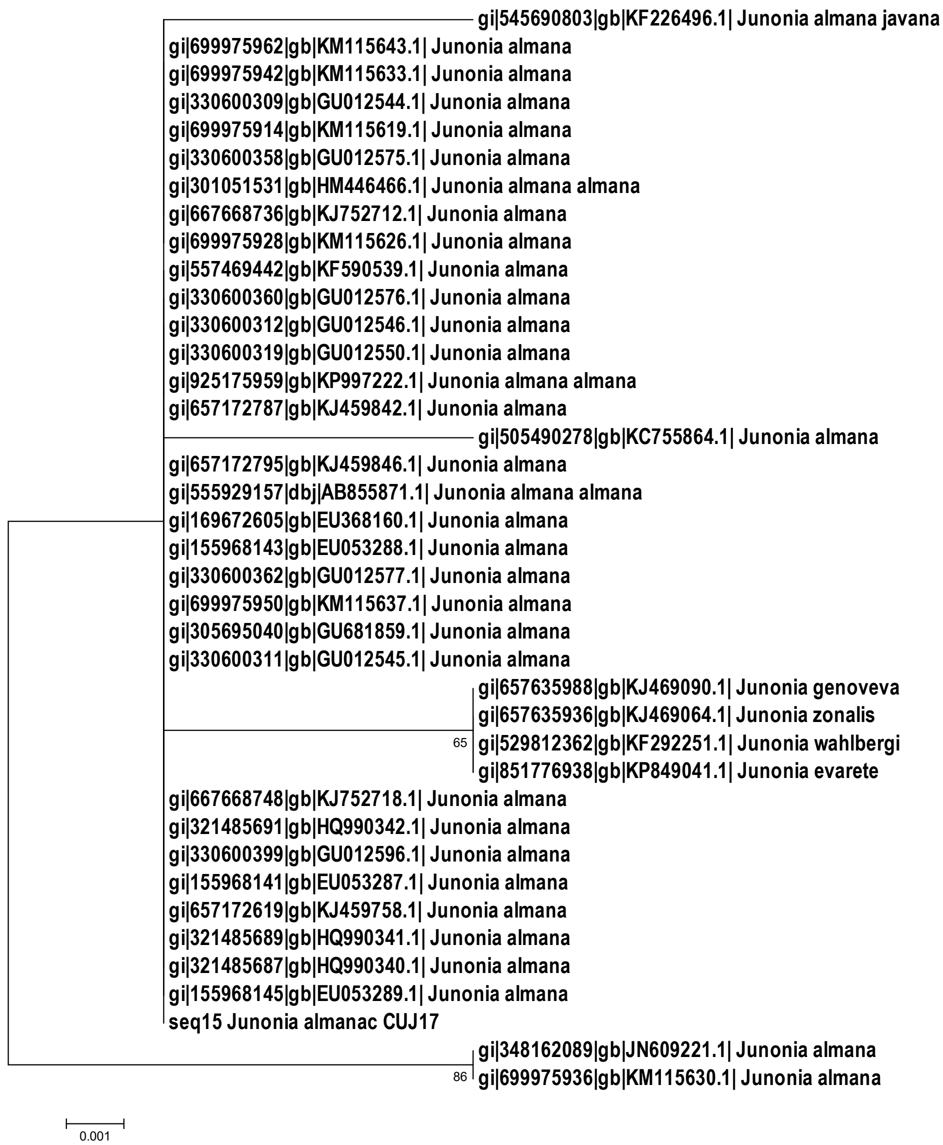

Fig. 123. NJ-tree with Phylogenetic relationship of *J. almana* CUJ17.

Discussion

The *J. almana* CUJ17 BLAST results showed 100% similarity to 21 sequences of GenBank nucleotide database. Hence, the COI sequence of CUJ17 is useful as a molecular barcode for identifying the species. Whereas many other sequences of *J. almana* gave 99.85% to 99.04% similarities and they were considered as the polymorphic novel variants. The phylogeny of *J. almana* was derived with NJ-tree developed with COI sequence isolated from CUJ17 (Fig.123). The *J. almana* CUJ17 was found to be placed in a clade with 31 samples involving *J. almana* GU012575, GU012576, GU012546 from Pune, India, KM115626, KJ757718, KJ757712 from Mizoram, India, KF590539 from Taiwan, AB855871 from Japan*,* and HQ990342 from Pakistan and HM446466 from P. R. China. KF226496 from Malaysia KC755864 from Pakistan, and HQ990341 from Pakistan, they were monophyletic sharing same ancestor. The JN609221 and KM115630 from Mizoram were with 99.04% similarity were found in the adjacent clade. The *J. genoveva* (KJ469090) from French Guiana a different species of the genus remain close to *J. almana*, formed a separate clade. The NJ-tree distance data revealed that the species diverged from their closely related species *J. genoveva* about 52,000 years ago. The data revealed that *J. almana* CUJ17 also has recent origin and they were related with South East Asia. The identification of closely related species *J. genoveva* (KJ469090) from French Guiana of South America indicates the origin of the genus from the Northern Asia near Bearings close to North America and they were distributed to South America through the land bridges (Condamine, 2013).

3.18. *Ypthima huebneri* Kirby, 1871 isolate CUJ18 (KT880654)

The specimen CUJ18 was identified as *Ypthima huebneri* Kirby, 1871 referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonym: *Ypthima howra* Moore, 1884

Ypthima catharina Butler, 1886

Ypthima jocularia Swinhoe, 1889

Ypthima huebneri is the common four ring which is a common species of Nymphalidae butterfly found in Asia.

Upper side; is greyish brown, fore wing; with comparatively large pre-apical ocellus, is black, bi-pupilled and yellow-ringed. Hind wing; with three ocelli, they are smaller, similar, uni-pupilled and post-discal ocelli. Under side; is greyish white in colour, not very densely covered with transverse short brown parallel lines. Fore wing; with a preapical ocellus as on the upper side, which is obscure, discal, sub terminal dull brown with transverse lines and a narrow brown ring round the ocellus, diffusely produced posteriorly. Hind wing; with one apical and three postdiscal posterior ocelli placed in a curved plane with traces of transverse brown discal and sub terminal lines. Antenna, head and thorax are greyish brown, the abdomen is slight paler in colour beneath (Fig. 124).

Many varieties of grasses are host plants including *Axonopus compressus* as larval food plant, the larvae are greenish in colour with a white mid dorsal line.

Fig. 124. The lower side of the *Y. huebneri* CUJ18, greyish brown, fore wing; with comparatively large pre-apical ocellus, is black, bi-pupilled and yellow-ringed. Hind wing; with three ocelli, they are smaller, similar, uni-pupilled and post-discal ocelli. Antenna, head and thorax; are greyish brown, the abdomen is slight paler in colour beneath.

Fig. 125. The habit of the *Y. huebneri* CUJ18.

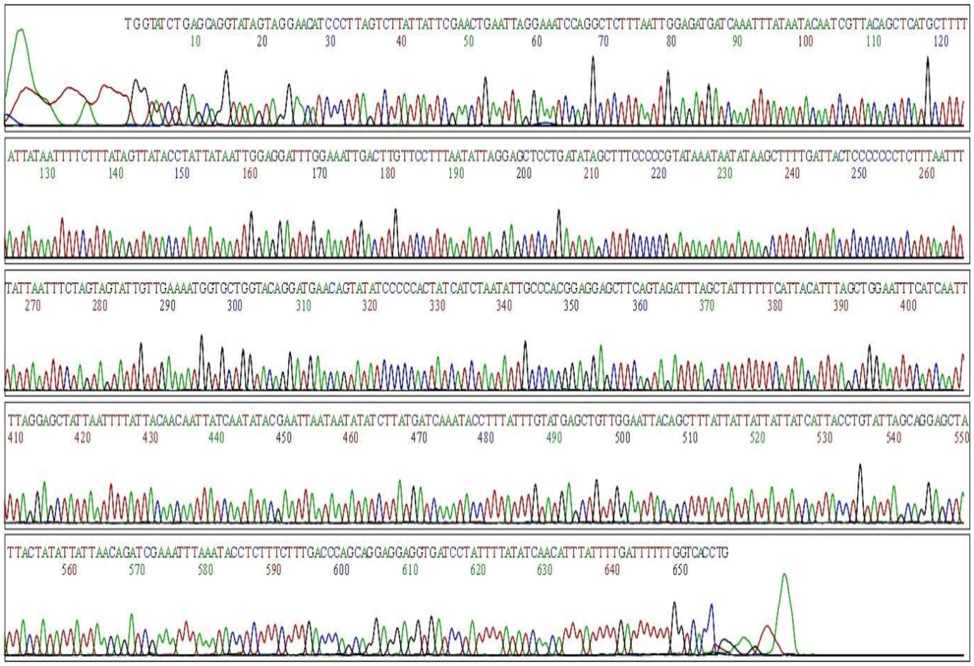

Fig. 126. The sequencing chromatogram forward DNA strand of *Y. huebneri* CUJ18.

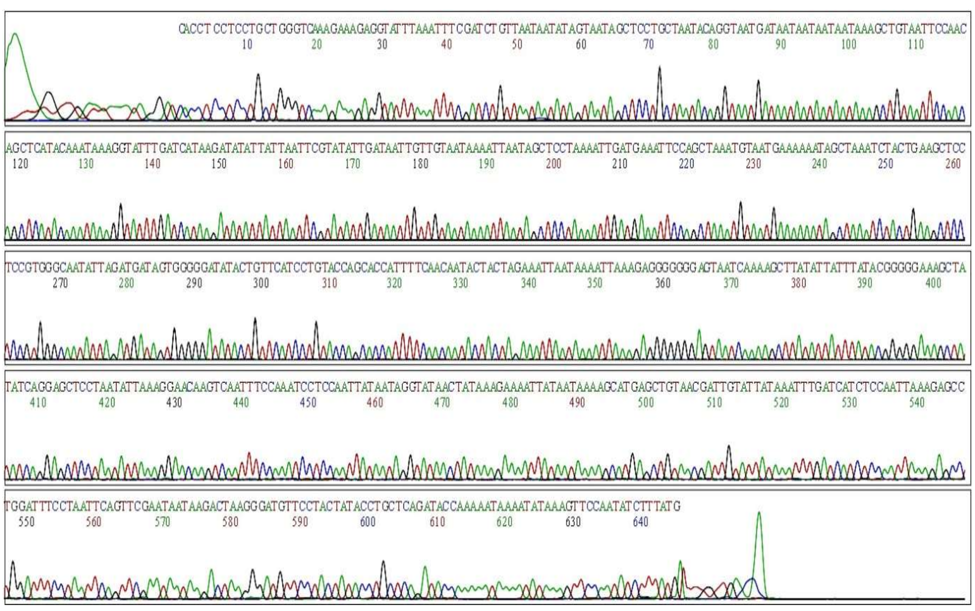

Fig. 127. The sequencing chromatogram of reverse DNA strand of *Y. huebneri* CUJ18.

The PCR amplification of partial COI sequence of *Y. huebneri* CUJ18 from North Kerala, India yielded a consensus with 603 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 126 to 130).

```
>Ypthima huebneri voucher CUJ18 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (603)
AACTTTATATTTTATTTTTGGTATCTGAGCAGGTATAGTAGGAACATCCCTTAGTCTTAT
TATTCGAACTGAATTAGGAAATCCAGGCTCTTTAATTGGAGATGATCAAATTTATAATAC
AATCGTTACAGCTCATGCTTTTATTATAATTTTCTTTATAGTTATACCTATTATAATTGG
AGGATTTGGAAATTGACTTGTTCCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCCCG
TATAAATAATATAAGCTTTTGATTACTCCCCCCCTCTTTAATTTTATTAATTTCTAGTAG
TATTGTTGAAAATGGTGCTGGTACAGGATGAACAGTATATCCCCCACTATCATCTAATAT
TGCCCACGGAGGAGCTTCAGTAGATTTAGCTATTTTTTCATTACATTTAGCTGGAATTTC
ATCAATTTTAGGAGCTATTAATTTTATTACAACAATTATCAATATACGAATTAATAATAT
ATCTTATGATCAAATACCTTTATTTGTATGAGCTGTTGGAATTACAGCTTTATTATTATT
ATTATCATTACCTGTATTAGCAGGAGCTATTACTATATTATTAACAGATCGAAATTTAAA
TAC
```

Fig.128. The partial sequence of mitochondrial COI gene of *Y. huebneri* CUJ18.

The conceptual translation of the consensus sequence of *Y. huebneri* CUJ18 gave the following peptide sequence (Fig. 129)

```
>seq16 Ypthima huebneri CUJ18
TLYFIFGIWAGMVGTSLSLIIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLILLISSSIVENGAGTGWTVYPPLSSNIAH
GGASVDLAIFSLHLAGISSILGAINFITTIINMRINNMSYDQMPLFVWAVGITALLLLLSL
PVLAGAITMLLTDRNLNTSFFDPAGGGDPILSSTFI
```

Fig. 129. The peptide sequence obtained from conceptual translation of consensus sequence of COI gene, DNA of *Y. huebneri* isolate CUJ18.

The nucleotide BLAST was conducted with the consensus sequence derived from of *Y. huebneri* CUJ18. The details about similarities of sequences obtained and alignment details were represented below.

S.I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|330600393|gb|GU012593.1|Ypthima huebneri voucher F516*	100.00	641	0	0	1	641	3	643	1184
2	*gi|330600459|gb|GU012632.1|Ypthima huebneri voucher F206*	100.00	639	0	0	1	639	12	650	1181
3	*gi|657172607|gb|KJ459752.1|Ypthima huebneri isolate F510*	100.00	591	0	0	35	625	1	591	1092
4	*gi|330600410|gb|GU012603.1|Ypthima huebneri voucher F400*	99.85	658	0	1	2	659	1	657	1208
5	*gi|330600343|gb|GU012567.1|Ypthima huebneri voucher F461*	99.84	640	0	1	20	659	1	639	1175
6	*gi|330600366|gb|GU012579.1|Ypthima huebneri voucher F362*	99.84	636	1	0	9	644	1	636	1170
7	*gi|657172669|gb|KJ459783.1|Ypthima huebneri isolate F340*	99.84	625	0	1	35	659	1	624	1147
8	*gi|572169451|gb|KF733789.1|Ypthima ceylonica*	97.72	659	14	1	1	659	1	658	1133
9	*gi|330600336|gb|GU012562.1|Ypthima huebneri voucher F443*	96.84	632	4	16	19	634	1	632	1050
10	*gi|321485837|gb|HQ990415.1|Ypthima asterope voucher NIBGE BUT-00101*	95.30	659	30	1	1	659	1	658	1044
11	*gi|699059687|gb|KM111660.1|Ypthima huebneri*	95.18	622	29	1	38	659	1	621	981
12	*gi|592971293|dbj|AB915952.1|Ypthima huebneri, isolate: ep101*	94.82	618	31	1	42	659	1	617	963
13	*gi|592971291|dbj|AB915951.1|Ypthima huebneri , isolate: ep100*	94.82	618	31	1	42	659	4	620	963
14	*gi|557469638|gb|KF590553.1|Ypthima akragas isolate N344*	93.32	659	43	1	1	659	1499	2156	976
15	*gi|557355880|dbj|AB859081.1|Ypthima multistriata ganus , isolate: ep74*	93.12	610	41	1	50	659	4	612	893
16	*gi|410520595|gb|JX185819.1|Ypthima motschulskyi*	93.02	659	45	1	1	659	1	658	961
17	*gi|557355922|dbj|AB859102.1|Ypthima sordida , isolate: ep87*	92.87	617	43	1	43	659	1	616	894
18	*gi|146762258|gb|EF545711.1|Ypthima chinensis voucher H-030*	92.87	645	45	1	15	659	1	644	935
19	*gi|85014092|gb|DQ338876.1|Ypthima confuse*	92.74	634	45	1	26	659	1	633	915
20	*gi|557355920|dbj|AB859101.1|Ypthima phania , isolate: ep90*	92.55	617	45	1	43	659	1	616	883
21	*gi|557355914|dbj|AB859098.1|Ypthima masakii , isolate: ep28*	92.43	634	47	1	26	659	4	636	904
22	*gi|374880155|gb|JQ578180.1|Hamadryas ferentina*	92.20	628	47	2	1	627	1	627	887

Table22. The BLAST hit Table of *Y. huebneri* CUJ18.

The consensus sequence of *Y. huebneri* CUJ18 COI sequence BLAST results showed 100% similarity to *Y. huebneri* GU012632, GU012593, GU012603, KJ459783, KJ459752 and GU012567 from Pune. While 99.85% similarity to GU012603 from Pune, India. However, GU012579 from Pune with 99.84% similarity, and GU012562 from Pune have 97.84% similarity. The *Y. ceylonica* KF733789 from Tamilnadu, India, with 97.72% similarity.

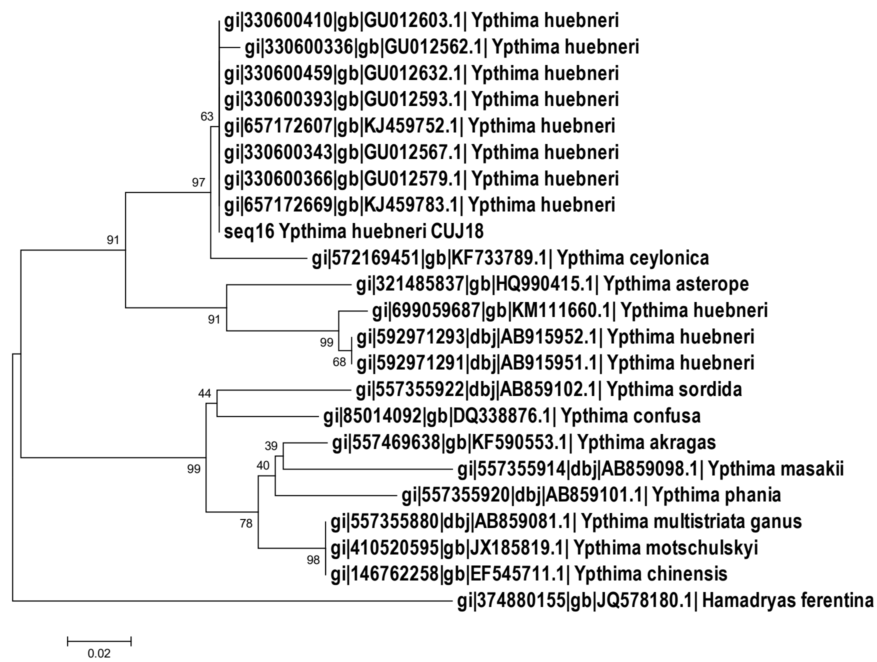

Fig. 130. NJ-tree with Phylogenetic relationship of *Y. huebneri* CUJ18.

The phylogeny of *Y. huebneri* CUJ18 with NJ-tree (Fig. 130) showed CUJ18 in a clade representing 8 samples *viz*; GU012632, GU012593, GU012603, KJ459783, KJ459752, GU012567, GU012603, GU012579, GU012562 and GU012562 from Pune, are monophyletic sharing the same ancestor. The *Y.*

ceylonica (KF733789) from Tamilnadu, India, with 97.72% similarity and found in the adjacent clade.

Discussion

The COI sequence of *Y. huebneri* CUJ18 showed 100% similarity to *Y. huebneri* GU012632, GU012593, GU012603, KJ459783, KJ459752 and GU012567 from Pune, India. Therefore, the COI sequence of *Y. huebneri* CUJ18 will be useful as a molecular barcode for identifying the species. While GU012603 with 99.85% similarity, GU012579 with 99.84% similarity, and GU012562 with 97.84% similarity from Pune are polymorphic variants. The *Y. ceylonica* (KF733789) from Tamilnadu, India, with 97.72% similarity is the closely related species of CUJ18 in the sub-continent. The NJ-tree developed from similar sequences obtained from database showed CUJ18 in a single clade representing 8 samples *viz*; GU012632, GU012593, GU012603, KJ459783, KJ459752 and GU012567 from Pune. The same clade also included GU012603, GU012579, GU012562 and GU012562 from Pune, India, they are monophyletic sharing the same ancestor (Fig. 130). The *Y. ceylonica* (KF733789) from Tamilnadu, India, found in the adjacent clade. The *Y. ceylonica* (KF733789) from Tamilnadu, India is a different species in the genus represented in a separate clade close to *Ypthima huebneri* CUJ18. The NJ-tree distance data revealed that the species was diverged from their closely related species *Y. ceylonica* about 2,000 years ago. The data revealed that *Y. huebneri* CUJ18 also has recent origin and they were related with South East Asia. The identification of closely related species *Y. ceylonica*. (KF733789) from Tamilnadu of South India is originated in Sri Lanka and they were distributed to South India through land bridges.

3.19. ***Tirumala limniace*** **(Cramer, 1775) isolate CUJ19 (KT880655)**

The specimen CUJ19 was identified as *Tirumala limniace* (Cramer, 1775) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio limniace* Cramer, [1775]

Danais limniace fruhstorferi van Eecke, 1915

Danaida limniace kuchingana Moulton, 1915

The *Tirumala limniace* is the blue tiger butterfly found in India that belongs to the Danaid group of the Nymphalidae.

Upper side; black, with bluish-white semi hyaline spots and streaks. Fore wing; inter space 1 two streaks, sometimes coalescent, with a spot beyond cell; a streak from base and an outwardly indented spot at its apex. A large oval spot at base of inter space 2, another at base of inter space 3. A smaller spot beyond it towards termen. Five obliquely placed pre apical streaks, and somewhat irregular sub terminal and terminal series of spots, the latter the smaller.

Hind wing; interspaces 1b, 1a, and 1 with streaks from base, double in the latter two, cell with a forked broad streak, the lower branch with a hook like slender loop, at base of 4 and 5 a broad elongate streak, at base of 6 a quadrate spot; beyond these again a number of scattered unequal sub terminal and terminal spots Under side; basal two-thirds of fore wing; dusky black, the apex and hind wing, olive-brown; the spots and streaks much as on the upper side. Antennae, head and thorax black, the latter two spotted and streaked with, white; abdomen dusky above, ochraceous spotted with white beneath (Fig 131)

Fig 131. The lower side of the *T. limniace* CUJ19. Black, with bluish-white semi hyaline spots and streaks. Hind wing; interspaces 1b, 1a, and 1 with streaks from base, double in the latter two, cell with a forked broad streak, the lower branch with a hook, like slender loop, at base of 4 and 5 a broad elongate streak, at base of 6 a quadrate spot; beyond these again a number of scattered unequal sub terminal and terminal spots

. Fig. 132. The lower side of the *T. limniace* isolate CUJ19.

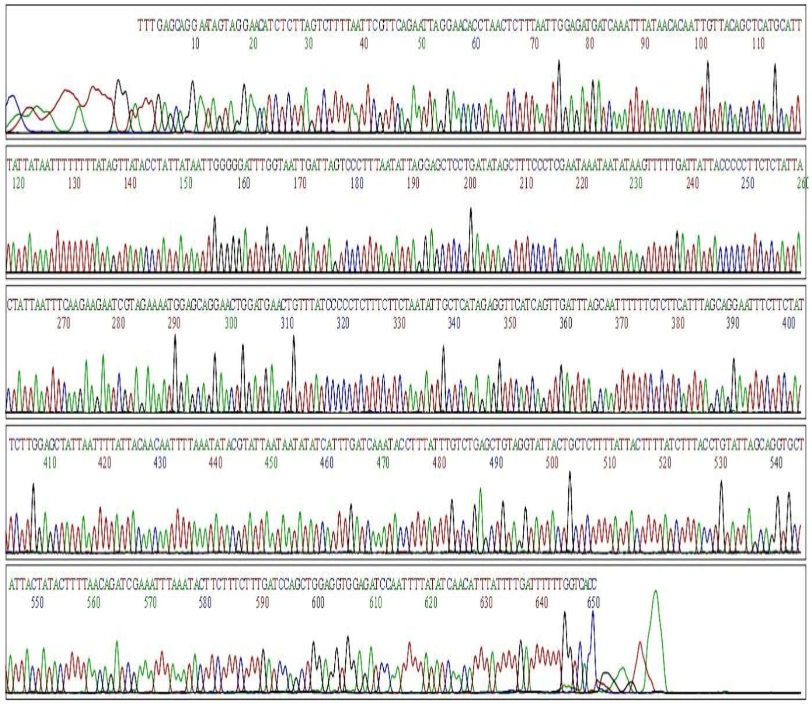

Fig. 133. The sequencing chromatogram of forward DNA strand of *T. limniace* CUJ19.

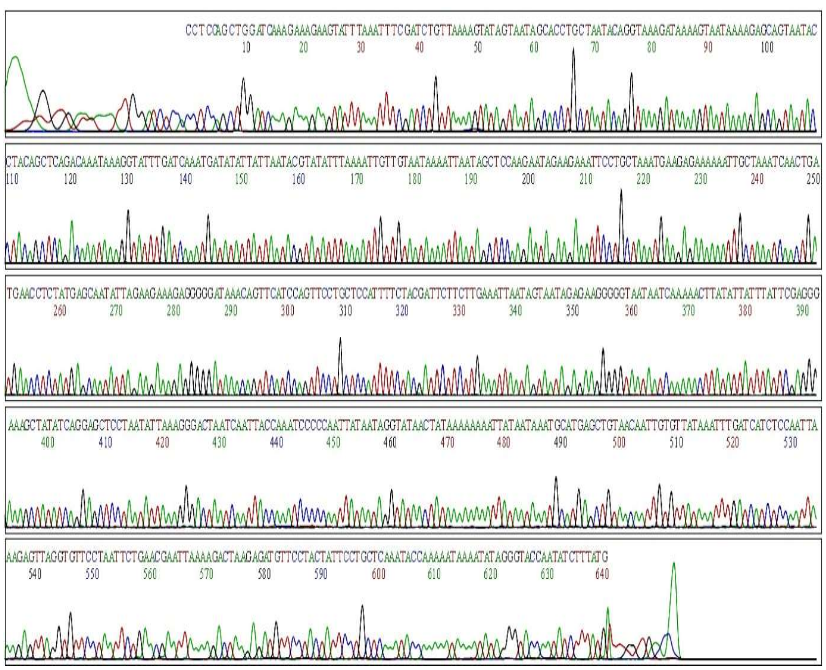

Fig.134. The sequencing chromatogram of reverse DNA strand of *T. limniace* CUJ19.

The larvae are light green with dark cross lines and have horn like structures on third and twelfth segments. The larval food plants involve *Asclepias curassavica*, *Calotropis gigantea*, *Tylophora indica* and *Wattakaka volubilis*.

The PCR amplification of partial COI sequence of *T. limniace* CUJ19 from Kottiyoor ($11^0$52'42"N, $75^0$51'30"E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 133 to 137).

```
>Tirumala limniace voucher CUJ19 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
TACCCTATATTTTATTTTTGGTATTTGAGCAGGAATAGTAGGAACATCTCTTAGTCTTTT
AATTCGTTCAGAATTAGGAACACCTAACTCTTTAATTGGAGATGATCAAATTTATAACAC
AATTGTTACAGCTCATGCATTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGG
GGGATTTGGTAATTGATTAGTCCCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCTCG
AATAAATAATATAAGTTTTTGATTATTACCCCCTTCTCTATTACTATTAATTTCAAGAAG
AATCGTAGAAAATGGAGCAGGAACTGGATGAACTGTTTATCCCCCTCTTTCTTCTAATAT
TGCTCATAGAGGTTCATCAGTTGATTTAGCAATTTTTTCTCTTCATTTAGCAGGAATTTC
TTCTATTCTTGGAGCTATTAATTTTATTACAACAATTTTAAATATACGTATTAATAATAT
ATCATTTGATCAAATACCTTTATTTGTCTGAGCTGTAGGTATTACTGCTCTTTTATTACT
TTTATCTTTACCTGTATTAGCAGGTGCTATTACTATACTTTTAACAGATCGAAATTTAAA
TACTTCTTT
```

Fig.135. The partial sequence of mitochondrial COI gene of *T. limniace* CUJ19.

The conceptual translation of the consensus sequence of *T. limniace* CUJ19 gave the following peptide sequence.

```
>seq17 Tirumala limniace CUJ19
TLYFIFGIWAGMVGTSLSLLIRSELGTPNSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLLLLISSSIVENGAGTGWTVYPPLSSNIAH
SGSSVDLAIFSLHLAGISSILGAINFITTILNMRINNMSFDQMPLFVWAVGITALLLLLSL
PVLAGAITMLLTDRNLNTS
```

Fig. 136. The peptide sequence obtained from conceptual translation of consensus sequence of cytochrome oxidase subunit I gene, DNA of *T. limniace* CUJ19.

The nucleotide BLAST was conducted with the consensus sequence derived from of *T. limniace* CUJ19. The details about similarities of sequences obtained and alignment details were represented below.

S. I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|657172745|gb|KJ459821.1|Tirumala limniace isolate F704*	99.84	621	1	0	35	655	1	621	1142
2	*gi|451329339|gb|KC306727.1|Tirumala limniace voucher ZN2*	99.84	615	1	0	11	625	1	615	1131
3	*gi|321485871|gb|HQ990432.1|Tirumala limniace voucher NIBGE BUT-00121*	99.70	658	2	0	1	658	1	658	1205
4	*gi|330600455|gb|GU012630.1|Tirumala limniace voucher F8*	99.67	606	2	0	25	630	1	606	1109
5	*gi|662117031|gb|KJ784473.1|Tirumala limniace*	99.54	658	3	0	1	658	1520	2177	1199
6	*gi|330600445|gb|GU012622.1|Tirumala limniace voucher F424*	99.51	608	3	0	27	634	1	608	1107
7	*gi|321485869|gb|HQ990431.1|Tirumala limniace voucher NIBGE BUT-00120*	99.39	658	4	0	1	658	1	658	1194
8	*gi|305695072|gb|GU681875.1|Tirumala limniace voucher NIBGE IMB-00134*	99.38	647	4	0	6	652	1	647	1173
9	*gi|396576490|gb|JX261943.1|Tirumala limniace voucher BUZOOUGC-Tl*	99.37	634	4	0	25	658	1	634	1149
10	*gi|657172661|gb|KJ459779.1|Tirumala limniace isolate F426*	99.36	624	4	0	35	658	1	624	1131
11	*gi|731772914|gb|KM507501.1|Tirumala limniace voucher A-11835*	99.20	621	5	0	38	658	1	621	1120
12	*gi|330600267|gb|GU012520.1|Tirumala limniace voucher F59*	99.17	603	5	0	28	630	1	603	1086
13	*gi|197359535|gb|EU792479.1|Tirumala limniace isolate SEC04BT05*	98.94	660	5	2	1	658	4	663	1179
14	*gi|330600451|gb|GU012626.1|Tirumala limniace voucher F197*	98.10	632	7	5	20	646	1	632	1096
15	*gi|342845086|gb|JN266591.1|Tirumala hamata voucher 11ANIC-07133*	97.33	599	16	0	6	604	6	604	1018
16	*gi|545691111|gb|KF226650.1|Tirumala septentrionis septentrionis voucher UMKL-JJW0329*	97.24	653	18	0	6	658	6	658	1107
17	*gi|657172591|gb|KJ459744.1|Tirumala septentrionis isolate A34*	97.24	615	17	0	35	649	1	615	1042
18	*gi|54639849|gb|AF187764.2|Dymasia dymas voucher NW27-7*	90.39	635	57	4	26	658	1	633	832
19	*gi|735666238|gb|KM459152.1|Aricia cramera isolate 12O009*	90.37	654	61	2	6	658	6	658	857

Table 23. The BLAST hit Table of *T. limniace* CUJ19.

The consensus sequence of *T. limniace* CUJ19 BLAST against database and showed 99.39% similarity to the *T. limniace* (HQ990431) from Pakistan, 99.37% to *T. limniace* (JX261943) from Coimbatore, India, 99.36% to *T. limniace* (KJ459779) from Pune, 98.94% to *T. limniace* (EU792479) from Bangalore. The *T. hamata* (JN266591) from Australia has 97.33% and *T. serptentrionis* (KJ459744) from Pune with 97.24% similarities.

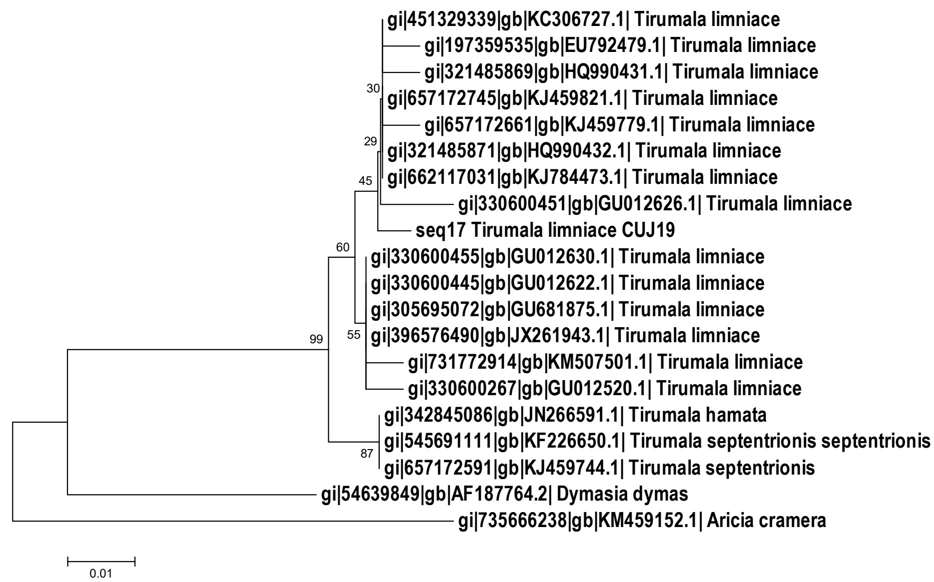

Fig. 137. NJ-tree with Phylogenetic relationship of *T. limniace* CUJ19.

The phylogeny of *T. limniace* CUJ19 was derived with NJ-tree developed from BLAST results (Fig. 137). *T. limniace* CUJ19 was found to be placed in an adjacent clade with 5 samples of *T. limniace* HQ990431 from Pakistan, JX261943 from Coimbatore, India, KJ459779 from Pune, and *T. limniace* EU792479 from Bangalore. The *T. hamata* JN266591 from Australia and *T. serptentrionis* (KJ459744) from Pune were found in different clade.

Discussion

The BLASTn results of *T. limniace* CUJ19 showed 99.39% similarity to the *T. limniace* HQ990431 from Pakistan, 99.37% to JX261943 from Coimbatore, India, 99.36% to KJ459779 from Pune and 98.94% to EU792479 from Bangalore. The sequences from database has some variations from CUJ19, therefore it is a novel sequence of the species. *T. hamata* JN266591 from Australia and *T. serptentrionis* (KJ459744) from Pune are with 97.33% and 97.24% similarities respectively. In the NJ-tree *T. limniace* CUJ19 found to be placed in an adjacent clade with 5 samples of *T. limniace* HQ990431 from Pakistan, JX261943 from Coimbatore, India, KJ459779 from Pune, and *T. limniace* EU792479 from Bangalore, all of them are monophyletic sharing a common ancestor. The *T. hamata* JN266591 from Australia and *T. serptentrionis* KJ459744 from Pune were found in a different clade, they were different species of the genus closely related to *T. limniace* CUJ19. The NJ-tree distance data revealed that the species was diverged from their closely related species *T. hamata* about 9,000 years ago. The data revealed that *T. limniace* CUJ19 also has recent origin and they were related to isolates from South East Asia.

3.20. *Melanitis leda* (Linnaeus, 1758) isolate CUJ20 (KT880656)

The specimen CUJ20 was identified as *Melanitis leda* (Linnaeus, 1758) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio leda* Linnaeus, 1758

Cyllo helena Westwood, 1851

Cyllo fulvescens Guénée, 1863

Melanitis ismene Butler, 1900

Melanitis leda africana Fruhstorfer, 1908

Melanitis leda africana f. zitenides Fruhstorfer, 1908

Melanitis leda ab. plagiata Aurivillius, 1911

The *Melanitis leda* is the common evening brown butterfly which is a common Nymphalidae species of Kerala, the flight of this species is erratic. They are frequent during dawn and dusk.

Fore wing; apex is sub-acute and termen slightly angulated just below apex. Upper side; brown, with two large sub apical black spots, each of them with a smaller spot outwardly of pure white and inwardly bordered with ferruginous interrupted lunule, the costal margin is narrow and pale. Hind wing; with dark, white-centered, tawny-ringed ocellus seen sub terminally in inter space 2, and the apical ocellus, others of the ocelli, are showing through on the underside. Under side; paler than upper, densely covered with transverse dark brown parallel lines, a discal curved dark brown narrow band on fore wing, a post-discal.

Fig. 138. The lower side of the *M. leda* CUJ20, brown, with two large sub apical black spots, under side a series of ocelli, four on the fore wing; in interspace 8, which is the largest and hind wing; six ocelli, among them the apical and sub tornal are the largest.

Fig. 139. The habit of the *M. leda* CUJ20.

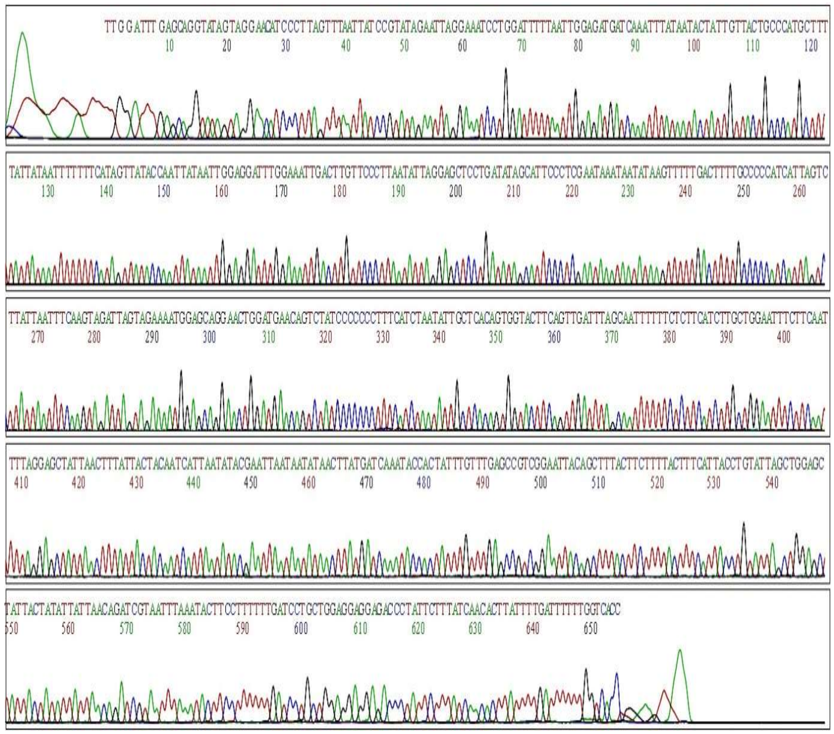

Fig.140. The sequencing chromatogram of forward DNA strand of *M. leda* CUJ20.

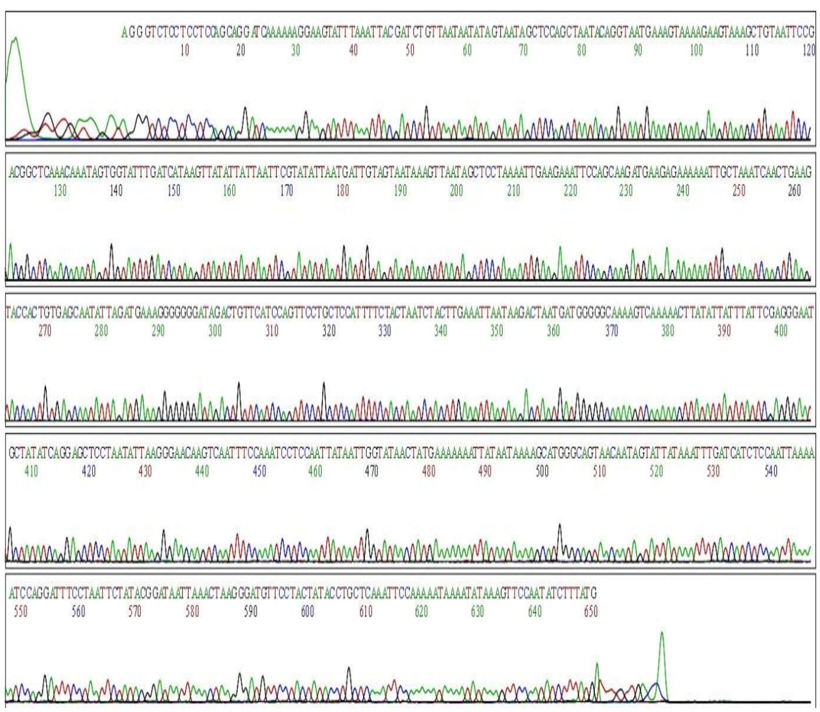

Fig.141. The sequencing chromatogram of reverse DNA strand of *M. leda* CUJ20.

Similar oblique band which is followed by a series of ocelli, four on the fore wing; in interspace 8, which is the largest and hind wing; six ocelli, among them the apical and sub tornal are the largest (Fig. 138).

Many grasses act as the larval host plants, it is a known pest of paddy, the larvae are shining green with two red coloured horns on the head the lateral sides are marked with white lines, the tail end is also found bifid.

The PCR amplification of partial COI sequence of *M. leda* CUJ20 from Nilambur ($11^0$18’58”N, $76^0$12’32”E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 140 to 144).

```
>Melanitis leda voucher CUJ20 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
AACTTTATATTTTATTTTTGGAATTTGAGCAGGTATAGTAGGAACATCCCTTAGTTTAAT
TATCCGTATAGAATTAGGAAATCCTGGATTTTTAATTGGAGATGATCAAATTTATAATAC
TATTGTTACTGCCCATGCTTTTATTATAATTTTTTTCATAGTTATACCAATTATAATTGG
AGGATTTGGAAATTGACTTGTTCCCTTAATATTAGGAGCTCCTGATATAGCATTCCCTCG
AATAAATAATATAAGTTTTTGACTTTTGCCCCCATCATTAGTCTTATTAATTTCAAGTAG
ATTAGTAGAAAATGGAGCAGGAACTGGATGAACAGTCTATCCCCCCCTTTCATCTAATAT
TGCTCACAGTGGTACTTCAGTTGATTTAGCAATTTTTTCTCTTCATCTTGCTGGAATTTC
TTCAATTTTAGGAGCTATTAACTTTATTACTACAATCATTAATATACGAATTAATAATAT
AACTTATGATCAAATACCACTATTTGTTTGAGCCGTCGGAATTACAGCTTTACTTCTTTT
ACTTTCATTACCTGTATTAGCTGGAGCTATTACTATATTATTAACAGATCGTAATTTAAA
TACTTCCTT
```

Fig.142. The partial sequence of mitochondrial COI gene of *M. leda* CUJ20.

The conceptual translation of the consensus sequence of *M, leda* CUJ20 gave the following peptide sequence (Fig. 143).

```
>seq18 Melanitis leda CUJ20

TLYFIFGIWAGMVGTSLSLIIRMELGNPGFLIGDDQIYNTIVTAHAFIMIFFMVMP
IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLVLLISSSLVENGAGTGWTV
YPPLSSNIAHSGTSVDLAIFSLHLAGISSILGAINFITTIINMRINNMTYDQMPLF
VWAVGITALLLLLSLPVLAGAITMLLTDRNLNTS
```

Fig. 143. The peptide sequence obtained from conceptual translation of consensus

sequence of COI gene, DNA of *M. leda* CUJ20.

The nucleotide BLAST was conducted with the consensus sequence derived from of *M. leda* CUJ20 and CUJ21. The details about similarities of sequences obtained and alignment details were represented below.

S.I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|321121697|gb|HQ962136.1|Melanitis leda leda voucher YB-KHC184*	100.00	658	0	0	1	658	1	658	1216
2	*gi|330600439|gb|GU012619.1|Melanitis leda voucher F121*	100.00	645	0	0	5	649	1	645	1192
3	*gi|330600327|gb|GU012554.1|Melanitis leda voucher F383*	100.00	627	0	0	8	634	1	627	1158
4	*gi|657172763|gb|KJ459830.1|Melanitis leda isolate T27*	100.00	624	0	0	35	658	1	624	1153
5	*gi|657172695|gb|KJ459796.1|Melanitis leda isolate F109*	100.00	624	0	0	35	658	1	624	1153
6	*gi|657172675|gb|KJ459786.1|Melanitis leda isolate F316*	100.00	624	0	0	35	658	1	624	1153
7	*gi|699059583|gb|KM111608.1|Melanitis leda voucher meled1*	100.00	621	0	0	38	658	1	621	1147
8	*gi|321121705|gb|HQ962140.1|Melanitis leda leda voucher YB-KHC244*	100.00	602	0	0	1	602	1	602	1112
9	*gi|657172739|gb|KJ459818.1|Melanitis leda isolate F604*	100.00	600	0	0	35	634	1	600	1109
10	*gi|451964312|gb|KC433403.1|Melanitis leda voucher M.LID1*	100.00	539	0	0	120	658	1	539	996
11	*gi|321121715|gb|HQ962145.1|Melanitis leda leda voucher YB-KHC273*	99.85	658	1	0	1	658	1	658	1210
12	*gi|657172791|gb|KJ459844.1|Melanitis leda isolate T76*	99.84	624	1	0	35	658	1	624	1147
13	*gi|657172769|gb|KJ459833.1|Melanitis leda isolate T31*	99.84	624	1	0	35	658	1	624	1147
14	*gi|657172767|gb|KJ459832.1|Melanitis leda isolate T29*	99.84	624	1	0	35	658	1	624	1147
15	*gi|657172667|gb|KJ459782.1|Melanitis leda isolate F351*	99.84	624	1	0	35	658	1	624	1147
16	*gi|657172599|gb|KJ459748.1|Melanitis leda isolate A56*	99.84	624	1	0	35	658	1	624	1147
17	*gi|657172545|gb|KJ459721.1|Melanitis leda isolate A2*	99.84	624	1	0	35	658	1	624	1147

18	*gi|657172571|gb|KJ459734.1|Melanitis leda isolate F631*	99.84	615	1	0	35	649	1	615	1131
19	*gi|330600325|gb|GU012553.1|Melanitis leda voucher F368*	99.84	611	0	1	37	646	1	611	1122
20	*gi|657172809|gb|KJ459853.1|Melanitis leda isolate F36*	99.68	624	2	0	35	658	1	624	1142
21	*gi|657172615|gb|KJ459756.1|Melanitis leda isolate F571*	99.68	624	2	0	35	658	1	624	1142
22	*gi|330600449|gb|GU012624.1|Melanitis leda voucher F398*	99.54	651	1	2	1	649	3	653	1184
23	*gi|330600409|gb|GU012602.1|Melanitis leda voucher F392*	98.33	660	0	11	5	653	1	660	1147
24	*gi|344188064|gb|JN278828.1|Melanitis leda voucher 11ANIC-07155*	97.87	658	14	0	1	658	1	658	1138
25	*gi|29408952|gb|AY090207.1|Melanitis leda voucher NW66-6*	97.47	633	16	0	26	658	1	633	1081
26	*gi|657172597|gb|KJ459747.1|Melanitis phedima isolate A47*	95.12	615	30	0	35	649	1	615	970
27	*gi|334850201|gb|JF905446.1|Melanitis leda*	94.99	658	33	0	1	658	1485	2142	1033
28	*gi|85013858|gb|DQ338759.1|Gnophodes chelys*	92.42	633	48	0	26	658	1	633	911
29	*gi|341604982|gb|JN197306.1|Calisto batesi voucher McGuire09-CAL-Sat78*	90.88	658	60	0	1	658	1	658	883
30	*gi|341604974|gb|JN197302.1|Calisto archebates voucher McGuire09-CAL-Sat90*	90.58	658	60	2	2	658	2	658	870

Table 24. The BLAST hit table of *M. leda* CUJ20.

.

The consensus sequence of *M. leda* CUJ20, BLAST against database and showed 100% similarity to *M. leda* GU012619, GU012554, KJ459832, KJ459818, KJ459796, KJ459786, KJ459782 and KJ459721) from Pune, India and (HQ962145, HQ962136 and HQ962140) from Thailand and 97.87% similarity with *M. leda* (JN278828), from Australia. The *M. phedima* (KJ459747) from Pune has 95.12% similarity to CUJ20.

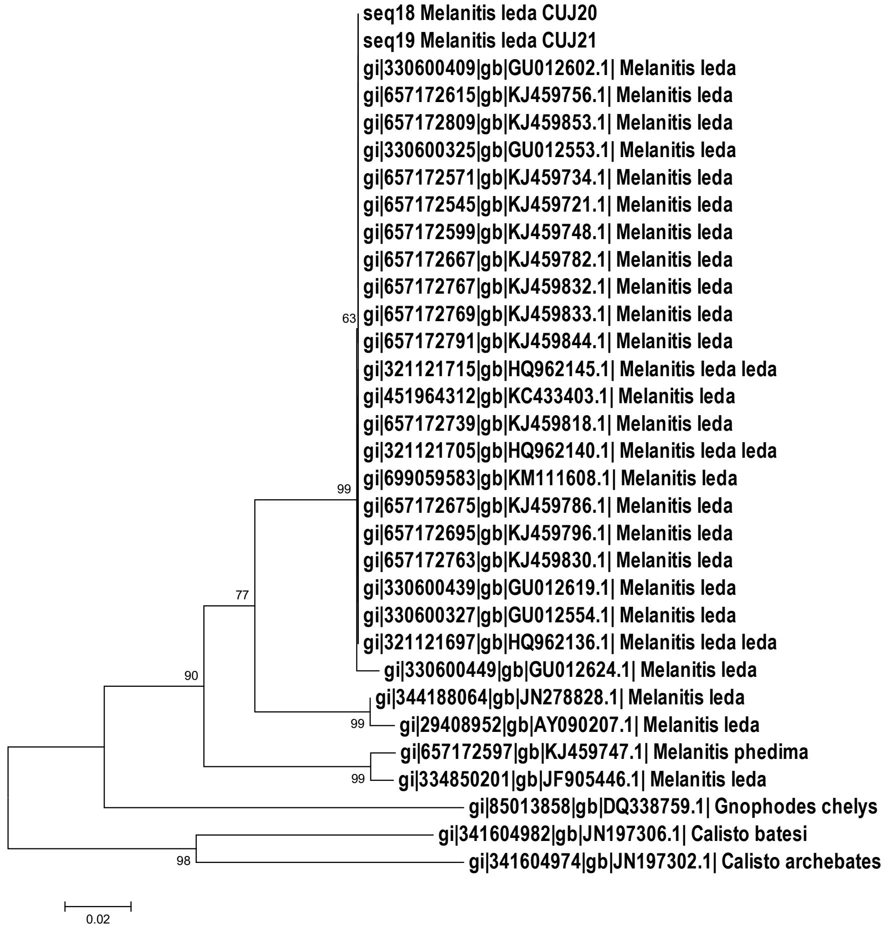

Fig. 144. NJ-tree with Phylogenetic relationship of *M. leda* CUJ20 AND CUJ21.

Phylogeny of *M. leda* CUJ20 was derived with NJ-tree developed from BLAST results (Fig. 144). The CUJ20 and CUJ21 were found to be placed in a clade with 11 samples viz; GU012619 GU012554, KJ459832, KJ459818, KJ459796, KJ459786, KJ459782 and KJ459721 from Pune, India and HQ962145, HQ962136, HQ962140 from Thailand with 100% similarity. However the polymorphic variants *M. leda* JN278828, from Australia and India were placed in a different clade. The *M. phedima* KJ459747 from Pune was placed in the adjacent clade.

Discussion

The BLAST results showed 100% similarity to *M. leda* CUJ20 to GU012619, GU012554, KJ459832, KJ459818, KJ459796, KJ459786, KJ459782 and KJ459721 from Pune, India, the HQ962145, HQ962136 and HQ962140 from Thailand, hence it can be a molecular barcode helping species identification. The *M. leda* JN278828, from Australia with 97.87% similarity is a polymorphic geographical variant of CUJ20. The *M. phedima* KJ459747 from Pune has 95.12% similarity to CUJ20 is the closely related species. Phylogeny of CUJ20 was derived with NJ-tree developed from the sequences obtained from BLAST hit results (Fig. 144). The *M. leda* CUJ20 was found to be placed in a clade with 11 samples *viz*; GU012619 GU012554, KJ459832, KJ459818, KJ459796, KJ459786, KJ459782 and KJ459721 from Pune, India and HQ962145, HQ962136, HQ962140 from Thailand, and were monophyletic sharing common ancestor. However *M. leda* JN278828 from Australia with 97.87% similarity was placed in a different clade. The *M. phedima* KJ459747 from Pune with 95.12% similarity was placed in the adjacent clade, which is a different species of the genus remain close to *M. leda* CUJ20 formed an adjoining clade. The NJ-tree distance data revealed that the species was originated from their closely related species *M. phedima* about 54,000 years ago. The data revealed that *M. leda* CUJ20 also has a recent origin and they were related to isolates from South East Asia.

3.21. *Acraea violae* (Fabricius, 1793) isolate CUJ22 (KT880658)

The specimen CUJ22 was identified as *Acraea violae (*Fabricius, 1793*)* referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonym: *Papilio violae* Fabricius, 1793

The *Acraea violae* is the Tawny coster butterfly which is a Nymphalidae common in grassland and scrub habitats. This species is the only representative of the tribe *Acraeini* from our region.

Upper side of the fore wing; transverse black spot in cell, and another oblique, irregular and broader at the disco-cellulars, a discal series of spots in interspaces 1, 3, 4, 5, 6 and 10, the apex and termen are black. Upper four spots of the discal series obliquely inclined outwards, the lower two inwards obliquely; the black edging to apex and termen posteriorly narrowing, but with linear slender-projections inwards in the interspaces (Fig. 145).

Hind wing; series of five black spots basaly, similar spot beyond in middle of cell, and a subcostal black spot above it, a discal series of obscure blackish spots, a minute post discal black dot in interspaces 4 and 6 respectively; finally, a broad black terminal band medially traversed by a series of small spots of the ground-colour. Most of the macular black markings are vague, being only the spots on the upper side seen by the transparency of the wing-membrane; the inner edge of the black terminal band crenulated (Fig. 146). Under side; ground-colour paler tawny yellow, for e wing; pale whitish on the apex, with the black markings as on the dorsal side but somewhat blurred and diffuse.

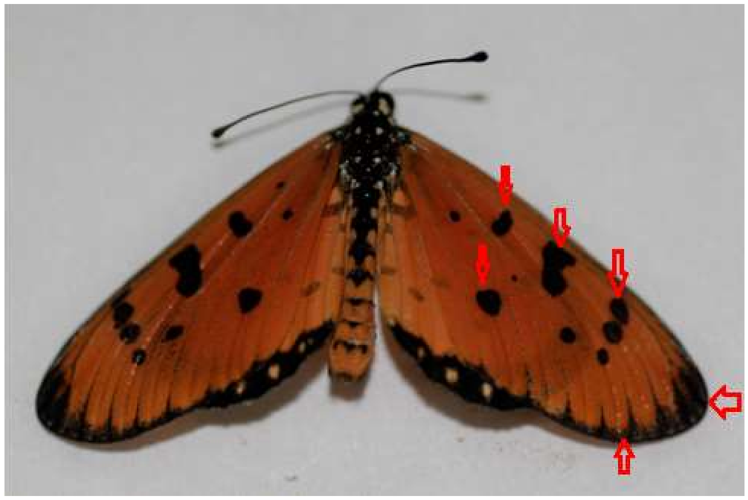

Fig. 145. The upper side of the *A. violae* CUJ22, fore wing; transverse black spot in cell, and another oblique, irregular and broader at the disco-cellulars; a discal series of spots in interspaces 1, 3, 4, 5, 6 and 10, apex and termen are black, the black edging to apex and termen posteriorly narrowing, but with linear slender - projections inwards in the interspaces.

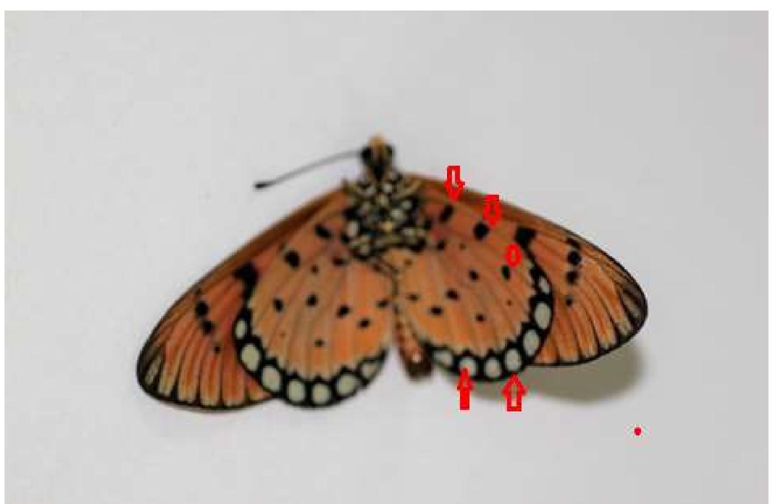

Fig. 146. The lower side of the *A. violae* CUJ22, a subcostal black spot above it, a discal series of obscure blackish spots, a minute post discal black dot in interspaces 4 and 6 respectively; a broad black terminal band medially traversed by a series of small spots of the ground-colour. The inner edge of the black terminal band crenulated.

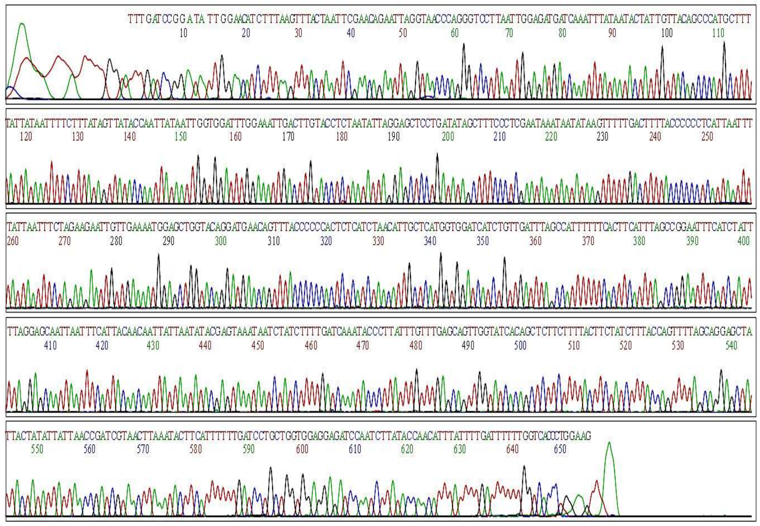

Fig.147. The sequencing chromatogram of forward DNA strand of *A. violae* CUJ22.

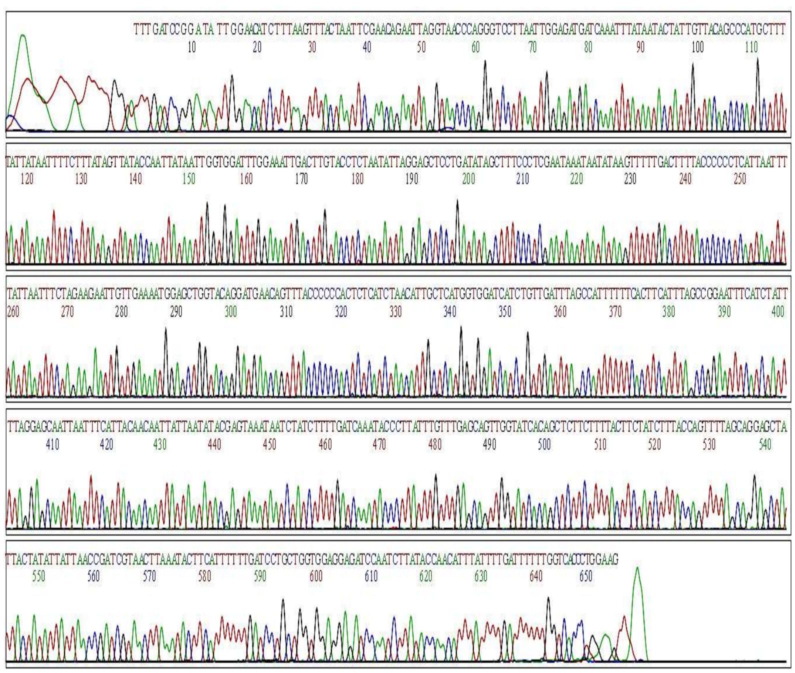

Fig.148. The sequencing chromatogram of reverse DNA strand of *A.violae* CUJ22.

Hind wing; the black spots and black terminal band as on the dorsal side, but the spots more clearly defined, none obscure; the series of spots traversing the black terminal margin very much larger and white-not tawny; the base of the wing black, separated from the basal transverse series of black spots by two or three large whitish spots.

Antennae; black, head and thorax; black, spotted with ochraceous and white. Abdomen anteriorly black, posteriorly ochraceous yellow with narrow transverse black lines; beneath, the palpi, thorax and abdomen; ochraceous, the thorax spotted with ochraceous, the abdomen with a longitudinal line of black at base.

The larval food plants involve *Passiflora edulis, Adenia bondala, Hibiscus cannabinus* and *Passiflora foetida* the larvae are chocolate coloured.

The PCR amplification of partial COI sequence of *A. violae* CUJ22 from Cheemeni ($12^0$13'52"N, $75^0$14'50"E) yielded a consensus with 622 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 147-151)

```
>Acraea violae voucher CUJ22 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (622)
ATAAAGATATTGGAACTTTATATTTTATTTTTGGTATTTGATCCGGAATAATTGGAACAT
CTTTAAGTTTACTAATTCGAACAGAATTAGGTAACCCAGGGTCCTTAATTGGAGATGATC
AAATTTATAATACTATTGTTACAGCCCATGCTTTTATTATAATTTTCTTTATAGTTATAC
CAATTATAATTGGTGGATTTGGAAATTGACTTGTACCTCTAATATTAGGAGCTCCTGATA
TAGCTTTCCCTCGAATAAATAATATAAGTTTTTGACTTTTACCCCCCTCATTAATTTTAT
TAATTTCTAGAAGAATTGTTGAAAATGGAGCTGGTACAGGATGAACAGTTTACCCCCCAC
TCTCATCTAACATTGCTCATGGTGGATCATCTGTTGATTTAGCCATTTTTTCACTTCATT
TAGCCGGAATTTCATCTATTTTAGGAGCAATTAATTTCATTACAACAATTATTAATATAC
GAGTAAATAATCTATCTTTTGATCAAATACCCTTATTTGTTTGAGCAGTTGGTATCACAG
CTCTTCTTTTACTTCTATCTTTACCAGTTTTAGCAGGAGCTATTACTATATTATTAACCG
ATCGTAACTTAAATACTTCATT
```

Fig.149 The partial sequence of mitochondrial COI gene DNA of *A. violae* CUJ22.

The conceptual translation of the consensus sequence of *A. violae* CUJ22 gave the following peptide sequence

```
>seq20 Acraea violae CUJ22

GSTKHKDIGTL7FIFGIWSGMIGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAF
IMIFFMVMPIMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLILLISSSIVE
NGAGTGWTVYPPLSSNIAHGGSSVDLAIFSLHLAGISSILGAINFITTIINMRVNN
LSFDQMPLFVWAVGITALLLLLSLPVLAGAITMLLTDRNLNTS
```

Fig. 150. The peptide sequence obtained from conceptual translation of sequence of COI gene of *A. violae* CUJ22

The nucleotide BLAST was conducted with the consensus sequence derived from *A. violae* CUJ22. The details about similarities of sequences obtained and alignment are represented below.

S. I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|657172657|gb|KJ459777.1|Acraea violae isolate F472*	100.00	633	0	0	62	694	1	633	1170
2	*gi|395758909|gb|JX226067.1|Acraea violae voucher BUZOOUGC-Av*	100.00	610	0	0	56	665	1	610	1127
3	*gi|657172649|gb|KJ459773.1|Acraea violae isolate F486*	99.68	633	2	0	62	694	1	633	1158
4	*gi|330600283|gb|GU012529.1|Acraea violae voucher F74*	99.18	612	2	3	56	665	1	611	1099
5	*gi|529367725|gb|KF395497.1|Acraea andromacha voucher 11ANIC-07827*	95.59	658	29	0	28	685	1	658	1055
6	*gi|167882675|gb|EU275524.1|Acraea camaena voucher NW160-12*	93.54	681	44	0	16	696	1	681	1014
7	*gi|167882683|gb|EU275528.1|Acraea endoscota voucher NW160-18*	92.25	697	53	1	16	712	1	696	987
8	*gi|758277351|gb|KP074832.1|Heliconius pardalinus butleri voucher MJ2021*	90.98	687	60	2	17	702	1	686	924
9	*gi|758277231|gb|KP074792.1|Heliconius elevatus voucher 503*	90.48	693	64	2	5	696	1	692	913
10	*gi|758276223|gb|KP074314.1|Heliconius luciana*	90.48	693	64	2	5	696	1	692	913

	voucher AN3									
11	*gi|758277303|gb|KP074816.1|Heliconius hecale voucher 71*	90.28	679	64	2	17	694	1	678	887
12	*gi|167882667|gb|EU275520.1|Acraea acerata voucher NW160-6*	90.26	698	65	3	16	712	1	696	909
13	*gi|758277246|gb|KP074797.1|Heliconius ethilla voucher 3014*	89.87	681	67	2	17	696	1	680	874
14	*gi|378830637|gb|JQ392684.1|Taygetis sosis voucher gsm213*	89.85	680	69	0	17	696	1	680	874
15	*gi|383867277|gb|JQ347260.1|Athyma sulpitia*	89.73	701	67	5	14	712	1479	2176	891
16	*gi|378830593|gb|JQ392662.1|Taygetis laches voucher PM04-17*	89.72	681	70	0	16	696	1	681	870
17	*gi|167882715|gb|EU275544.1|Acraea pseudegina voucher NW160-9*	89.56	699	68	5	16	712	1	696	881

Table 25. The BLAST hit table of *A. violae* CUJ22.

The nucleotide BLAST of COI sequence of *A. violae* CUJ22 showed 100% similarity to the *A. violae* KJ459777 from Pune and JX226067 from Coimbatore, India and 99.68% similarity with KJ459773 from Pune. Another *A. violae* isolate GU012529 from Pune gave 99.18% similarity. The *A. andromacha* KF395497 from Australia has 95.59% similarity and *A. camaena* EU275524 from Brazil showed 93.54% similarity.

The phylogeny of *A. violae* CUJ22 derived with NJ-tree from the similar sequences (Fig. 151). The *A. violae* CUJ22 was found to be placed in a clade with 2 samples involving KJ459777 from Pune and JX226067 from Coimbatore, India. The *A. violae* KJ459773 and GU012529 from Pune were placed in a different clade. The *A. andromacha* (KF395497), from Australia and *A. camaena* EU275524 from Brazil formed the adjacent clade.

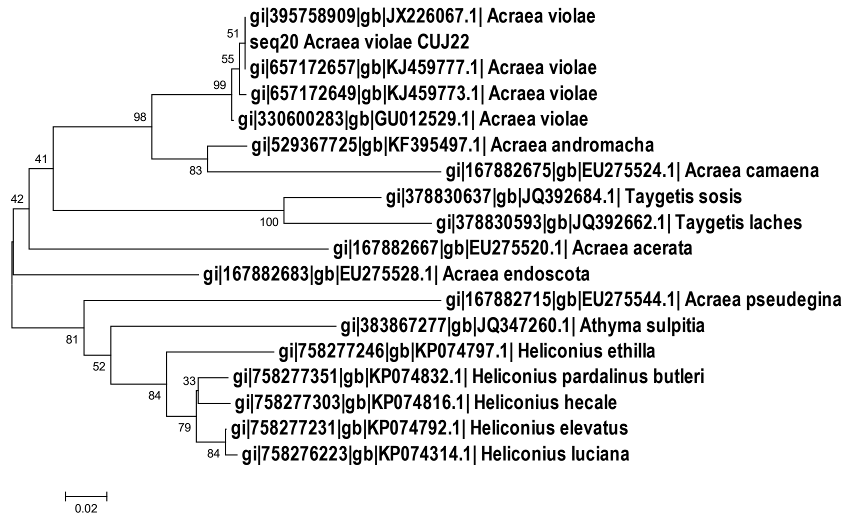

Fig. 151. The NJ-tree with Phylogenetic relationship of *A. violae* CUJ22.

Discussion

The nucleotide BLAST of COI sequence from *A. violae* CUJ22 showed 100% similarity to the *A. violae* KJ459777 from Pune and JX226067 from Coimbatore, India, so it is a molecular barcode helping species identification. The two isolates from Pune KJ459773 and GU012529 gave 99.68 and 99.18% similarities, they are polymorphic variants of CUJ22. The *A. andromacha* KF395497 from Australia has 95.59% similarity and *A. camaena* EU275524 from Brazil showed 93.54% similarity. In the NJ-tree *A. violae* CUJ22 was placed in a clade with two samples involving KJ459777 from Pune and JX226067 from Coimbatore, India they were monophyletic sharing same ancestor. The *A. violae* KJ459773 from Pune, with 99.68% similarity and GU012529 from Pune with 99.18% similarity was placed in a different clade. The *A. andromacha* KF395497, from Australia with 95.59% similarity and *A. camaena* EU275524 from Brazil with

93.54% similarity formed the adjacent clade. The *A. andromacha* KF395497, from Australia with 95.59% similarity is a different species of the genus remain close to *A. violae* CUJ22, in a separate clade. The NJ-tree distance data revealed that the species was diverged from their closely related species *A. andromacha* about 38,000 years ago. Most of the species in the genus *Acraea* were seen in Africa the *Acraea violae* is the only one species of the genus found in India and Sri Lanka the other two species outside Africa include *A. issoria* in South East Asia and *A. andromacha* from Australia.

3.22. *Junonia lemonias* Linnaeus, 1758 isolate CUJ26 (KT880659)

The specimen CUJ26 was identified as *Junonia lemonias* Linnaeus, 1758 referring to the identical morphological features of the species described by Bingham C. T. (Bingham, 1907).

Synonyms; *Precis lemonias* Fruhstorfer, 1912

Junonia lemonias, the lemon pansy, is a common Nymphalid butterfly found in South Asia It is found in gardens and open wooded areas, and planes

Upper side of the wings; is brown with numerous eye-spots, black and lemon-yellow spots and lines. Under side; dull brown, a number of wavy lines and spots in varying shades of brown and black, also an eyespot on the lower side of the fore wing. The markings are distinct and vivid and the wing shape is more rounded. The lemon pansy is a very active butterfly and can be seen basking with its wings open facing the sun. It sits very low to the ground and can be approached easily.

It feeds with its wings half open It is a fairly strong flier and flies close to the ground with rapid wing beat and often returns to settle back in the same spot (Fig. 152 and 153). The larval food plants involve *Corchorus capsularis, Hygrophila auriculata, Sida rhombifolia* and *Cannabis sativa*. The larvae were ash coloured with small spines and white markings.

The PCR amplification of partial COI sequence of *J. lemonias* CUJ26 from Thamarassery (11^{0}24'23"N, 75^{0}55'36"E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented below (Fig.154-158)

Fig. 152. The upper side of the *J. lemonias* CUJ26, brown coloured with three eye-spots two on fore wing and one on hind wing, black and lemon-yellow spots and lines on the wings.

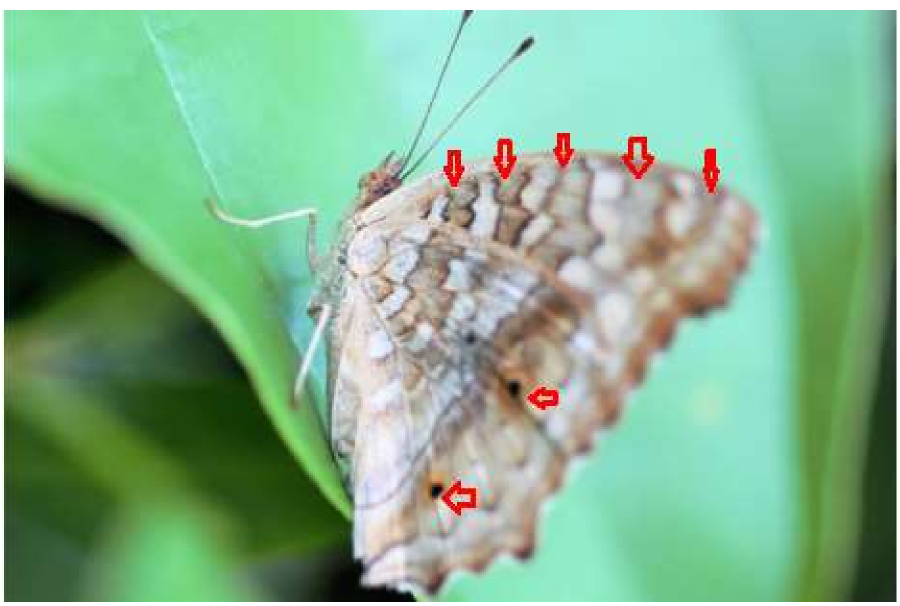

Fig.153. The lower side of the *J. lemonias* CUJ26, dull brown, a number of wavy lines and spots are present in varying shades of brown and black, also an eyespot on the lower side of the fore wing and another on hind wing.

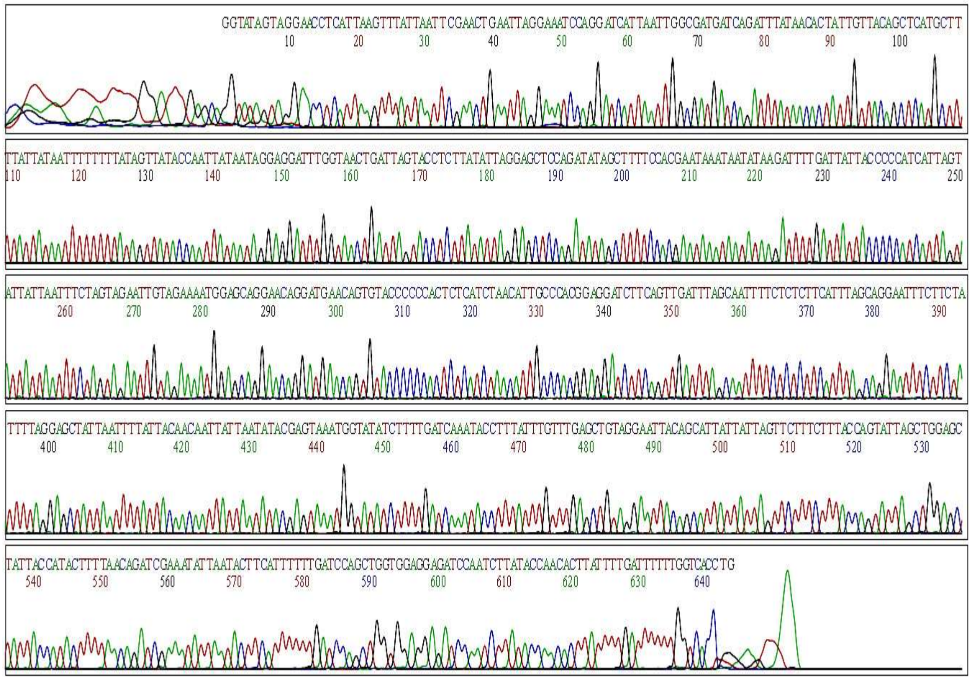

Fig.154. The sequencing chromatogram of forward DNA strand of *J. lemonias* CUJ26.

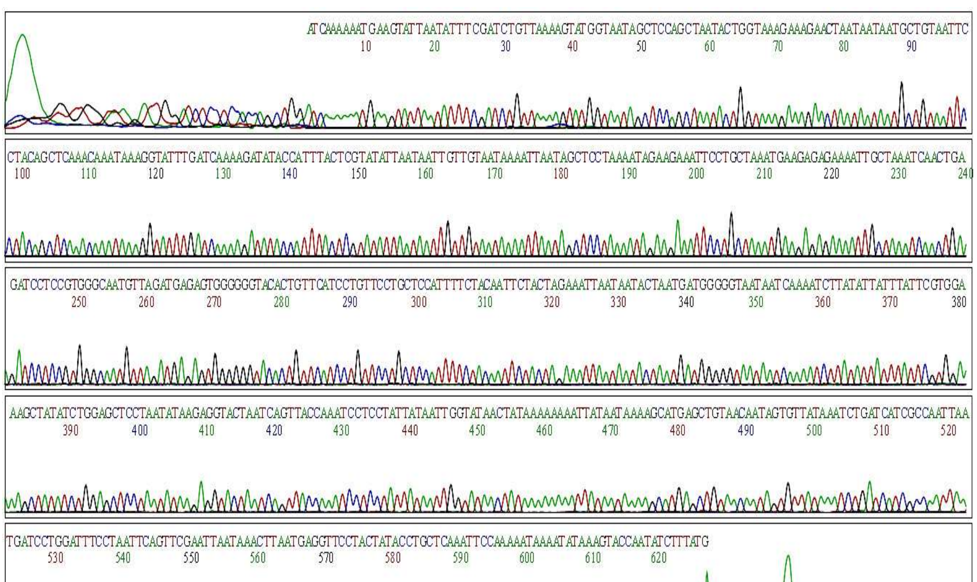

Fig.155. The sequencing chromatogram of reverse DNA strand of *J. lemonias* CUJ26.

```
>Junonia lemonias voucher CUJ26 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
TACTTTATATTTTATTTTTGGAATTTGAGCAGGTATAGTAGGAACCTCATTAAGTTTATT
AATTCGAACTGAATTAGGAAATCCAGGATCATTAATTGGCGATGATCAGATTTATAACAC
TATTGTTACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCAATTATAATAGG
AGGATTTGGTAACTGATTAGTACCTCTTATATTAGGAGCTCCAGATATAGCTTTTCCACG
AATAAATAATATAAGATTTTGATTATTACCCCCATCATTAGTATTATTAATTTCTAGTAG
AATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTGTACCCCCCACTCTCATCTAACAT
TGCCCACGGAGGATCTTCAGTTGATTTAGCAATTTTCTCTCTTCATTTAGCAGGAATTTC
TTCTATTTTAGGAGCTATTAATTTTATTACAACAATTATTAATATACGAGTAAATGGTAT
ATCTTTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACAGCATTATTATTAGT
TCTTTCTTTACCAGTATTAGCTGGAGCTATTACCATACTTTTAACAGATCGAAATATTAA
TACTTCATT
```

Fig.156. The partial sequence of mitochondrial COI gene DNA of *J. lemonias* CUJ26.

The conceptual translation of the consensus sequence of *J. lemonias* CUJ26 gave the following peptide sequence.

```
>seq21 Junonia lemonias CUJ26

TLYFIFGIWAGMVGTSLSLLIRTELGNPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMMGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLVLLISSSIVENGAGTGWTV
YPPLSSNIAHGGSSVDLAIFSLHLAGISSILGAINFITTIINMRVNGMSFDQMPLF
VWAVGITALLLVLSLPVLAGAITMLLTDRNINTS
```

Fig.157. The peptide sequence obtained from conceptual translation of consensus sequence of COI gene, DNA of *J. lemonias* CUJ26

The nucleotide BLAST was conducted with the consensus sequence derived from of *Junonia lemonias* CUJ26.The details about similarities of sequences obtained and alignment details were represented below.

S. I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|893640295|gb|KP941756.1|Junonia lemonias*	100.00	658	0	0	1	658	1494	2151	1216
2	*gi|321121851|gb|HQ962213.1|Junonia lemonias lemonias voucher YB-KHC6544*	100.00	658	0	0	1	658	1	658	1216
3	*gi|375004897|gb|JN698955.1|Junonia lemonias isolate*	100.00	657	0	0	2	658	13	669	1214

	MZUbp76									
4	*gi|330600431|gb|GU012615.1|Junonia lemonias voucher F169*	100.00	649	0	0	10	658	1	649	1199
5	*gi|330600277|gb|GU012526.1|Junonia lemonias voucher F70*	100.00	639	0	0	1	639	1	639	1181
6	*gi|155968183|gb|EU053308.1|Junonia lemonias voucher NW101-15*	100.00	633	0	0	26	658	1	633	1170
7	*gi|657172559|gb|KJ459728.1|Junonia lemonias isolate A18*	100.00	624	0	0	35	658	1	624	1153
8	*gi|330600251|gb|GU012511.1|Junonia lemonias voucher F20*	100.00	624	0	0	11	634	1	624	1153
9	*gi|330600422|gb|GU012610.1|Junonia lemonias voucher F337*	100.00	621	0	0	23	643	1	621	1147
10	*gi|925175961|gb|KP997223.1|Junonia lemonias lemonias voucher MMJ-08*	100.00	604	0	0	24	627	1	604	1116
11	*gi|395758905|gb|JX226065.1|Junonia lemonias voucher BUZOOUGC-Jl*	99.84	627	0	1	15	641	1	626	1151
12	*gi|155968185|gb|EU053309.1|Junonia lemonias voucher NW81-4*	99.68	633	2	0	26	658	1	633	1158
13	*gi|330600354|gb|GU012573.1|Junonia lemonias voucher N313*	99.54	650	0	2	4	650	1	650	1181
14	*gi|155968189|gb|EU053311.1|Junonia lemonias voucher NW97-9*	99.53	633	3	0	26	658	1	633	1153
15	*gi|169672603|gb|EU368159.1|Junonia lemonias isolate sheyan4*	99.09	657	6	0	2	658	1	657	1181
16	*gi|155968187|gb|EU053310.1|Junonia lemonias voucher NW97-10*	98.74	635	4	4	26	658	1	633	1125
17	*gi|197359552|gb|EU792494.1|Junonia lemonias isolate SEC19LP05*	97.58	619	9	6	41	658	19	632	1055
18	*gi|330600330|gb|GU012556.1|Junonia orithya voucher F387*	97.16	634	17	1	1	633	8	641	1070
19	*gi|344179386|gb|JN274489.1|Junonia villida voucher 11ANIC-07800*	97.11	658	19	0	1	658	1	658	1110
20	*gi|442535842|gb|KC158391.1|Junonia hierta voucher NIBGE BUT-00272*	96.51	658	23	0	1	658	1	658	1088
21	*gi|821183359|gb|KM288090.1|Junonia evarete voucher fled8*	95.90	658	27	0	1	658	24	681	1074
22	*gi|657635938|gb|KJ469065.*	95.90	658	27	0	1	658	1	658	1066

	1|Junonia zonalis isolate LCB163									

Table 26. The BLAST hit table of *J. lemonias* CUJ26.

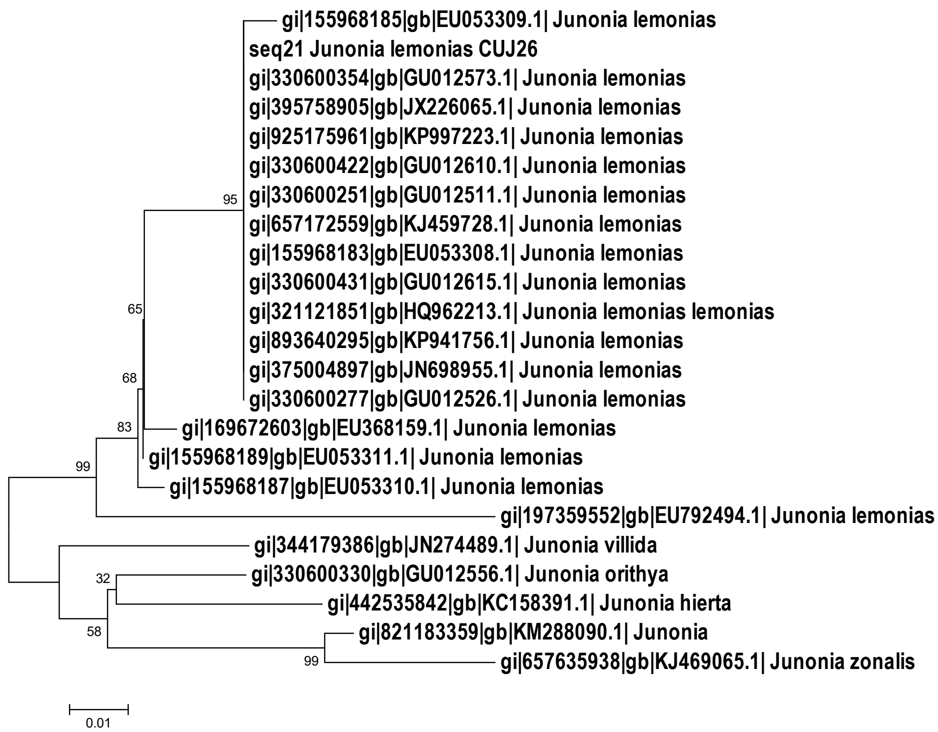

Fig. 158. The NJ-tree with Phylogenetic relationship of *J. lemonias* CUJ26.

The COI sequence of *J. lemonias* CUJ26, BLAST result showed 100% similarity to that of *J. lemonias* KP941756, KP997223, HQ962213, JN698955, EU053308, GU012615, GU012526, KJ459728, GU012511, GU012573, and GU012610 from Pune, Coimbatore, Mizoram and Thailand. Whereas many other samples of the species showed similarities from 99.84 to 98.58% with CUJ26. The *J. orithya* GU012556 from Pune with 97.16% similarity and *J. vilida* JN274489 from Australia with 97.11% similarity were the two closely related species.

The phylogeny of *J. lemonias* CUJ26 was derived with NJ-tree developed from the similar sequences obtained from database (Fig. 158). The *J. lemonias* CUJ26 was found to be placed in a clade with 12 samples involving *J. lemonias* KP941756 from Pune, KP997223 from Coimbatore, HQ962213 from Thailand, JN698955 from Mizoram, EU053308, EU053309, GU012615, GU012526, KJ459728, GU012511, GU012573, GU012610 from Pune and JX226065 from Coimbatore with 100% similarity. The EU053311, EU368159 from P. R. China, EU053310, EU053310, and EU792494 from Bangalore with 98.74% to 99.68% were found in a different clade. The *J. orithya* GU012556, from Pune, with 97.16% similarity and *J. vilida* JN274489 from Australia with 97.11% similarity were placed in a different clade. The *J. orithya* GU012556 from Pune and *J. vilida* JN274489 from Australia were different species of the genus remain close to *J. lemonias* CUJ26, in a separate clade.

Discussion

The COI sequence of *J. lemonias* CUJ26 showed 100% similarity to that of 11 samples of *J. lemonias* sequences from database. The COI sequence of CUJ26 is useful as a molecular barcode for identifying the species from database sequences. While many samples of the species showed similarities from 99.84 to 98.58%, they were polymorphic novel variants of the species. The *J. orithya* GU012556 from Pune with 97.16% similarity and *J. vilida* JN274489 from Australia with 97.11% similarity were the two closely related species. The phylogeny of *J. lemonias* CUJ26 was derived with NJ-tree developed from the similar sequences obtained from database (Fig. 158). The *J. lemonias* CUJ26 was found to be placed in a clade with 12 samples involving that *J. lemonias* KP941756 from Pune, KP997223 from

Coimbatore, HQ962213 from Thailand, JN698955 from Mizoram, EU053308, EU053309, GU012615, GU012526, KJ459728, GU012511, GU012573, GU012610 from Pune and JX226065 from Coimbatore with 100% similarity, they were monophyletic sharing same ancestor. The EU053311, EU368159 from P. R. China, EU053310, the EU053310, and EU792494 from Bangalore were found in a different clade. The *J. orithya* GU012556, from Pune, India and *J. vilida* JN274489 from Australia were placed in an adjacent clade. The *J. orithya* GU012556 from Pune and *J. vilida* JN274489 from Australia were different species of the genus remain close to *J. lemonias*. The NJ-tree distance data revealed that the species was diverged from their closely related species *J. villida* about 40,000 years ago. The *J. lemonias* is common in India, Myanmar and Sri Lanka the genus has representation in both South East Asia and Africa.

3.23. *Danaus chrysippus* (Linnaeus, 1758) isolate CUJ27 (KT880660)

The specimen CUJ27 was identified as *Danaus chrysippus* (Linnaeus, 1758) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms; *Papilio aegyptius* Von Schreber, 1759
Papilio alcippus Cramer, 1777
Papilio asclepiadis Gagliardi, 1811
Limnas alcippoides Moore, 1883

Danaus chrysippus, also known as the plain tiger is a medium-sized butterfly widespread in Asia and Africa It belongs to the family, Nymphalidae its coloration is mimicked by many species.

The body; black with many white spots, the wings; tawny, the upper side; brighter and richer than the underside. The apical half of the fore wing is black with a white band. The hind wing; three black spots around the centre, has a thin border of black enclosing a series of semi-circular white spots.

The larval food plants involve *Calotropis gigantean, Asclepias curassavica* and *Cryptolepis buchananii*, the larvae are ash coloured with a bluish tint and rings of black and yellow.

The PCR amplification of partial COI sequence of *D. chrysippus* CUJ27 from Thamarassery ($11^0$25'48"N, $76^0$01'17"E) yielded a consensus with 573 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in Fig. 160 to 164.

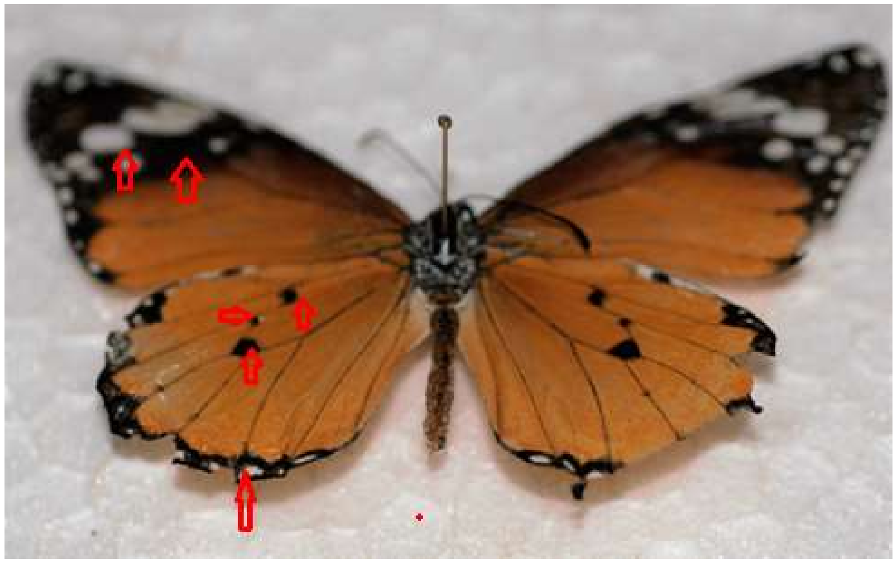

Fig. 159. The upper side of the *D. chrysippus* CUJ27, apical half of the fore wing is black with a white band. The hind wing; three black spots around the centre, has a thin border of black enclosing a series of semi-circular white spots.

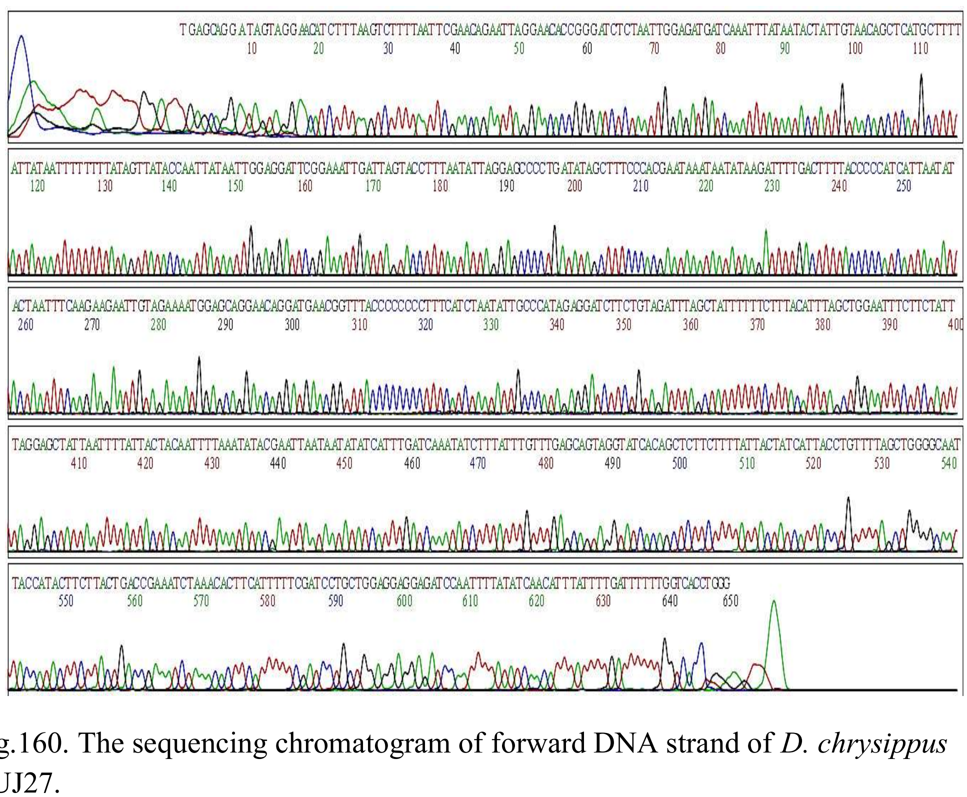

Fig.160. The sequencing chromatogram of forward DNA strand of *D. chrysippus* CUJ27.

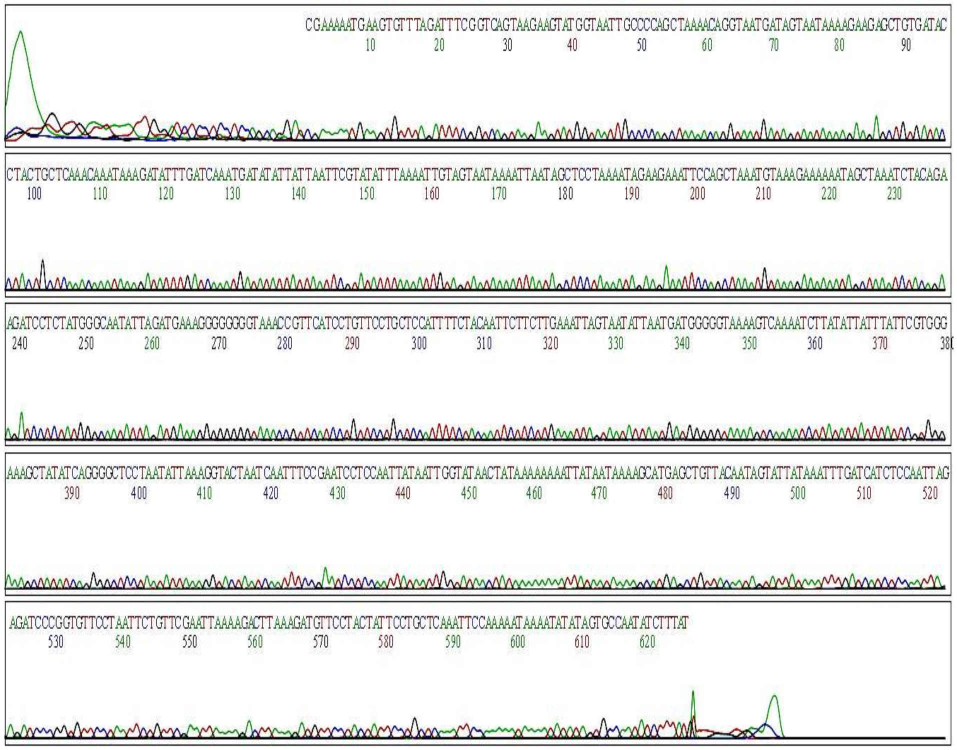

Fig.161. The sequencing chromatogram of reverse DNA strand of *D. chrysippus* CUJ27.

```
>Danaus chrysippus voucher CUJ27 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (573)
CACTATATATTTTATTTTTGGAATTTGAGCAGGAATAGTAGGAACATCTTTAAGTCTTTT
AATTCGAACAGAATTAGGAACACCGGGATCTCTAATTGGAGATGATCAAATTTATAATAC
TATTGTAACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCAATTATAATTGG
AGGATTCGGAAATTGATTAGTACCTTTAATATTAGGAGCCCCTGATATAGCTTTCCCACG
AATAAATAATATAAGATTTTGACTTTTACCCCCATCATTAATATTACTAATTTCAAGAAG
AATTGTAGAAAATGGAGCAGGAACAGGATGAACGGTTTACCCCCCCCTTTCATCTAATAT
TGCCCATAGAGGATCTTCTGTAGATTTAGCTATTTTTTCTTTACATTTAGCTGGAATTTC
TTCTATTTTAGGAGCTATTAATTTTATTACTACAATTTTAAATATACGAATTAATAATAT
ATCATTTGATCAAATATCTTTATTTGTTTGAGCAGTAGGTATCACAGCTCTTCTTTTATT
ACTATCATTACCTGTTTTAGCTGGGGCAATTAC
```

Fig.162. The partial sequence of mitochondrial COI gene DNA of *D. chrysippus* CUJ27

The conceptual translation of the consensus sequence of *D. chrysippus* CUJ27 gave the following peptide sequence.

```
>seq22 Danaus chrysippus voucher CUJ27
TMYFIFGIWAGMVGTSLSLLIRTELGTPGSLIGDDQIYNTIVTAHAFIMIFFMVMPIMIGG
FGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLMLLISSSIVENGAGTGWTVYPPLSSNIAH
SGSSVDLAIFSLHLAGISSILGAINFITTILNMRINNMSFDQMSLFVWAVGITALLLLLSL
PVLAGAIT
```

Fig. 163. The peptide sequence obtained from conceptual translation of consensus sequence of COI gene, DNA of *D. chrysippus* CUJ27.

The nucleotide BLAST was conducted with the consensus sequence derived from of *D. chrysippus* CUJ27. The details about similarities of sequences obtained and alignment details were represented below.

S. I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|915122298|gb|KP007618.1|Danaus chrysippus chrysippus voucher ANIC:MFB-10-P022*	100.00	658	0	0	1	658	1	658	1216
2	*gi|915122272|gb|KP007603.1|Danaus chrysippus chrysippus voucher MCZ:DL-10-Z011*	100.00	658	0	0	1	658	1	658	1216
3	*gi|915122266|gb|KP007600.1|Danaus chrysippus chrysippus voucher MCZ:DL-10-Z008*	100.00	658	0	0	1	658	1	658	1216
4	*gi|915122242|gb|KP007587.1|Danaus chrysippus alcippus voucher RMBR:CE-019*	100.00	658	0	0	1	658	1	658	1216
5	*gi|915122240|gb|KP007586.1|Danaus chrysippus alcippus voucher RMBR:CE-013*	100.00	658	0	0	1	658	1	658	1216
6	*gi|915122232|gb|KP007582.1|Danaus chrysippus alcippus voucher RMBR:CE-007*	100.00	658	0	0	1	658	1	658	1216
7	*gi|915122230|gb|KP007581.1|Danaus chrysippus alcippus voucher RMBR:CE-005*	100.00	658	0	0	1	658	1	658	1216
8	*gi|672349312|gb|KJ817359.1|Danaus chrysippus isolate DcAt-1*	100.00	658	0	0	1	658	1	658	1216
9	*gi|657172603|gb|KJ459750.1|Danaus chrysippus isolate F133*	100.00	615	0	0	35	649	1	615	1136
10	*gi|330600271|gb|GU012522.1|Danaus chrysippus voucher F61*	100.00	615	0	0	18	632	1	615	1136
11	*gi|915122288|gb|KP007613.1|Danaus chrysippus chrysippus voucher RMBR:ID-P037B*	99.85	658	1	0	1	658	1	658	1210
12	*gi|915122286|gb|KP007612.1|Danaus chrysippus chrysippus voucher RMBR:ID-P037A*	99.85	658	1	0	1	658	1	658	1210

13	*gi|915122284|gb|KP007611.1|Danaus chrysippus dorippus voucher MCZ:DL-13-P002*	99.85	658	1	0	1	658	1	658	1210
14	*gi|915122326|gb|KP007634.1|Danaus chrysippus bataviana voucher MCZ:RM12*	99.70	658	2	0	1	658	1	658	1205
15	*gi|915122324|gb|KP007632.1|Danaus chrysippus cratippus voucher MCZ:RM08*	99.70	658	2	0	1	658	1	658	1205
16	*gi|915122322|gb|KP007631.1|Danaus chrysippus gelderi voucher MCZ:RM06*	99.70	658	2	0	1	658	1	658	1205
17	*gi|915122294|gb|KP007616.1|Danaus chrysippus chrysippus voucher RMBR:LD-08-A01*	99.70	658	2	0	1	658	1	658	1205
18	*gi|915122370|gb|KP007706.1|Danaus petilia voucher RMBR:RE-08-J015*	97.11	657	19	0	2	658	2	658	1109
19	*gi|915122368|gb|KP007705.1|Danaus petilia voucher RMBR:RE-08-J014*	97.11	657	19	0	2	658	2	658	1109
20	*gi|915122234|gb|KP007583.1|Danaus chrysippus chrysippus voucher RMBR:CE-008*	96.78	653	21	0	6	658	6	658	1090
21	*gi|915122342|gb|KP007666.1|Danaus gilippus cleothera voucher NWC:NW153-15*	94.67	657	35	0	2	658	2	658	1020

Table 27. The BLAST hit table of *D. chrysippus* CUJ27.

The COI sequence of *D. chrysippus* CUJ27 BLAST against database showed 100% similarity to *D. chrysippus* KP007618 from India, KP007603 and KP007600 from Myanmar, KP007587 and KP007586 from Togolese Republic, KP007582, KP007581 from Republic of Cameroon, and KJ817359 from Bangalore, KJ459750 and GU012522 from Pune. Whereas KP007613 and KP007612 from Philippines and KP007611 from Republic of Kenya have 99.85% similarity. However KP007634 from West Nusa Tunggara of Indonesia, KP007632 from East Nusa Tunggara of Timor, KP007631 from Sulawesi Indonesia and KP007616 from Thailand showed 99.70% similarity. The *D. petilia* KP007706 and *D. petilia* KP007705 from Australia were with 97.11% similarity.

The variations were clearly reflected in the NJ-tree developed from the sequences obtained from database (Fig. 164). Thus, 12 samples of *Danaus* *chrysippus* with 99.70 to 100% similarity from different geographical locations including the one under present investigation CUJ27 were found to be placed in a single monophyletic clade sharing common ancestor. The *D. petilia* KP007706 and KP007705 from Australia were with 97.11% similarity seen in a distant clade.

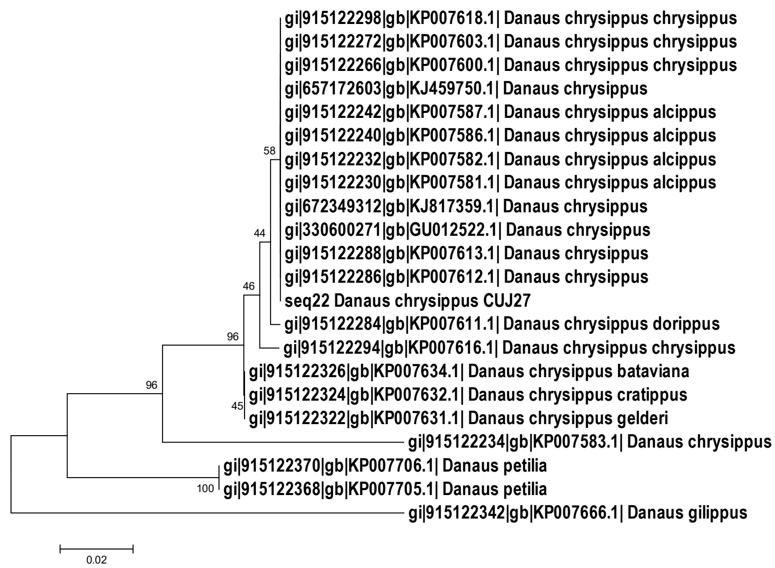

Fig. 164 NJ-tree with Phylogenetic relationship of *D. chrysippus* CUJ27.

Discussion

The COI sequence of *D. chrysippus* CUJ27 BLAST against database showed 100% similarity to *D. chrysippus* KP007618 from India, and many other sequences from Myanmar, Togolese Republic, Republic of Cameroon, Bangalore and Pune, India. Therefore it has the power to recognise the species from database, and useful as a molecular barcode. Whereas it has 99.85 – 99.70% similarity with samples from Philippines, Republic of Kenya, West Nusa Tunggara of Indonesia, East Nusa Tunggara of Timor, Sulawesi Indonesia and Thailand. They are novel polymorphic sequences of the species with nucleotide substitutions. The sequences of The *D. petilia* KP007706 and *D. petilia* KP007705 are the closely related species from Australia they were with 97.11% similarity. The variations were clearly reflected in the NJ-tree developed from the sequences obtained from database (Fig. 164). Thus, 12 samples of *D. chrysippus* with 99.70 to 100% similarity from different geographical locations including the one under present investigation CUJ27 were found to be placed in a single monophyletic clade sharing common ancestor. The *D. petilia* KP007706 and KP007705 from Australia were with 97.11% similarity seen in a distant clade. The *D. petilia* was a different species of the genus remain close to *D. chrysippus* CUJ27. The NJ-tree distance data revealed that the species was diverged from their closely related species *D. petilia* about 90,000 years ago. The *D. chrysippus* is common in Africa, Arabia, India, Myanmar, Sri Lanka, Thailand, Indonesia, Timor, Philippines and Australia. The species has representation in both South East Asia, Arabia and Africa. The analysis of COI gene sequence isolated from *D. chrysippus* CUJ27 revealed the association of this sample towards African samples than South East Asia.

3.24. *Castalius rosimon* (Fabricius, 1775) isolate CUJ29 (KT880661)

The specimen CUJ29 was identified as *Castalius rosimon* (Fabricius, 1775) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonym; *Papilio rosimon* Fabricius, 1775

The *Castalius rosimon* is the common Pierrot which is a small butterfly found in South Asia that belongs to the lycaenids, it is very common in Kerala. Upper side; mainly white fore wing; the costa, apex and termen edged with black, the edging much broader on apex and termen; base outwards for a short distance less densely overlaid with metallic blue scales which cover and make indistinct.

Under side; primarily white, fore wing; a long oblique black band from base outwards to the costa; below it and obliquely placed an irregular black somewhat conical mark; following these are two outwardly oblique, medially interrupted, black macular bands; the inner of the two extended from costa along the discocellulars, is then widely interrupted below its posterior portion that is formed of two elongate coalescent spots and touches the inner sub terminal transverse line of elongate spots just above the tornus. The outer, obliquely placed line is sub apical and medially broken, the middle portion consisting of a quadrate spot is shifted outwards; finally, two parallel sub-terminal transverse series of black elongate spots, the inner series of broad, more or less rectangular spots. Cilia of both fore and hind wings white alternated with black at the apices of the veins; filamentous short tail to the hind wing black tipped.

With white antennae; head, thorax and abdomen black, the shafts of the antennae ringed with white, the head between the eyes and behind them white; beneath: the palpi, thorax and abdomen white, the last barred broadly with white on the sides (Fig. 165). The larval food plants involve *Ziziphus mouritiana, Ziziphus rugosa* and *Ziziphus xylopyrus*, the larvae are bright green in coloured.

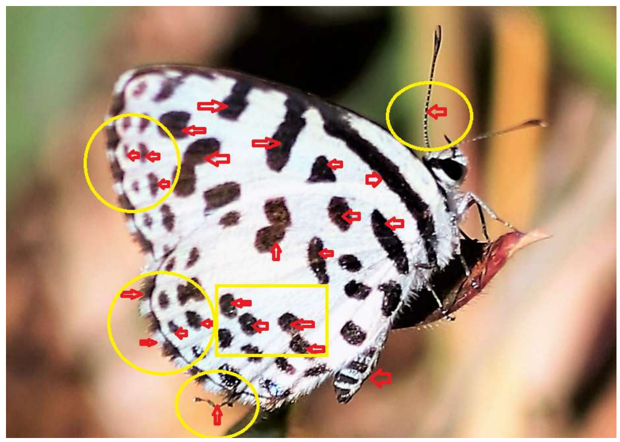

Fig. 165. The lower side of the *C. rosimon* CUJ29, a long oblique black band from base outwards to the costa, two outwardly oblique, medially interrupted, black macular bands, two elongate coalescent spots and touches the inner sub terminal transverse line, the outer, obliquely placed line is sub apical and medially broken, a quadrate spot is shifted outwards, two parallel sub-terminal transverse series of black elongate spots, the inner series of broad, more or less rectangular spots, the outer series of more linear spots. Hind wing: a transverse basal black band, with an elongate black spot below it on the dorsum, four black posterior spots two and two, each pair coalescent and placed *en echelon*. Cilia of both fore and hind wings white alternated with black at the apices of the veins, the shafts of the antennae ringed with white.

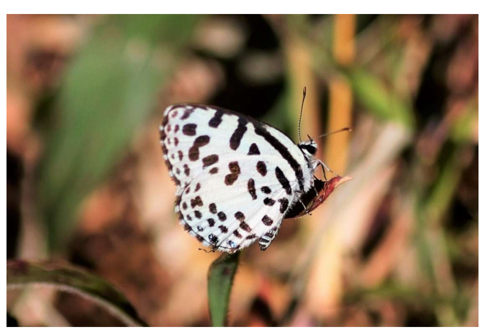

Fig. 166. The habit of the *C. rosimon* CUJ29.

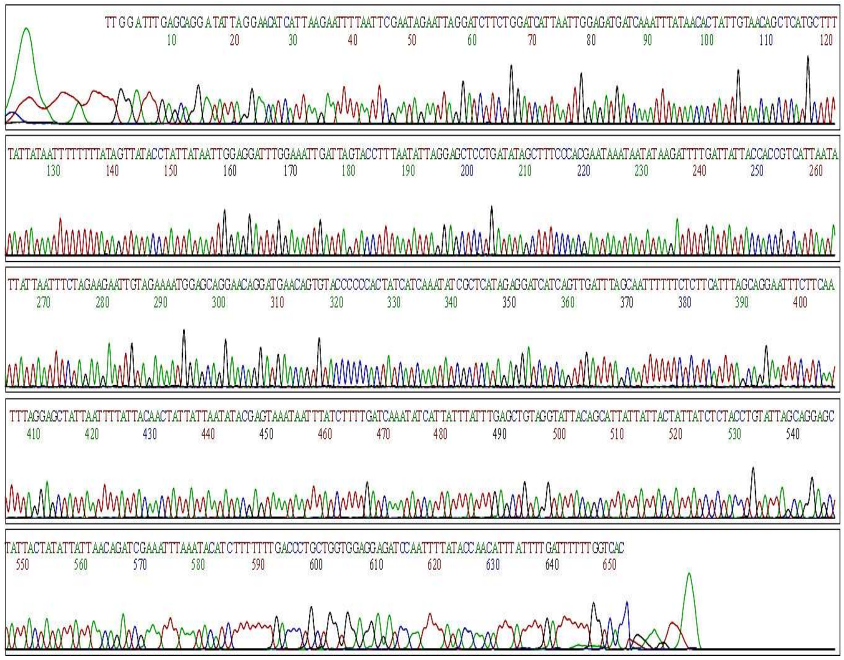

Fig.167. The sequencing chromatogram of forward DNA strand of *C. rosimon* C UJ 29.

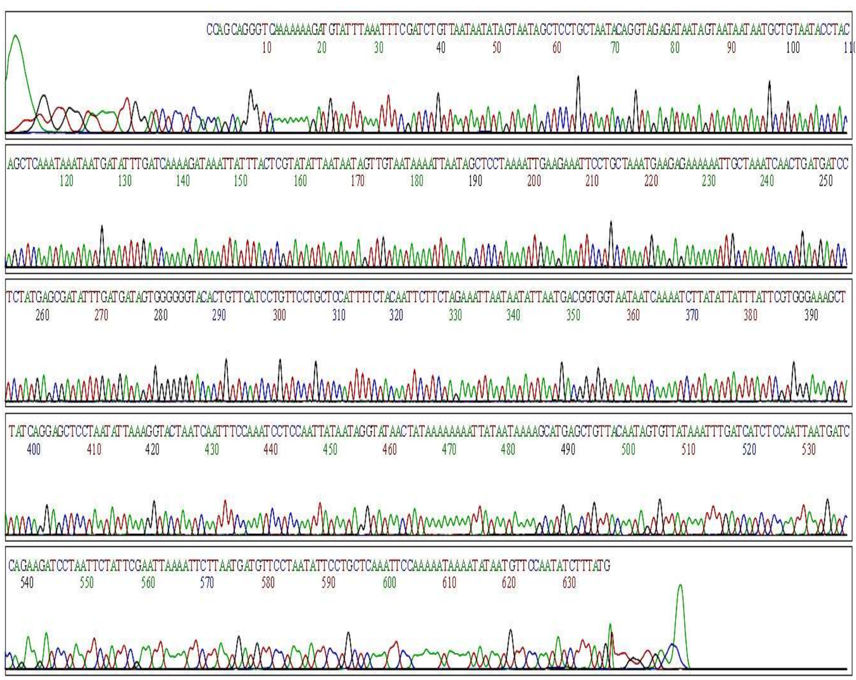

Fig.168. The sequencing chromatogram of reverse DNA strand of *C. rosimon* CUJ29.

The PCR amplification of partial COI sequence of *C. rosimon* CUJ29 from Aravanchal ($12^0$13'01"N, $75^0$16'49"E) yielded a consensus with 603 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 167-171).

```
>Castalius rosimon voucher CUJ29 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (603)
AACATTATATTTTATTTTTGGAATTTGAGCAGGAATATTAGGAACATCATTAAGAATTTT
AATTCGAATAGAATTAGGATCTTCTGGATCATTAATTGGAGATGATCAAATTTATAACAC
TATTGTAACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGG
AGGATTTGGAAATTGATTAGTACCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCACG
AATAAATAATATAAGATTTTGATTATTACCACCGTCATTAATATTATTAATTTCTAGAAG
AATTGTAGAAAATGGAGCAGGAACAGGATGAACAGTGTACCCCCCACTATCATCAAATAT
CGCTCATAGAGGATCATCAGTTGATTTAGCAATTTTTTCTCTTCATTTAGCAGGAATTTC
TTCAATTTTAGGAGCTATTAATTTTATTACAACTATTATTAATATACGAGTAAATAATTT
ATCTTTTGATCAAATATCATTATTTATTTGAGCTGTAGGTATTACAGCATTATTATTACT
ATTATCTCTACCTGTATTAGCAGGAGCTATTACTATATTATTAACAGATCGAAATTTAAA
TAC
```

Fig. 169. The partial sequence of mitochondrial COI gene DNA of *C. rosimon* CUJ29.

The conceptual translation of the consensus sequence of *C. rosimon* CUJ29 gave the following peptide sequence.

>seq23 *Castalius rosimon* CUJ29

```
TLYFIFGIWAGMLGTSLSILIRMELGSSGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLMLLISSSIVENGAGTGWTV
YPPLSSNIAHSGSSVDLAIFSLHLAGISSILGAINFITTIINMRVNNLSFDQMSLF
IWAVGITALLLLLSLPVLAGAITMLLTDRNLNT
```

Fig. 170. The peptide sequence obtained from conceptual translation of consensus sequence of COI gene, DNA of *C. rosimon* CUJ29.

The nucleotide BLAST was conducted with the consensus sequence derived from of *C. rosimon* CUJ29. The details about similarities of sequences obtained and alignment details were represented below.

S.I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	q. end	S. start	S. end	Bit score
1	*gi|675401125|gb|KJ402129.1|Castalius rosimon isolate F194*	99.84	612	1	0	35	646	1	612	1125
2	*gi|321121969|gb|HQ962272.1|Castalius rosimon voucher YB-KHC6828*	99.70	658	2	0	1	658	1	658	1205
3	*gi|264687314|gb|GQ129016.1|Castalius rosimon voucher MCZ:MWT93B024*	99.70	658	2	0	1	658	1	658	1205
4	*gi|675401153|gb|KJ402143.1|Castalius rosimon isolate F504*	99.68	624	2	0	35	658	1	624	1142
5	*gi|675401086|gb|KJ402109.1|Castalius rosimon isolate OR3*	99.68	618	2	0	35	652	1	618	1133
6	*gi|675401327|gb|KJ402230.1|Castalius rosimon isolate F432*	99.68	618	2	0	35	652	1	618	1131
7	*gi|675401078|gb|KJ402105.1|Castalius rosimon isolate BLADA7*	99.67	612	2	0	35	646	1	612	1120
8	*gi|675401369|gb|KJ402251.1|Castalius rosimon isolate F314*	99.67	597	2	0	35	631	1	597	1092
9	*gi|679567239|gb|KJ934113.1|Castalius rosimon voucher ILL_194*	99.64	548	2	0	1	548	1	548	1002
10	*gi|675401243|gb|KJ402188.1|Castalius rosimon isolate F14*	99.36	621	4	0	35	655	1	621	1125
11	*gi|328488271|gb|JF262052.1|Euphilotes centralis voucher ADW05I826*	94.98	657	33	0	2	658	1	657	1031
12	*gi|328488289|gb|JF262058.1|Philotiella leona voucher ADW05I823*	94.67	657	35	0	2	658	1	657	1020
13	*gi|328488286|gb|JF262057.1|Philotes sonorensis voucher KED05I833*	94.65	654	35	0	5	658	1	654	1014
14	*gi|870901603|gb|KP870794.1|Phengaris arion voucher RVcoll.10-C430*	94.53	658	36	0	1	658	1	658	1016
15	*gi|336112580|gb|HQ918159.1|Maculinea teleius voucher TERU5*	94.53	658	36	0	1	658	1	658	1016
16	*gi|336112550|gb|HQ918149.1|Maculinea arion voucher RV08R116*	94.53	658	36	0	1	658	1	658	1016
17	*gi|264687323|gb|GQ129019.1|Eicochrysops hippocrates voucher MCZ:TL97W513*	94.53	658	36	0	1	658	1	658	1016
18	*gi|296792102|gb|GU688436.1|Phengaris teleius voucher BC ZSM Lep 30500*	94.53	658	36	0	1	658	1	658	1016
19	*gi|321485867|gb|HQ990430.1|Tarucus rosacea voucher NIBGE BUT-00119*	94.38	658	37	0	1	658	1	658	1011
20	*gi|300200514|gb|HM391889.1|Phengaris teleius voucher BC ZSM Lep 28464*	94.38	658	37	0	1	658	1	658	1011
21	*gi|304271071|gb|HQ004707.1|Maculinea arion voucher RVcoll.08-M307*	94.38	658	37	0	1	658	1	658	1011

Table 28. The BLAST hit table of *C. rosimon* CUJ29.

The consensus sequence *C. rosimon* CUJ29 BLAST against database and showed 99.84% similarity to that *C. rosimon* KJ402129 from Pune. Whereas HQ962272 from Thailand and GQ129016 from Malaysia with 99.70% similarity. While KJ402143, KJ402109 and KJ402230 from Pune with 99.68% similarity, KJ402105 and KJ402251 from Pune with 99.67% similarity. However KJ934113 from Thailand with 99.64% similarity and KJ402188 from Pune with 99.36% similarity. The *Tarucus risacea* HQ990430 from Pakistan with 94.38% similarity.

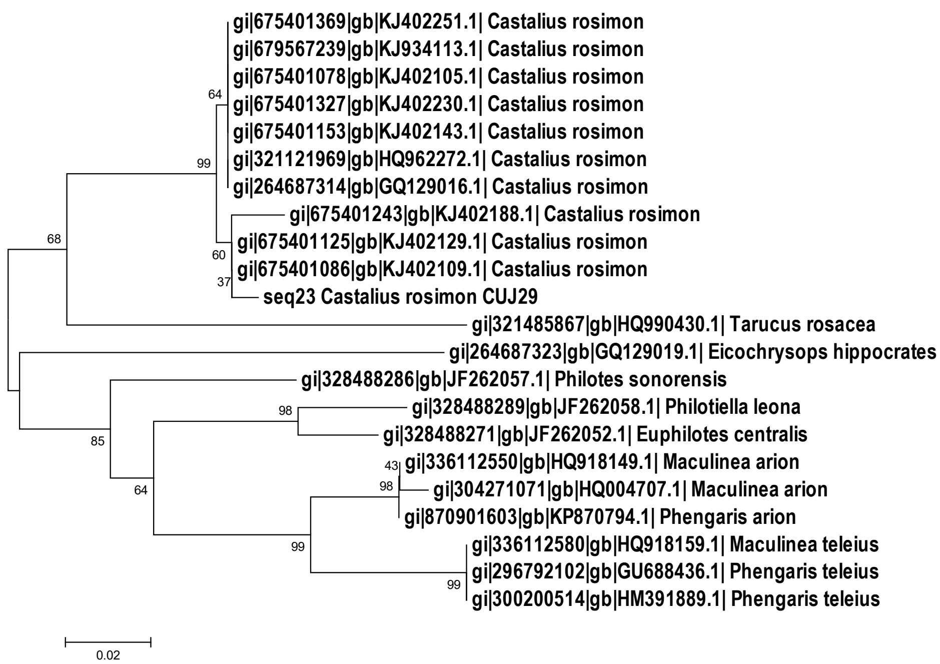

Fig. 171. NJ-tree with Phylogenetic relationship of *C. rosimon* CUJ29.

The phylogeny of *C. rosimon* CUJ29 was derived with NJ-tree developed from the similar sequences obtained from BLAST hit results (Fig. 171). The *C. rosimon* CUJ29 was found to be placed in a clade with 3 samples involving KJ402129, KJ402109 and KJ402188 from Pune with 99.36% similarity. The

HQ962272 from Thailand and GQ129016 from Malaysia with 99.70% similarity, KJ402143 and KJ402230 with 99.68% similarity and KJ402105 and KJ402251 from Pune with 99.67% similarity, KJ934113from Thailand with 99.64% similarity were placed in an adjacent clade. The *Tarucus risacea* HQ990430 from Pakistan with 94.38% similarity found in a separate clade.

Discussion

The COI sequence of *C. rosimon* CUJ29 BLAST against database and showed 99.84% similarity to that *C rosimon* KJ402129 from Pune. Hence the sequence is useful as a molecular barcode identifying the species. CUJ29 showed 0.16% divergence from KJ402129, therefore CUJ29 is a novel sequence. Many other samples were found in the database from Thailand, Malaysia, and Pune with 99.70 to 99.36% similarity. The *Tarucus risacea* (HQ990430) from Pakistan with 94.38% similarity is the closely related species of *C. rosimon*. The phylogeny of *C. rosimon* CUJ29 was derived with NJ-tree developed from the similar sequences identified from BLAST hit results (Fig. 171). The CUJ29 was found to be placed in a clade with 3 samples involving KJ402129, KJ402109 and KJ402188 from Pune, they were monophyletic sharing same ancestor. The HQ962272 from Thailand and GQ129016 from Malaysia, KJ402143, KJ402230, KJ402105 and KJ402251 from Pune, KJ934113 from Thailand were placed in an adjacent clade. The *Tarucus risacea* HQ990430 from Pakistan was a different species remain close to *C. rosimon* CUJ29 found in a separate clade. The NJ-tree distance data revealed that the species was diverged from *Tarucus risacea* HQ990430 about 94,000 years ago. The *C. rosimon* is common in India, Myanmar, Sri Lanka and Thailand, the members of genus were common along Indonesia, Timor, Philippines and Australia. The related species *Tarucus risacea* has representation in both South Asia, Arabia and Africa. The analysis of COI gene sequence isolated from *C. rosimon* CUJ29 revealed the association of this sample with African and South East Asian forms.

3.25. *Euploea core* (Cramer, 1780) isolate CUJ31 (KT880662)

The specimen CUJ31 was identified as *Euploea core* (Cramer, 1780) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms: *Papilio core* Cramer, (1780)

The *Euploea core* is the common crow butterfly which is a common butterfly found in Kerala, belongs to the crows and tigers subfamily danainae. *E. core* is a glossy black butterfly with rows of white spots on the margins of its wings *E. core* is a slow, steady flier and is unpalatable to predators, the toxins sequested by their caterpillars from food plants make it unpalatable. It has less enemies and is usually observed gliding through the air with a minimum effort.

Upper side; dark brown, broadly paler along terminal margins; fore and hind wing, sub terminal and terminal series of white spots; on fore wing the spots more or less oval, curved inwards opposite apex, the latter series often incomplete, not reaching apex, the spots smaller, often there is a small costal spot, and very rarely a spot in apex of cell and one or more discal spots. On the hind wing the inner series of spots are elongate, the outer conical. Under side similar, but ground-colour more uniform; cell, costal and discal spots on both fore and hind wing nearly always present. The larval food plants involve *Nerium oleander, Nerium odorum, streblus asper, Asclepias curassavica, Mimusops elengi, Tylophora indica* and *Cryptolepis buchananii*, the larvae are chocolate brawn coloured.

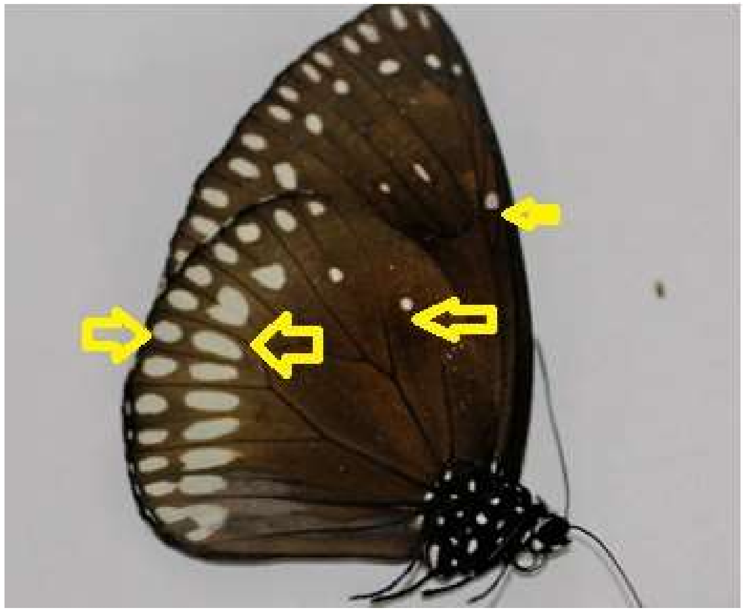

Fig. 172. The lower side of the *E. core* CUJ31. Upper side; dark brown, broadly paler along terminal margins, sub terminal and terminal series of white spots; on fore wing the spots more or less oval, the hind wing the inner series of spots are elongate, the outer conical.

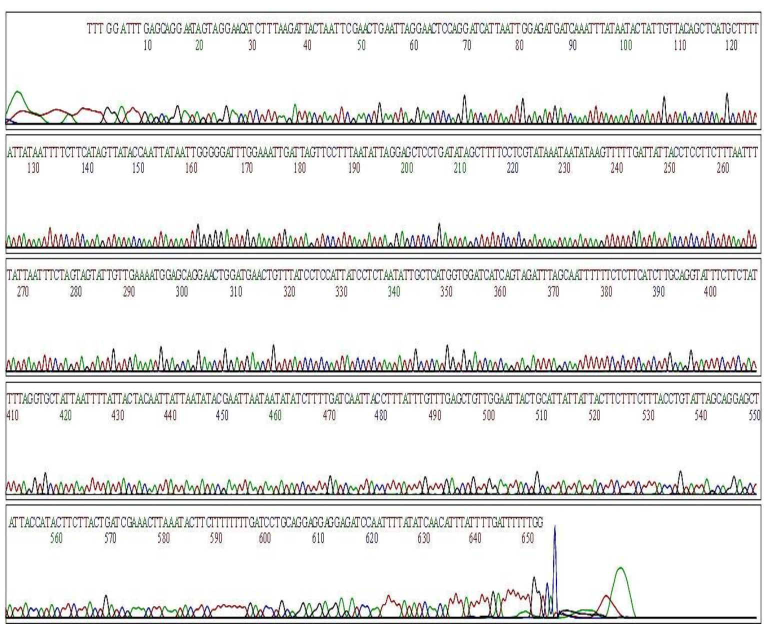

Fig. 173. The sequencing chromatogram of forward DNA strand of *E. core* CUJ31.

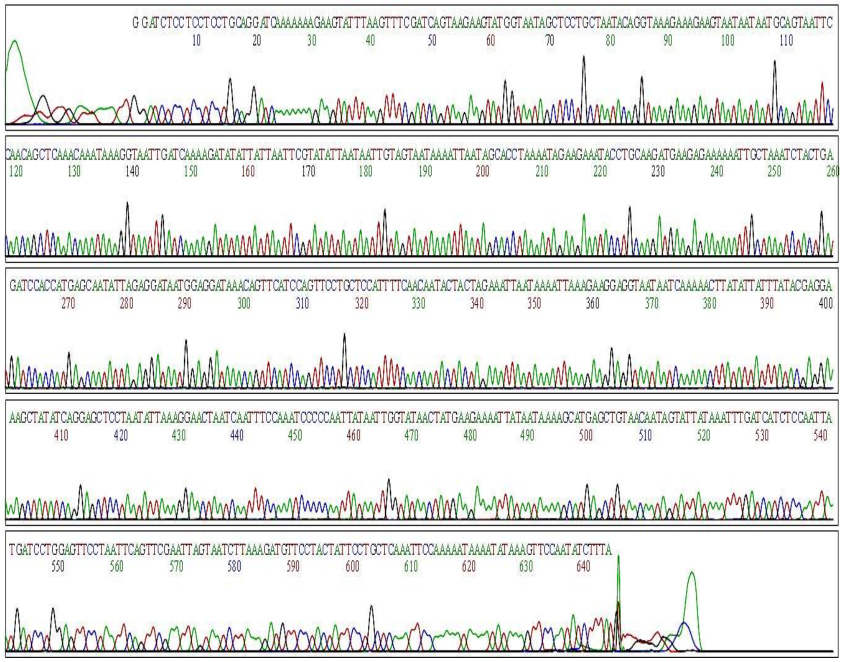

Fig. 174. The sequencing chromatogram of reverse DNA strand of *E. core* CUJ31.

The PCR amplification of partial COI sequence of *E.core* CUJ31 from Thalasseri ($11^0$46’42”N, $75^0$28’14”E) yielded a consensus with 609 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table (table 29), and NJ-tree are presented in (Fig. 173 to 177).

```
>Euploea core voucher CUJ31 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (609)
AACTTTATATTTTATTTTTGGAATTTGAGCAGGAATAGTAGGAACATCTTTAAGATTACT
AATTCGAACTGAATTAGGAACTCCAGGATCATTAATTGGAGATGATCAAATTTATAATAC
TATTGTTACAGCTCATGCTTTTATTATAATTTTCTTCATAGTTATACCAATTATAATTGG
GGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGAGCTCCTGATATAGCTTTTCCTCG
TATAAATAATATAAGTTTTTGATTATTACCTCCTTCTTTAATTTTATTAATTTCTAGTAG
TATTGTTGAAAATGGAGCAGGAACTGGATGAACTGTTTATCCTCCATTATCCTCTAATAT
TGCTCATGGTGGATCATCAGTAGATTTAGCAATTTTTTCTCTTCATCTTGCAGGTATTTC
TTCTATTTTAGGTGCTATTAATTTTATTACTACAATTATTAATATACGAATTAATAATAT
ATCTTTTGATCAATTACCTTTATTTGTTTGAGCTGTTGGAATTACTGCATTATTATTACT
TCTTTCTTTACCTGTATTAGCAGGAGCTATTACCATACTTCTTACTGATCGAAACTTAAA
TACTTCTTT
```

Fig.175. The partial sequence of mitochondrial COI gene DNA of *E. core* CUJ31.

The conceptual translation of the consensus sequence of *E. core* CUJ31 gave the following peptide sequence.

```
>seq24 Euploea core CUJ31

TLYFIFGIWAGMVGTSLSLLIRTELGTPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLILLISSSIVENGAGTGWTV
YPPLSSNIAHGGSSVDLAIFSLHLAGISSILGAINFITTIINMRINNMSFDQLPLF
VWAVGITALLLLLSLPVLAGAITMLLTDRNLNTS
```

Fig. 176. The peptide sequence obtained from conceptual translation of consensus sequence of COI gene, DNA of *E. core* CUJ3.

The nucleotide BLAST was conducted with the consensus sequence derived from of *E. core* CUJ31. The details about similarities of sequences obtained and alignment details were represented below (table 29)

S. I. No.	**Subject Ids**	**% Identity**	**Alignment length**	**Mismatches**	**Gap opens**	**Q. start**	**Q. end**	**S. start**	**S. end**	**Bit score**
1	*gi|451329343|gb|KC306729.1|Euploea core voucher ZN4*	100.00	469	0	0	14	482	1	469	867
2	*gi|330600433|gb|GU012616.1|Euploea core voucher F171*	99.85	648	0	1	12	659	1	647	1190
3	*gi|330600413|gb|GU012605.1|Euploea core voucher F320*	99.84	641	0	1	11	651	1	640	1177
4	*gi|330600386|gb|GU012589.1|Euploea core voucher F437*	99.84	641	0	1	3	643	1	640	1177
5	*gi|330600347|gb|GU012569.1|Euploea core voucher F80*	99.84	639	0	1	1	639	13	650	1173
6	*gi|657172555|gb|KJ459726.1|Euploea core isolate A15*	99.84	625	0	1	35	659	1	624	1147
7	*gi|657172579|gb|KJ459738.1|Euploea core isolate A22*	99.84	616	0	1	35	650	1	615	1131
8	*gi|330600263|gb|GU012518.1|Euploea core voucher F56*	99.84	607	0	1	27	633	1	606	1114
9	*gi|330600407|gb|GU012601.1|Euploea core voucher F391*	99.83	579	1	0	7	585	1	579	1064
10	*gi|657172779|gb|KJ459838.1|Euploea core isolate T60*	99.68	625	1	1	35	659	1	624	1142
11	*gi|657172617|gb|KJ459757.1|Euploea core isolate F575*	99.50	598	2	1	35	632	1	597	1086
12	*gi|330600349|gb|GU012570.1|Euploea core voucher F358*	98.60	643	0	9	1	635	12	653	1129
13	*gi|342845096|gb|JN266596.1|Euploe*	98.58	632	8	1	1	632	1	631	1116

	a alcathoe voucher 11ANIC-07149									
14	*gi|545690667|gb|KF226428.1|Euploea eyndhovii gardineri voucher UMKL-JJW0194*	97.27	659	17	1	1	659	1	658	1116
15	*gi|657172593|gb|KJ459745.1|Euploea sylvester isolate A35*	96.94	620	16	3	35	653	1	618	1037
16	*gi|295149285|gb|GU365909.1|Euploea corinna voucher QL7*	96.66	659	21	1	1	659	48	705	1094
17	*gi|545690669|gb|KF226429.1|Euploea midamus chloe voucher UMKL-JJW0195*	96.51	659	22	1	1	659	1	658	1088

Table 29. The BLAST hit table of *E.core* CUJ31.

E. core used in the present study CUJ31 shared 100% sequence similarity with the sequence from Mizoram KC306729. While the similarity was 99.85% with several specimens from Pune (GU012616, GU012605, GU012589, GU012569, KJ459726, KJ459738 and GU012518). However sequence similarity of other representatives of same species from Pune ranged from 99.60 to 99.84%. The sequence similarity of related species *E. alcathoe* (JN266596) from Australia (98.58%) and *E.sylvester* (KJ459745) from Pune, India (96.94%) diffred significantly (Table 29).

There variations were clearly reflected in the NJ-tree developed from the sequences obtained from database. Thus *E. core* from different geographical locations including the one under present investigation where the sequence similarity ranged from 99.50 to 100% formed a single monophyletic clade sharing a common ancestor were as the closely related species with less than 99.10% sequence similarity formed distinct clades of their own (Fig. 177).

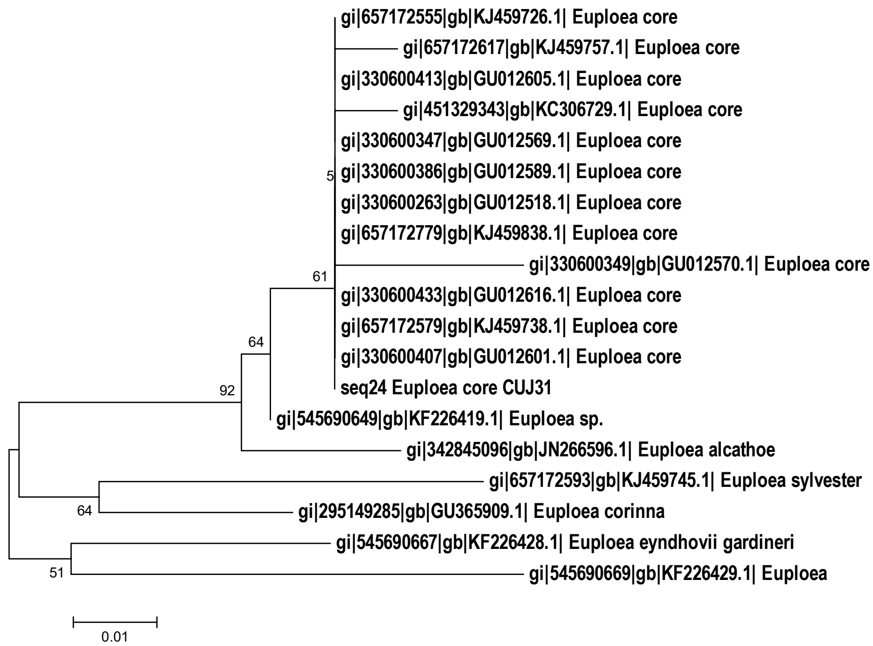

Fig. 177 NJ-tree with Phylogenetic relationship of *E. core* CUJ31.

Discussion

E.core used in the present study CUJ31 shared 100% sequence similarity with the sequence from Mizoram KC306729. The COI sequence isolated from CUJ31 is a molecular barcode which can identify the species from database sequences. However, sequence similarity of other representatives of same species from Pune ranged from 99.60 to 99.85%. They were novel sequences of the species from similar geographical locations. The sequence similarity of related species *E. alcathoe* JN266596 from Australia and *E.sylvester* KJ459745 from Pune, India diffred significantly (Table 29), there variations were clearly reflected in the NJ-tree, the *E. core* from different geographical locations including CUJ31 formed a single monophyletic clade sharing a common ancestor. While the closely related species formed a distinct clades of their own (Fig. 177). The NJ-tree distance data revealed that the species was diverged from their closely related species *E. alcathoe* about 17,000 years ago. The *Euploea core* is common in India, Myanmar, Sri Lanka and Thailand, the members of genus were common along Indonesia, Timor, Philippines and Australia. The related species *E. core* has representation in India, Sri Lanka, Myanmar and South East Asia. The analysis of COI gene sequence isolated from *E. core* CUJ31 revealed the association of this sample to South East Asian forms.

3.26. *Luthrodes pandava* (Horsfield, 1829) isolate CUJ33 (KT880664)

The specimen CUJ33 was identified as *Luthrodes pandava* (Horsfield, 1829) referring to the identical morphological features of the species described by Bingham C. T. (1907).

Synonyms; *Chilades pandava* (Horsfield, 1829)

Lycaena pandava Horsfield, (1829)

Catochrysops nicola Swinhoe, 1885

Catochrysops bengalia de Nicéville, 1885

Catochrysops vapanda Semper, 1890

The *Luthrodes pandava* the Plain Cupid butterfly is a species of Lycaenid butterfly found in India, Ceylon, Burma, Indo-china, Peninsular Malaysia, Singapore, Taiwan, Java, Sumatra and the Philippines. They are among the few butterflies that breed on plants of the cycad family. Upper side; lavender-blue, fore wing; costa narrowly and terminal margin more broadly fuscous brown, the latter with in addition an anticiliary black line; cilia light brown transversely traversed close to but not at their bases by a dark brown line. Hind wing: costa narrowly fuscous brown; a sub-terminal series of black spots outwardly edged by a white line; the spot in interspace 2 the largest and inwardly crowned more or less broadly with ochraceous yellow; an anticiliary black line and the cilia as on the fore wing.

Under side: greyish brown fore and hind wings: the following transverse darker brown markings on each wing, the markings edged on the inner and outer sides with white lines a short bar across the discocellulars, a discal catenulated band, the posterior two elongate spots of which on the fore wing are *en Echelon*, the white edgings on the inner side to both sub-terminal bands on the hind wing are more or less lunular the other dark brown. Antennae black, shafts ringed with white; head, thorax and abdomen brown, the head and thorax clothed with bluish hairs; beneath: palpi, thorax and abdomen whitish.

The PCR amplification of partial COI sequence of *Luthrodes pandava* CUJ33 from Aravanchal ($12^0$12’56”N, $75^0$16’52”E) yielded a consensus with 603 bp. The chromatograms, DNA sequence obtained, its conceptual translation, BLASTn hit table, and NJ-tree are presented in (Fig. 178 to 182).

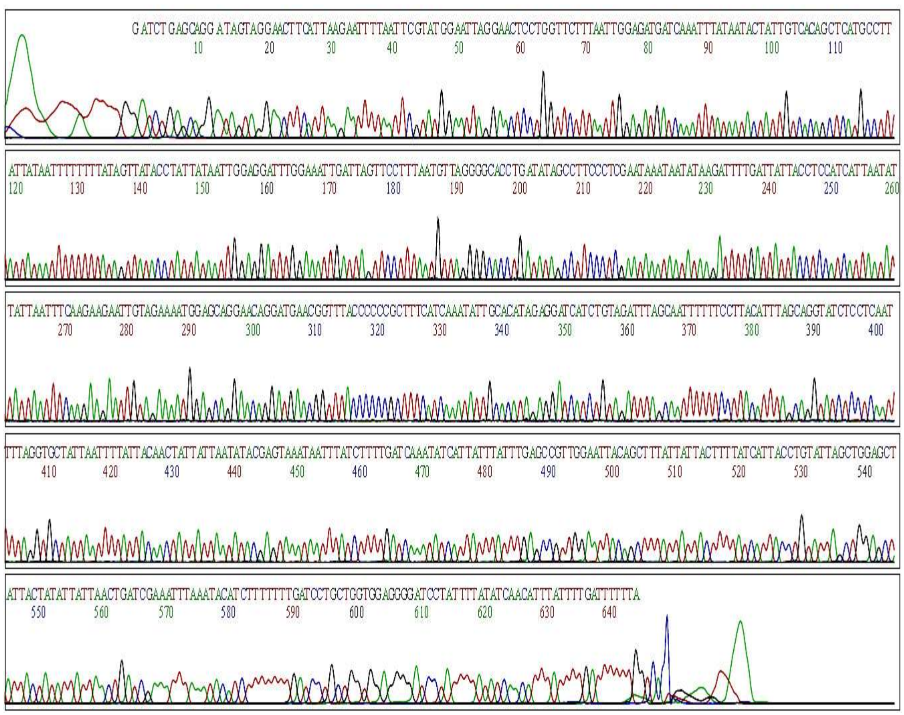

Fig. 178. The sequencing chromatogram of forward DNA strand of *L. pandava* CUJ33.

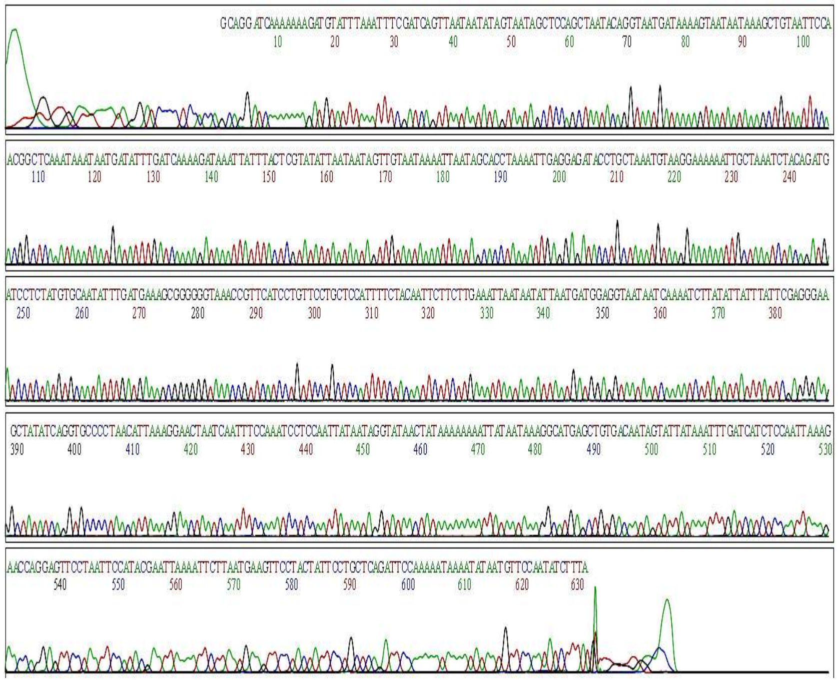

Fig. 179. The sequencing chromatogram of reverse DNA strand of *L. pandava* CUJ33.

```
>Luthrodes pandava voucher CUJ33 cytochrome oxidase subunit I
gene, partial cds; mitochondrial (603)
AACATTATATTTTATTTTTGGAATCTGAGCAGGAATAGTAGGAACTTCATTAAGAATTTT
AATTCGTATGGAATTAGGAACTCCTGGTTCTTTAATTGGAGATGATCAAATTTATAATAC
TATTGTCACAGCTCATGCCTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGG
AGGATTTGGAAATTGATTAGTTCCTTTAATGTTAGGGGCACCTGATATAGCCTTCCCTCG
AATAAATAATATAAGATTTTGATTATTACCTCCATCATTAATATTATTAATTTCAAGAAG
AATTGTAGAAAATGGAGCAGGAACAGGATGAACGGTTTACCCCCCGCTTTCATCAAATAT
TGCACATAGAGGATCATCTGTAGATTTAGCAATTTTTTCCTTACATTTAGCAGGTATCTC
CTCAATTTTAGGTGCTATTAATTTTATTACAACTATTATTAATATACGAGTAAATAATTT
ATCTTTTGATCAAATATCATTATTTATTTGAGCCGTTGGAATTACAGCTTTATTATTACT
TTTATCATTACCTGTATTAGCTGGAGCTATTACTATATTATTAACTGATCGAAATTTAAA
TAC
```

Fig. 180. The partial sequence of mitochondrial COI gene DNA of *L. pandava* CUJ33.

The conceptual translation of the consensus sequence of *L. pandava* CUJ33 gave a peptide sequence.

>seq26 *Luthrodes pandava* CUJ33

```
TLYFIFGIWAGMVGTSLSILIRMELGTPGSLIGDDQIYNTIVTAHAFIMIFFMVMP
IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLMLLISSSIVENGAGTGWTV
YPPLSSNIAHSGSSVDLAIFSLHLAGISSILGAINFITTIINMRVNNLSFDQMSLF
IWAVGITALLLLLSLPVLAGAITMLLTDRNLNT
```

Fig. 181. The peptide sequence obtained from conceptual translation of consensus sequence of COI gene, DNA of *L. pandava* CUJ33.

The nucleotide BLAST was conducted with the consensus sequence derived from of *L. pandava* CUJ33. The details about similarities of sequences obtained and alignment details were represented below.

S.I. No.	Subject Ids	% Identity	Alignment length	Mismatches	Gap opens	Q. start	Q. end	S. start	S. end	Bit score
1	*gi|599175914|gb|KJ131016.1| Luthrodes pandava voucher ILL_171*	99.82	548	1	0	1	548	18	565	1007
2	*gi|675401209|gb|KJ402171.1| Luthrodes pandava isolate N306*	99.52	624	3	0	35	658	1	624	1136
3	*gi|675401361|gb|KJ402247.1| Luthrodes pandava isolate N304*	99.35	612	4	0	35	646	1	612	1109
4	*gi|557838136|gb|KF668149.1| Luthrodes pandava voucher JM-38*	99.35	611	4	0	48	658	1	611	1107
5	*gi|675401337|gb|KJ402235.1| Luthrodes pandava isolate N280*	99.34	609	4	0	35	643	1	609	1103
6	*gi|284812669|gb|GU076254.1 |Chilades pandava voucher L1-5265*	99.33	599	4	0	1	599	39	637	1085
7	*gi|321121961|gb|HQ962268.1 |Chilades pandava voucher YB-KHC6794*	99.24	658	5	0	1	658	1	658	1188
8	*gi|316994162|gb|GU372585.1 |Chilades pandava*	99.24	658	5	0	1	658	1	658	1188
9	*gi|264687120|gb|GQ128951.1 |Chilades pandava voucher MCZ:MWT93A009*	99.24	657	5	0	2	658	1	657	1186
10	*gi|284812853|gb|GU076346.1 |Chilades pandava voucher L1-8495*	99.17	599	5	0	1	599	39	637	1079
11	*gi|284812675|gb|GU076257.1 |Chilades pandava voucher*	99.17	599	5	0	1	599	39	637	1079

	L1-5270									
12	*gi|284812551|gb|GU076195.1 |Chilades pandava voucher Tai35*	99.17	599	5	0	1	599	39	637	1079
13	*gi|284812549|gb|GU076194.1 |Chilades pandava voucher Tai24*	99.00	599	6	0	1	599	39	637	1074
14	*gi|284812587|gb|GU076213.1 |Chilades pandava voucher L1-3106*	98.83	599	7	0	1	599	39	637	1068
15	*gi|675401341|gb|KJ402237.1| Luthrodes pandava isolate N277*	98.70	617	4	4	35	649	1	615	1092
16	*gi|227436341|gb|FJ663704.1| Chilades galba phiala voucher 2005-LOWA-615*	94.84	659	32	2	1	658	1	658	1027
17	*gi|675401251|gb|KJ402192.1| Chilades parrhasius isolate F28*	94.71	624	33	0	35	658	1	624	970
18	*gi|227435443|gb|FJ663255.1| Plebejus sarta voucher 2005-LOWA-348*	94.23	659	36	2	1	658	1	658	1005
19	*gi|264687218|gb|GQ128984.1 |Madeleinea tintarrona voucher MCZ:RV03V182*	93.93	659	38	2	1	658	1	658	994
20	*gi|304271727|gb|HQ005035.1 |Polyommatus daphnis voucher RV-07-E425*	93.79	660	37	4	1	658	1	658	989

Table 30. The BLAST hit table of *L. pandava* CUJ33.

L. pandava in the present study CUJ33 showed 99.82 to 98.83% sequence similarity to that *L. pandava* KJ131016, KJ402171, KJ402247, KF668149, KJ402235, GU076254, HQ962268, GU372585, GQ128951, GU076346, GU076257, GU076195, GU076194, KJ402237 and GU076213 from Goa, Pune, Egypt, Thailand, Republic of Korea, Malaysia and Taiwan. The sequence similarity of related species *Chilades galba phiala* FJ663704 from Uzbekistan with 99.84% and that of *C. parrhasius* KJ402192 from Pune, is 94.71%.

The *L. pandava* from different geographical locations including the one under present investigation CUJ33 ranged from 99.82 to 98.83% were included in adjacent clades. The CUJ33 was found to be placed in a clade with KJ131016 from

Goa, India, with 99.82% similarity, they were monophyletic sharing same ancestor. The *Chilades galba phiala* FJ663704 from Uzbekistan with 94.84% similarity and *C. parrhasius* KJ402192 from Pune, with 94.71% similarity were found in an adjacent clade and were the related species of *L. pandava* CUJ33.

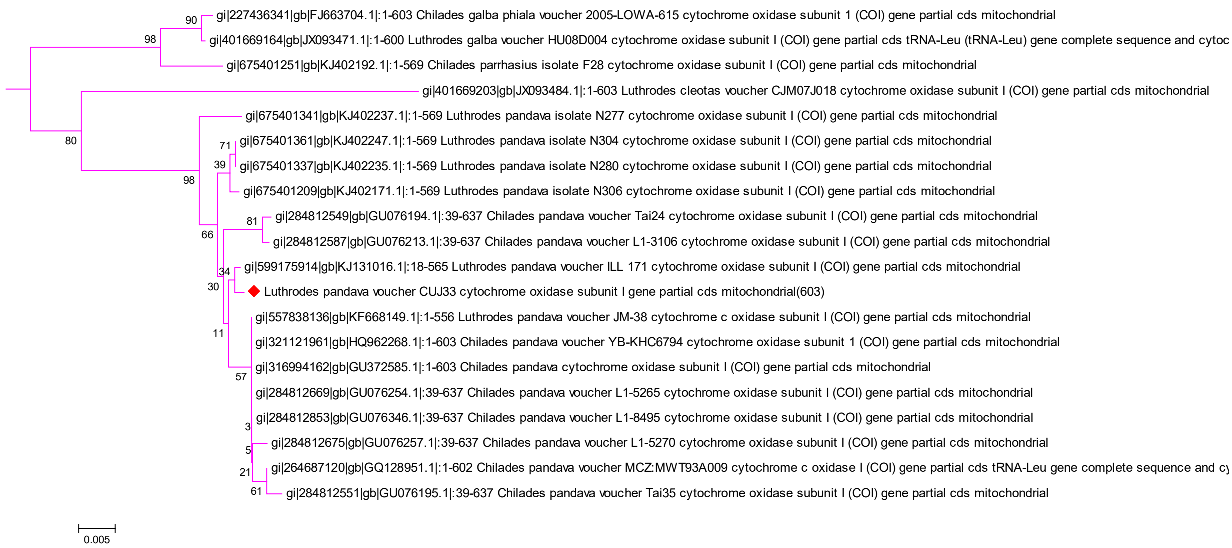

Fig. 182 NJ-tree with Phylogenetic relationship of *L. pandava* CUJ33.

Discussion

L. pandava in the present study CUJ33 showed 99.82 to 98.83% sequence similarity to that of *L. pandava* sequences KJ131016, KJ402171, KJ402247, KF668149, KJ402235, GU076254, HQ962268, GU372585, GQ128951, GU076346, GU076257, GU076195, GU076194, KJ402237 and GU076213 from the database. These sequences were from Goa, Pune, Egypt, Thailand, Republic of Korea, Malaysia and Taiwan. Never found a sequence with 100% similarity to that of CUJ33 hence it is a novel sequence of the species. The sequence similarity of related species *Chilades galba phiala* FJ663704 from Uzbekistan with 94.84% and that of *C. parrhasius* KJ402192 from Pune, is 94.71%. This reveals the existence of a significant barcoding gap between COI sequences of CUJ33 and closely related species *C. galba phiala* FJ663704, hence it can be useful as a molecular bacode for identification of *L. pandava*. The variations were clearly reflected in the NJ-tree developed from similar sequences from the database (Fig. 182). The *L. pandava* from different geographical locations including the one under present investigation CUJ33 were included in adjacent clades. The CUJ33 was found to be placed in a clade with KJ131016 from Goa, India, they were monophyletic sharing same ancestor. The *C. galba phiala* FJ663704 from Uzbekistan and *C. parrhasius* KJ402192 from Pune, were found in an adjacent clade and were the related species of *L. pandava* CUJ33. The NJ-tree distance data revealed that the species was diverged from their closely related species *Chilades galba* about 5,800 years ago. The *L. pandava* is common in India, Myanmar, Sri Lanka and Thailand, the members of genus were common along Indonesia, Timor, Philippines and Australia. The related species of *L. pandava* has representation in India, Sri Lanka, Myanmar and South East Asia the members of genus also found in Africa and South America. The analysis of COI gene sequence isolated from *L. pandava* CUJ33 revealed the close association of this sample to South East Asian forms.

Chapter 4

Summary and conclusions

This chapter summarizes the results of the present study. The butterfly samples for this study collected from different locations of Kasaragod, Kannur, Kozhikode and Malappuram districts in North Kerala, belonging to Western Ghats, over a period of three years. They belongs to four families of the super family Papilionoidea *viz*; Papilionidae, Nymphalidae, Pieridae and Lycaenidae. The partial coding region of their mitochondrial COI gene was amplified through PCR and sequenced. The summery of the results are presented below.

S. I.	Voucher No.	GenBank Accession number.	Name	Genera	Family	Length of DNA	Affinity found
1	CUJ1	HQ424201	*Troides minos*	Troides	Papilionidae	658	S.E. Asia
2	CUJ2	HQ424202	*Atrophaneura aristolochiae*	Atrophaneura *	Papilionidae	658	S.E. Asia
3	CUJ3	KT880639	*Graphium agamemnon*	Graphium	Papilionidae	609	S.E. Asia
4	CUJ4	KT880640	*Papilio polytes*	Papilio	Papilionidae	603	S.E. Asia
5	CUJ5	KT880641	*Papilio polymnestor*	Papilio	Papilionidae	603	S.E. Asia
6	CUJ6	KT880642	*Hypolimnas bolina*	Hypolimnas	Nymphalidae	609	S.E. Asia
7	CUJ7	KT880643	*Papilio clytia*	Papilio	Papilionidae	609	S.E. Asia
8	CUJ8	KT880644	*Pachliopta hector*	Pachliopta*	Papilionidae	622	S.E. Asia
9	CUJ9	KT880645	*Pachliopta aristolochiae*	Pachliopta*	Papilionidae	609	S.E. Asia
10	CUJ10	KT880646	*Papilio liomedon*	Papilio	Papilionidae	609	S.E. Asia
11	CUJ11	KT880647	*Delias eucharis*	Delias	Pieridae	609	S.E. Asia
12	CUJ12	KT880648	*Eurema hecabe*	Eurema	Pieridae	609	S.E. Asia
13	CUJ13	KT880649	*Eurema hecabe*	Eurema	Pieridae	609	S.E. Asia
14	CUJ14	KT880650	*Orsotriaena medus*	Orsotriaena	Nymphalidae	609	S.E. Asia
15	CUJ15	KT880651	*Cynitia lepidea*	Cynitia	Nymphalidae	609	S.E. Asia
16	CUJ16	KT880652	*Junonia iphita*	Junonia	Nymphalidae	609	S.E. Asia
17	CUJ17	KT880653	*Junonia almana*	Junonia	Nymphalidae	609	S.E. Asia
18	CUJ18	KT880654	*Ypthima huebneri*	Ypthima	Nymphalidae	603	S.E. Asia
19	CUJ19	KT880655	*Tirumala limniace*	Tirumala	Nymphalidae	609	S.E. Asia
20	CUJ20	KT880656	*Melanitis leda*	Melanitis	Nymphalidae	609	S.E. Asia
21	CUJ21	KT880657	*Melanitis leda*	Melanitis	Nymphalidae	609	S.E. Asia

22	CUJ22	KT880658	*Acraea violae*	Acraea	Nymphalidae	622	S.E. Asia
23	CUJ26	KT880659	*Junonia lemonias*	Junonia	Nymphalidae	609	S.E. Asia
24	CUJ27	KT880660	*Danaus chrysippus*	Danaus	Nymphalidae	573	Africa
25	CUJ29	KT880661	*Castalius rosimon*	Castalius	Lycaenidae	603	S.E. Asia
26	CUJ31	KT880662	*Euploea core*	Euploea	Nymphalidae	609	S.E. Asia
27	CUJ32	KT880663	*Troides minos*	Troides	Papilionidae	609	S.E. Asia
28	CUJ33	KT880664	*Luthrodes pandava*	*Luthrodes*	Lycaenidae	603	S.E. Asia

*Belonging to same genus

Table 31. The nucleotide size of partial coding region of COI DNA sequenced from different samples with their GenBank Accession numbers, Genera and Family they belong (S. E. Asia: South East Asia).

Many butterflies of Western Ghats are endemic and not found from any other regions of the world. For the present study, multiple samples from 24 species were collected from different locations. Among the 28 butterflies barcoded, two are rare and endemic to Western Ghats. They are the *Troides minos* and *Papilio liomedon.* The *P. liomedon* is a threatened species of butterfly and is protected by incorporating in Schedule one of Indian Wild Life (Protection) Act, 1972. The *Pachliopta hector* and *Cynitia lepidea,* DNA barcoded in the present study are also threatened species that are also included in the Schedule I and II of Wild Life (Protection) Act of 1972 respectively.

The BLAST hit table from NCBI showed 100% identity with COI sequence of *Troides minos* CUJ32 (KT880663**)**, and the 20 samples of closely related species with variations from 98.18 to 95.88%, *T. minos* CUJ1 (HQ424201) was a novel sequence and the first time deposit of COI sequence of *T. minos* in GenBank (table 5). The NJ-tree derived with the top related sequences, the CUJ1 and CUJ32 were found to be placed at the same clade monophyletic with 100% similarity. There was no instance of nucleotide polymorphism, and were same species. The NJ-tree distance data revealed that the species diverged from their closely related species *T.*

aeacus (EU625344) about 90,000 years ago.

The BLAST hit table of *Atrophaneura aristolochiae* CUJ2 (HQ424202) showed 100% identity with the 3 samples of *Pachliopta aristolochiae* (KJ195272, KF226554 and KJ195257) sequences deposited in GenBank (Tables 6 and 7). The nine other samples of same species showed variations from 99.85 to 99.08%. The NJ-tree derived with the sequences of top related GenBank accessions (Fig. 14), the CUJ2 found placed at the same clade with 2 samples of *P. aristolochiae*. The *A. aristolochiae* CUJ2, (HQ424202) isolated from Alakode, Kannur District has only 99.54% similarity to CUJ9 collected from Cheemeni, Kasaragod District, this is an instance of nucleotide polymorphism. The multi sequence alignments (Fig.16) showed single nucleotide polymorphism between CUJ2 and CUJ9 involving C to T transition and C to A transversion, The C to A mutation in CUJ2 resulted in Amino acid substitution of Methionine instead of Leucine. The NJ-tree distance data revealed that the species was diverged from their closely related species *A. alcinous* (KJ195255) about 80,000 years ago. The *Pachliopta hector* diverged from *Pachliopta aristolochiae* 40,000 years after their evolution.

The nucleotide BLAST with *Graphium agamemnon* CUJ3 revealed 100% identity of CUJ3 with *G. agamemnon* (KJ195270) from Pune (Table 8). The phylogeny of CUJ3 was derived with NJ-tree (Fig. 31), the CUJ3 found placed in the same clade with *G. agamemnon* (KJ195270). The *G. macfarlanei* (KF396538) from Australia with 95.24% identity found in the adjacent clade more close to the species. The NJ-tree distance data revealed that species *G. agamemnon* diverged from its closely related *G. macfarlanei* (KF396538) from Australia about 74,000 years ago and the CUJ3 showed divergence of about 56,000 years from members of same species.

The BLASTn hit table derived from the nucleotide BLAST with *Papilio polytes* CUJ4 (KT880640) at NCBI revealed 100% identity of CUJ4 with the 5 samples among 20 of *P. polytes* sequences deposited in GenBank (Table 9). The phylogeny of CUJ4 was derived with NJ-tree (Fig. 38), the CUJ4 found placed at the same clade with 5 samples of *P. polytes*. The *P. memnon* (HM246453) from P. R. China and *P. protenor* (HM246460) from P. R. China with 94.58% identity found in the adjacent clade more close to the species. The NJ-tree distance data revealed that the species diverged from their closely related species about 1,50,000 years ago.

The BLASTn hit table derived from the nucleotide BLAST with *Papilio polymnestor* CUJ5 (KT880641) at NCBI revealed 100% identity of CUJ5 with the 1 sample of *P. polymnestor* (KJ195275) from Pune, among the 6 sequences of the species deposited in GenBank other 4 sequences were with 99.84% and the remaining 1 sequence with 99.83% similarity (table 10). The phylogeny of CUJ5 was derived with NJ-tree (Fig. 45), the CUJ5 was found placed at the same clade with 3 samples of *P. polymnestor.* The *P. memnon* (HQ962218) isolated from Thailand with 99.67% identity found in the adjacent clade more close to the species. The NJ-tree distance data revealed that the species was diverged from their closely related species about 6,000 years ago and also from *P. deiphobus* (JF681019) from Indonesia about 60,000 years ago.

The BLAST Hit table with *Hypolimnas bolina* CUJ6 (KT880642) obtained from the NCBI showed 100% identity with the 11 samples of *H. bolina* sequences deposited in GenBank, the 3 sequences were with 99.68%, 99.20% and 98.63% similarity (Table 11). In the NJ-tree (Fig. 51), the CUJ6 was found placed in the same clade with11samples of *H. bolina*. The *H. diomea* (GQ240284) with 95.74% identity found in the adjacent clade more close to the species. The NJ-tree distance data revealed that the species was diverged from their closely related species about 1,00,000 years ago. They diverged from *Junonia almana* about 2,40,000 years ago.

The BLAST hit table with COI sequence of *Papilio clytia* CUJ7 showed 99.51% identity to 1 sample of *P. clytia (*AY457594) sequence deposited in GenBank (Table 12). The Neighbour Joining tree derived with the sequences of top related sequences (Fig. 57), the CUJ7 found placed at the same clade with 1 sample of *P. clytia* (AY457594) from Malaysia. The *P. torquatus tolmide* (JQ548606.1) from Costa Rica with 93.70% identity was found in the adjacent clade more close to the species. The NJ-tree distance data revealed that the species was diverged from their closely related species about 1,36,000 years ago.

The BLASTn with *Pachliopta hector* CUJ8 (KT880644) showed 100% identity with the two sample of *P. hector* (KF733790 and EU792488) sequences deposited both from Bangalore, India in GenBank (Table 13). The NJ-tree derived with the sequences of top related BLAST hit results (Fig. 64), the CUJ8 found placed at the same clade with 2 samples of *P. hector*. The *Graphium nomius* (KF733790) from Madurai, India also with 100% identity and found in the same clade more close to the species. The NJ-tree distance data revealed that the species was diverged from their closely related species *A. aristolochiae* about 59,000 years ago.

The BLASTn with *Papilio liomedon* CUJ10 (KT880646) the BLAST hit table showed 97.21% identity with the 2 samples of *Papilio demolion* (JF681020) from Thailand and *Papilio noblei* (JF747532) from P. R. China, 96.81% with *Papilio demoleus* (KF226558) from Malaysia, deposited in GenBank (Table 14). The COI sequence of *P. liomedon* was not reported earlier, it was a novel sequence and the first time report in the GenBank nucleotide database of NCBI. The NJ-tree developed from the top related results from BLAST hit table (Fig. 71), the CUJ10 was found placed at the same clade with *Papilio iswara* (KF226563) but evolved 19,000 years after *P.liomedon* and was diverged from the ancestors of *P. demoleus* (KF226558) from Malaysia about 15,500 years ago.

The BLASTn with *Delias eucharis* CUJ11 (KT880647) showed 100% identity with the 1 sample of *D. eucharis* (KJ422911) from Pune, India, deposited in GenBank (Table 15). The NJ-tree derived with the sequences of top related results from BLAST hit (Fig.77), the CUJ11 found placed at the same clade with 2 samples of *D. eucharis* one with 100% identity and another with 99.83% similarities found in the same clade more close to the species. The NJ-tree distance data revealed that the species was diverged from their closely related species *D. hyparete (*JX78952) about 82,000 years ago.

The BLASTn with *Eurema hecabe* CUJ12 and CUJ13 (KT880648 and KT880649), and the BLAST hit table from NCBI showed 100% identity with the 11 samples of *E. hecabe* sequences deposited in GenBank (table 16and 17). The ten other samples of same species showed variations from 99.85 to 98.78%. The NJ-tree derived with the sequences of top related results from BLAST hit (Fig. 84 and Fig. 93), the CUJ12 found placed at the same clade with 13 samples of *E. hecabe.* The CUJ13 isolated from Thalassery, Kannur District has only 99.54% similarity to CUJ12 collected from Aravanchal, Kannur District, this is an instance of nucleotide polymorphism in the species. The NJ-tree distance data revealed that the species was diverged from their closely related species *E. blanda* about 1,28,000 years ago. The CUJ12 was further diverged more than 10,000 years from CUJ13.

The BLAST hit table with *Orsotriaena medus* CUJ14 (KT880650) showed 100% identity with the 2 samples of *O. medus* (GU012586 and KJ459854) from Pune and 5 sequences of same species were with slight variability with identity from 99.84 to 99.38% among the sequences deposited in GenBank (table 18). The NJ-tree derived with the top related sequences from BLAST hit results (Fig. 102), The CUJ14 found placed at the same clade with 6 samples of *O. medus* with close identity and another in adjacent clade with 99.68% similarities. The NJ-tree distance data revealed that the species was diverged from their closely related species *O. jopas* (HM240615) about 35,000 years ago.

The BLAST hit table with *Cynitia lepidea* CUJ15 (KT880651) showed 100% identity with the 2 samples of *C. lepidea* (GU012547 and KJ459781) from Pune, and 6 sequences of same species were with slight variability from 99.85 to 99.39% among the sequences deposited in GenBank (Table 19). The NJ-tree was derived from the top related sequences from BLAST hit results, (Fig. 109) the

CUJ15 found placed at the same clade with 3 samples of *C. lepidea* with close identity and another 4 samples in adjacent clade with 99.52% similarities. The NJ-tree distance data revealed that the species was diverged from their closely related species *Tanaecia julii* (GQ864809) about 23,500 years ago.

The BLAST hit table with *Junonia iphita* CUJ16 (KT880652) showed 100% identity with the 6 samples of *J. iphita* and 6 sequences of same species were with slight variability from 99.85 to 97.42% among the sequences deposited in Gen**B**ank (table 20). The NJ-tree developed from the top related sequences from BLAST hit results (Fig. 116), the CUJ16 was found placed at the same clade with 10 samples of *J.iphita* with close identity and another 4 samples in adjacent clade up to 97.42% similarities. The NJ-tree distance data revealed that the species was diverged from their closely related species *J. hedonia* (KF226499) about 2,00,000 years ago.

The BLAST hit table with *Junonia almana* CUJ17 (KT880653) showed 100% identity with the 21 samples of *J. almanac,* another 13 sequences were with slight variability from 99.85 to 99.04% from the sequences deposited in GenBank (table 21). The NJ-tree, with related sequences from BLAST hit results (Fig. 123), the CUJ17 found placed at the same clade with 8 samples of *J.almana* having close identity and another in adjacent clade with 95.42% similarity. The NJ-tree distance data revealed that the species was diverged from their closely related species *J. genoveva* (KJ469090) from French Guiana of South America about 52,000 years ago.

The BLAST hit table with *Ypthima huebneri* CUJ18 (KT880654) showed

100% identity with the 3 samples of *Y. huebneri* and another 4 sequences with slight variability from 99.85 to 99.84% from the sequences deposited in GenBank (table 22). The NJ-tree derived from the top related sequences from BLAST hit results (Fig.130), the CUJ18 found placed at the same clade with 8 samples of *Y. huebneri* with close identity and few samples in adjacent clade having similarities up to 94.82%. The NJ-tree distance data revealed that the species was diverged from their closely related species *Y. ceylonica* (KF733789) from Tamilnadu of South India about 2,000 years ago.

The BLAST hit table with *Tirumala limniace* CUJ19 (KT880655) showed 99.84% identity with the 2 samples of *T. limniace* and 4 sequences of same species with slight variability in identity from 99.70 to 98.10% among the sequences in GenBank (table 23). The NJ-tree derived from the related sequences from BLAST hit results, (Fig. 137) the CUJ19 found placed at the same clade with 8 samples of *T. limniace* with close identity and few samples in adjacent clade with up to 98.10% similarities. The NJ-tree distance data revealed that the species was diverged from their closely related species *T. hamata* (JN266591) about 9,000 years ago.

The BLAST hit table with *Melanitis leda* CUJ20 (KT880656) showed 100% identity with the 10 samples of *M. leda* sequences deposited in GenBank (table 24). The 15 other samples of same species showed variations from 99.85 to 97.47%. The NJ-tree derived with the top related sequences from BLAST results (Fig.144), the CUJ20 was found in the same clade with 11 samples of *M. leda.* The CUJ20 isolated from Nilambur, Malappuram District has 100% similarity to CUJ21 collected from Aravanchal, Kannur District, there was no instance of nucleotide polymorphism between them and they were identical. The NJ-tree distance data revealed that the species was diverged from their closely related species *M. phedima* (KJ459747) about 54,000 years ago.

The BLAST hit table with *Acraea violae* CUJ22 (KT880658) showed 100% identity with the two samples of *A. violae* and two sequences with slight variability with identity from 99.68 to 99.18% among the sequences deposited in Gen**B**ank (table 25). The NJ-tree developed from top related sequences from BLAST hit results (Fig. 151), the CUJ22 found placed at the same clade with 2 samples of *A. violae* with close identity and 2 samples in adjacent clade with 99.68 to 99.18% similarities. The NJ-tree distance data revealed that the species diverged from their closely related species *A. andromacha* (KF395497) about 38,000 years ago. Most of the species in the genus *Acraea* were seen in Africa, the *A. violae* is the only one species of the genus seen in India and Sri Lanka.

The BLAST hit table with *Junonia lemonias* CUJ26 (KT880659) showed 100% identity with the10 samples of *J. lemonias* and seven sequences of same species were with slight variability with identity from 99.84 to 97.58% among the sequences deposited in GenBank (table 26). The NJ-tree derived from the top related sequences from BLAST hit results (Fig. 158), the CUJ26 found placed at the same clade with 12 samples of *J. lemonias* with close identity and 5 samples in adjacent clade with 99.84 and 97.58% similarities. The NJ-tree distance data revealed that the species was diverged from their closely related species *J. villida* (JN274489) about 40,000 years ago.

The BLAST hit table with COI sequence of *Danaus chrysippus* CUJ27 (KT880660) showed 100% identity with the10 samples of *D. chrysippus* and 7 sequences with slight variability in identity from 99.85 to 99.70% among the sequences deposited in GenBank (table 27). The NJ-tree derived from the top related sequences from BLAST hit results (Fig. 164), the CUJ27 found placed at the same clade with 12 samples of *D. Chrysippus* with close identity and 5 samples in adjacent clade with 99.85 to 99.70% similarities. The NJ-tree distance data revealed that the species was diverged from their closely related species *D. petilia* about 90,000 years ago. The analysis of COI gene sequence isolated from *D. chrysippus* CUJ27 revealed the association towards African samples than from South East Asia.

The BLAST hit table with the COI sequence of *Castalius rosimon* CUJ29 (KT880661) showed 99.84% identity with the 2 samples of *C. rosimon* and 4 other sequences of same species with slight variability in identity from 99.70 to 99.36% among the sequences deposited in GenBank (table 28). The NJ-tree derived from the top related sequences from BLAST hit results (Fig. 171), the CUJ29 found placed at the same clade with 3 samples of *C. rosimon* with close identity and few samples in adjacent clade up to 99.36% similarities. The NJ-tree distance data revealed that the species was diverged from their closely related species *Tarucus rosacea* about 94,000 years ago.

The BLAST hit table with COI sequence of *Euploea core* CUJ31 (KT880662) showed 100% identity with 1sample of *E. core* and 11 sequences with slight variability in identity from 99.85 to 98.60% among the sequences deposited in GenBank (table 29). The NJ-tree derived from the top 17 sequences from BLAST hit results (Fig. 177), the CUJ31 found placed at the same clade with 9 samples of *E. core* with close identity and 3 samples in adjacent clade with 99.85 to 98.60% similarities. The NJ-tree distance data revealed that the species was diverged from their closely related species *E. alcathoe* about 17,000 years ago.

The BLASTn was conducted with the COI sequence of *Luthrodes pandava* CUJ33 (KT880664) as query and the BLAST hit table showed 99.84% identity with 1sample of *L. pandava* KJ131016 and 15 sequences of same species were with slight variability from 99.84 to 98.70% among the sequences deposited in GenBank (table 30). The NJ-tree was derived from the sequences of BLAST hit results (Fig. 182), the CUJ33 found to be placed in a single clade with *L. pandava* KJ131016 they are monophyletic. Many samples found in a different clade with 13 samples of *L. pandava* with close identity up to 98.70% similarities. The NJ-tree distance data revealed that the species diverged from its closely related species *Chilades galba* about 5,800 years ago.

The partial coding sequence of COI gene isolated from 24 species of butterflies have the power to recognise the species and suitable as a molecular barcode to identify species and an effective tool in phylogenetic analysis. The Fig. 183 demonstrates the power of partial coding sequences of mitochondrial COI gene for phylogeny analysis.

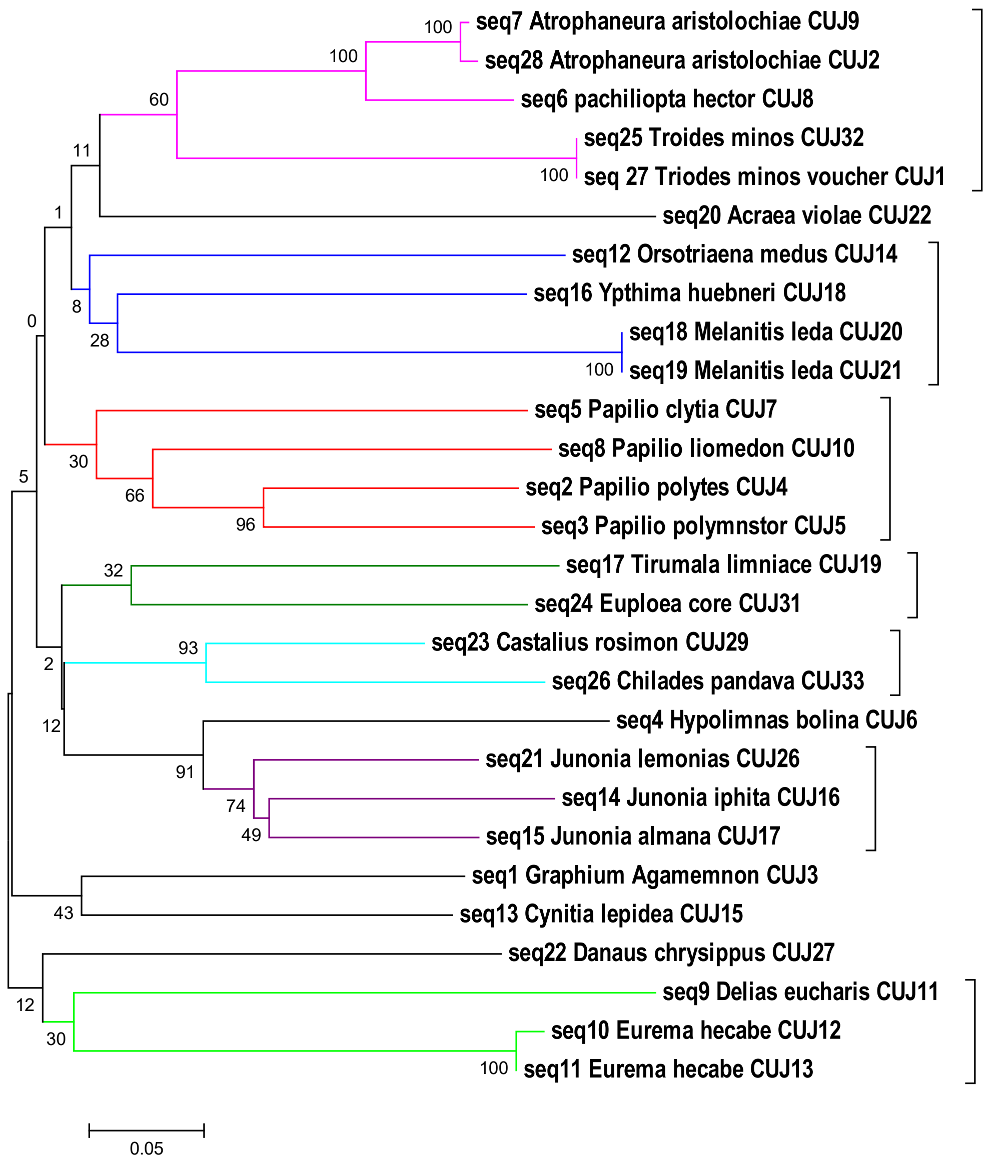

Fig. 183. The NJ–tree derived from COI sequences with Phylogenetic relationships of the 24 species of *Papilionoids* from North Kerala. This represents the samples according to their taxonomic relationships in their related clade.

The Western Ghats formed around 70 million years ago during the movement of Indian subcontinent plate from the southern hemisphere to the tropic. Here a study of the divergence of 24 butterfly species from middle Western Ghats was done based on nucleotide substitution rates of their COI gene sequences. The distance data obtained revealed that they were evolved recently. This supports the possibility of butterflies in Western Ghats were distributed either from North or South East Asia or from Africa. The present study revealed the affinities based on their partial coding sequences of the COI gene isolated from 24 species of butterflies from Western Ghats with the related sequences from GenBank nucleotide database. The CUJ27 have an African affinity, while others have affinity with South East Asia.

The present study identified two samples of *A. aristolochiae* CUJ2 (HQ424202) and *A. aristolochiae* isolate CUJ9 (KT880645) with dissimilar nucleotide and peptide sequences. The *A. aristolochiae* CUJ2 (HQ424202) from Alakkode of Kannur district, Kerala, has 99.54% identical with *A. aristolochiae* isolate CUJ9 from Cheemeni, Kasaragod District of Kerala. This is an instance of nucleotide polymorphism. The mutations involved were studied with multiple sequence alignments using ClustalW (Fig. 16). The two different single nucleotide substitutions were found; one with C to T transition at 1831th nucleotide of mitochondrial genome and another C to A transversion at 1898th nucleotide of the mitochondrial genome. *P. aristolochiae* (KJ195272) and *P. aristolochiae* (KJ195291) from Pune, India, where the samples identified from database with same mutation at 1831th nucleotide. The substitutions at 1898th nucleotide of CUJ2 make it a unique genotype. The T to C mutation is at low constraint sites and causes no peptide change, but the C to A mutation is at high constraint 1st codon position and therefore leads to peptide change (Fig. 16). Hence, it is necessary to clarify the taxonomic status of CUJ2 in future studies.

Nucleotide polymorphism was also identified in COI sequence of *E. hecabe* CUJ12 and *E .hecabe* CUJ13. They were collected respectively from Aravanchal and Thalassery belonging to Kannur district. The sequences showed 99% identity between them. The mutation identified was studied with multiple sequence alignments using ClustalW. Two nucleotide substitutions at two different positions were identified, both with G to A transition. The mutations are silent, without peptide change depicting single nucleotide polymorphism.

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