STUDIES ON THE GENETIC DIVERSITY OF WASPS OF THE FAMILY VESPIDAE FROM KERALA

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By

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Certificate

This is to certify that this thesis entitled "Studies on the Genetic Diversity of Wasps of the Family Vespidae from Kerala" is a bonafide research work done by Mrs. S. Kavitha in the laboratory of Molecular Biology of the Department of Zoology under my supervision and guidance, in partial fulfillment 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.

Dr. K.V. Lazar

(Supervising Teacher)

March 10, 2015

Declaration

I do hereby declare that this thesis entitled "Studies on the Genetic Diversity of Wasps of the Family Vespidae from Kerala" is an authentic record of the work carried out by me in the Division of Molecular Biology, Department of Zoology, University of Calicut, under the guidance of Dr. K.V. Lazar, and that no part of this thesis has been previously submitted for any other Degree.

S. Kavitha

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Dedicated to my Parents

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FOREWORD

By nearly any measure, the most successful animals on the planet are the arthropods. They have conquered land, sea and air, and make up over three-fourths of all currently known living and fossil organisms, or over one million species in all. Vespidae wasps are an important group of Neotropical insects, but usually neglected. Their social organization, notably expressed in the variation of nest architecture and worker's aggressiveness in defense of their colonies, attracts both the general public and particularly entomologists. Vespidae wasps have relevant ecological and economic roles in the biological control of pests and pollination of flowers. Some species are necrophagous, and play a considerable role in nutrient cycling by accelerating this process.

Determining the genetic diversity using molecular tools will help to define origin, evolution and phylogenetic relationships of species. The analysis of genetic diversity of insects like wasps has not been carried out much in India. Hence, the wasps of the family Vespidae (Phylum: Arthropoda> Class: Insecta> Order: Hymenoptera) was selected for the present study. The family is inturn divided into six subfamilies: Eumeninae, Masarinae and Euparagiinae (solitary wasps); Stenogastrinae (pre-social wasps); Vespinae and Polistinae (social wasps).

From Kerala, fifty-one species are hitherto reported but only a single molecular barcode data is available from family Vespidae. The cytochrome c oxidase

subunit 1 gene of the mitochondrial genome is the gene candidate used for molecular barcoding of Vespidae in the present study.

The major objectives of the present study were collection and genotyping of some Vespidae wasps of Kerala, the generation of molecular barcodes for the species and the study of the genetic structure of mitochondrial genes. The present work also had the objective of analyzing the phylogenetic relationship, evolutionary divergence and origin of each species along with the study of interspecies relationship of the selected species of wasps.

Eight wasps of the family Vespidae viz. *Vespa tropica, Rhynchium brunneum, Delta pyriforme, Phimenes flavopictum, Delta conoideum, Polistes strigosus, Ropalidia spatulata* and *Ropalida jacobsoni* were collected from various places of Kerala using small handnet and photographed. The total genomic DNA was isolated from the insects and mitochondrial cytochrome c oxidase subunit 1 gene was subjected to amplification by polymerase chain reaction using primers derived from the mitochondrial genes of eukaryotes. The PCR product was analyzed using agarose gel electrophoresis, viewed under a UV tranilluminator and photographed. The purified PCR product was sequenced by Sanger's DNA sequencing method using ABI 3730 automated sequencer (Applied Biosystems, ABI). The final sequence obtained was analyzed for its phylogeny and interspecies relationships.

The mitochondrial cytochrome c oxidase subunit 1 gene sequence of the eight selected wasps were amplified and sequenced in the present work. All the sequences obtained were novel and were deposited in the GenBank. These sequences can be used as the molecular barcodes of the species. The nucleotide and conceptual peptide sequences were searched for similarity using BLASTn and BLASTp.

The construction of phylogeney tree (N-J tree) to analyze the origin, mode of divergence and interrelationship between species was also performed. All the species showed close similarity to wasps of same genus or subfamily, suggesting the correct taxonomic postions of the collected species within the family Vespidae. The wasps of the same genus or subfamily were found to have a single origin. The social wasps of the subfamily Polistinae, Vespinae and solitary wasps of the subfamily Eumeninae were observed to have originated from different ancestors in the phylogeny analysis. A ClustalW alignment and a phylogeny tree construction was also carried out by considering the eight selected wasps. It showed the interspecies relationship pattern between them.

The data generated in the present work holds great possibilities for augmenting various fields like agriculture, vaccine and drug development. The molecular barcodes generated will allow the correct identification of the species even from degraded specimen, any body part or any stage in the life cycle of the species. The analysis of genetic diversity of Vespidae wasps will provide newer insights into

the understanding of origin, evolutionary patterns and mode of divergence of insects together with the application of its gene sequences in the field of systematics, and ecology.

INTRODUCTION

Class Insecta is the most prolific group in Kingdom Animalia which mastered the world and took full possession of it earlier to man's attempt for the same. The word 'insect' comes from a Latin word 'insectum' which means "cut up or divided into segments". It is further divided into 20 orders which include Hymenoptera (Triplehorn and Johnson, 2005).

Hymenoptera is a hyperdiverse order which is treated as a supreme success of Mesozoic radiations of insects (Grissell, 1999). It is a vast group, whose major part is recognized by the late Jurassic period, including 22 superfamilies and many of the 89 existing families, which appeared towards the later part of Cretaceous period (Rasnitsyn, 2002). They play a vital role in the terrestrial ecosystems as parasitoids, predators and pollinators.

Hymenoptera includes two subdivisions viz. Apocrita and Symphyta. Apocrita is again divided into Aculeata and Parasitica. The partition of Aculeata and Parasitica was based on behaviour, that is predatory behavior vs parasitic behaviour, but many of them had ectoparasitoid biology (Sharkey *et al.,* 2012).

The speciality of Aculeata is the possession of a diagnostic synapomorphy, which is the venom injecting apparatus called 'stinger', a conversion of the female ovipositor. The members of Aculeata include bees, ants and wasps. India holds a rich fauna of Aculeata (Kumar and Srinivasan, 2010).

The first complete account on aculeate hymenoptera (wasps, bees and ants, cuckoo wasps) of India, Burma and Ceylon was reported by Bingham (1897, 1908). Aculeata consist of three Superfamilies viz, Vespoidea, Apoidea and Chrysidoidea (Debevec *et al.*, 2012).

Vespidae is a large family of insects under the superfamily Vespoidea, which encompasses about 5000 species of wasps. Majority of the eusocial and some of the solitary wasps, having cosmopolitan distribution are coming under this family (Biosci *et al*., 2013). It is divided into six subfamilies, among which two are strictly eusocial species (Polistinae and Vespinae) and three includes solitary species (Euparagiinae, Masarinae and Eumeninae). Stenogastrinae includes solitary as well as social species.

The largest family is Eumeninae with nearly 3000 species in the world (Carpenter and Garcete-Barrett, 2002). Among the six subfamilies only Vespinae, Polistinae and Eumeninae are reported from Kerala (Lambert, 2002). Their colonies consist of males, workers and queens.

The wasps of subfamily Vespinae are very dangerous and highly poisonous to human being. A comprehensive study on species of Vespidae, Apidae (Superfamily: Apoidea) and Scolidae (Superfamily: Vespoidea) from India was made by Batra (1977), Das and Gupta (1989) and Gupta and Jonathan (2003) respectively.

Social wasps are vital agents for natural biocontrol in agricultural and natural ecosystems having a voracious feeding activity (Ghoneim, 2014). Some of the social

wasps are commonly observed in agro-ecosystems (Auad *et al.,* 2010; Brugger *et al.,* 2010). They also proved to be good pollinators of plants (Stephens, 2012).

Genetic variation is fundamental to the breeding success for all populations (Stiling, 2000). Decline of genetic diversity can unfavorably affect growth of the population and can renounce the recovery of endangered species. The analysis of the variations in the genetic makeup of a species by examining the DNA sequence is called 'Genotyping'and it is less erroneous compared to other traditional approaches.

Using molecular tools, the individual sequences are examined and compared with the related or unrelated sequences for the definite identification and comparison of genetic variation. Sequence changes in the genetic makeup of species is observed as their 'molecular barcodes' (Hajibabaei *et al.,* 2006). The utilisation of molecular tools for analysis in the field of Entomology, especially for species identification is becoming more common and relevant in recent years (Roques *et al.,* 2009).

For example, the identification of immature life stages, pest insects like fruit flies and medically important insects like mosquitoes (Dittrich-Schroder *et al.,* 2009; Sethusa *et al.,* 2014; Ashfaq *et al.,* 2014). The establishment of the DNA barcoding libraries can identify and distinguish various organisms which belong to different taxonomic positions. The essentiality of maintaining a DNA barcode library and thus their application in various analyses gained more and more importance by time.

Heraty *et al.* (2011) presented the first complete molecular based study of Hymenoptera. Mashhoor *et al.* (2013) reported the first molecular barcode data (Cytochrome c oxidase subunit 1) of a Vespidae wasp, *Eumenes petiolata* from Kerala. This was also the first report from India as well. It was pointed out in their study that, for an accurate identification, determination of interspecies relationship and delineation of phylogeny of Vespidae wasps of India, an assessment of the genetic diversity among the wasp fauna is a pre-requisite. Lopez-Osorio *et al.* (2014) sequenced the Vespidae CO1 sequence of *Vespa affinis*, which was the second report of a molecular barcode data of Vespidae family from India. Molecular barcoding data of wasps of India were not reported in any other studies, which would have been useful for their precise characterization.

A trustworthy and accessible classification of species is elementary to research in biodiversity and conservation biology. While an 8.7 million species have been described so far, it represents only a tiny fraction of the actual diversity present on Earth (Mora *et al.,* 2011).

Owing to the constant threat of loss of biodiversity, there is an increasing need to accelerate the pace of species discovery and taxonomic databasing (Godfray, 2002). Even the routine identification of known species seems difficult, often demanding a highly specialized knowledge, which is a limiting factor in ecological studies and biodiversity inventories (Monaghan *et al.,* 2005). In response, many new

proposals have called for a more prominent role of competent DNA-based methods in the delineation and identification of species (Kekkonen and Hebert, 2014).

In recent years, DNA based studies are increasing on a fast pace. Genbank now claims nearly 16,13,104 insect sequences (Vargas *et al*., 2014). About 3, 00,000 of those are of Vespidae genes, a significant fraction of them is being used in phylogenetic studies (http://www.ncbi.nlm. nih.gov/nuccore/?term=vespidae).

Studies on insect systematics have now examined around 50,000 protein-coding genes and nearly 5,00,000 protein sequences, among which 13 protein coding genes and 1988 protein sequences are of the family Vespidae (http://www.ncbi.nlm.nih.gov/protein/).

The markers commonly used in molecular taxonomy, molecular systematics, molecular phylogenetics, forensic and medical studies include restriction fragment length polymorphism (RFLP), simple sequence length polymorphism (SSLP), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), variable number tandem repeat (VNTR), microsatellite polymorphism or simple sequence repeat (SSR), single nucleotide polymorphism (SNP), short tandem repeat (STR), single feature polymorphism (SFP) or Diversity Arrays Technology (DArT) and restriction site associated DNA markers (RAD markers) (Fedorov *et al.*, 1999; Grechko, 2002; Belaj *et al.*, 2012).

The diversity of markers available has unquestionably furthered the cause of insect molecular systematics. The most commonly sequenced regions in insect systematics are mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA). As contiguous segments of DNA, they lend themselves for an easy comparison (Caterino *et al.,* 2000).

Insect mtDNA consists of thirty-seven genes including two ribosomal RNA (rRNA) genes, twenty-two transfer RNA (tRNA) genes and thirteen protein coding genes (Clay and Wolstenholme, 1985; Crozier and Crozier, 1993). It is present abundantly in cells and has a high nucleotide divergence and size variation. Hence, it was increasingly tapped as a storehouse of rich and rapidly evolving molecular markers, allowing evolutionary history to be studied in closely related populations and taxa (Harrison, 1989; Simon, 1991; Lopez-ozorio *et al.*, 2014).

It has a high rate of mutation and lower effective population size in comparison to nuclear DNA, which make mtDNA a potent tool to probe for substantiating reproductive isolation among lineages. This fact motivated a proposal to assign DNA-based species identification by analyzing a consistent segment of the mitochondrial genome. By this approach, a sequence library from taxonomically established voucher specimens can be used as DNA identifiers for species, in short, the DNA barcodes (Hebert *et al.,* 2003).

Virtually every animal molecular study involves mtDNA haplotyping in some stages. A 658 fragment of mitochondrial DNA called cytochrome c oxidase I (*COI*) was elected as a standardized tool in the DNA barcoding process in the recent years (Galtier and Duret, 2007). This is due to the fact that *CO1* appeared to be the most conservative protein-coding genes in the mitochondrial genome of animals, which was preferred for the evolutionary, phylogenetic and systematic studies (Folmer *et al.,* 1994; Goldstein and DeSalle, 2011). It can be quickly recovered from diverse species using a limited set of primers.

Some commonly used regions are present for both mitochondrial and ribosomal genes, but never consistent across all taxa. For mtDNA the most recurrently sequenced genes are CO1, COII, 16S rDNA and 12S rDNA. *CO1* and *COII* are being sequenced over the widest variety of taxa with homologous sequences.

The internal transcribed spacer (ITS) and 28S regions dominate in studies of Dipterans and Hymenopterans (Oh *et al.,* 2009). Phylogenetic studies using nuclear protein-coding genes are far less in number than of either mtDNA or nuclear rDNA. However, a few loci are becoming widely used, of which elongation factor-1α (EF-1α) has been the most popularly studied one (Walldorf and Hovemann, 1990; Ceccarelli and Crozier, 2007). Its sequences have proven very useful for studies among specious groups and genera (Caterino *et al.,* 2000).

DNA barcoding helps the species identification process by converting the expert taxonomic knowledge of key morphological characters to an easily accessible format. In addition to assigning specimens to an identified species, DNA barcoding can fasten the species discovery, as large sequence variations in animal mtDNA generally points to a new species status.

Still, it does possess some limitations like preservation of ancestral polymorphisms, pseudogenes, hybridization and the idiosyncrasies of mtDNA inheritance (Benasson, 2001; Moritz, 1994; Thalman, 2004; Will, 2005). Investigations on various vertebrate and invertebrate groups showed that COI barcodes discriminate more than 95% of species (Ward *et al.*, 2005; Hajibabaei *et al.*, 2006).

The potential taxonomic revisions motivated by DNA barcoding results have been left to experts for conformation based on morphology, behaviour and other salient features (Hebert *et al.*, 2004). It is to be anticipated that for a greater part of described taxa, a global DNA barcoding program will present a complete barcoding registry in the probable future, enabling the systematic discovery of enigmatic species. Since, 85% or more species is still unidentified, a great challenge lies in the potential application of DNA-based methods in the process of discovery and delineation of new species in poorly characterized taxa (Monaghan *et al.*, 2005).

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REVIEW OF LITERATURE

1. Morphotaxonomic and sociobiological studies in Vespidae

The earliest known description of Vespidae was made by Linnaeus. In 1758, he described 4 species of Vespidae wasps viz. *Vespa coraba, Sphex tropica* (presently, *Vespa tropica), Vespa gallicia* and *Vespa vulgaris* (presently, *Vespula vulgaris*). Following Linnaeus, De Geer (1773) and Fabricius (1793, 1798) made their contributions in the study of Vespidae.

Saussure (1852, 1867) published monographs on wasps of Vespidae family and studied taxonomy of Vespidae of Asia and Africa based on the collection of 'Leyden' museum, Holland.

Bingham (1888) published taxonomic notes on some bees and wasps of Burma in the '*Journal of National Society'*. The Vespidology of India, Ceylon and Burma was extensively dealt by Bingham (1897), in the book '*Fauna of British India including Ceylon and Burma'*. It laid a strong foundation to Vespidae systematics.

In 1905, Bingham reported the taxonomy of Vespidae, and also published notes on aculeate Hymenoptera of the Indian museum. Torre (1904) gave a detailed description of Vespidae wasps in his book '*Genera Insectorum'*.

The taxonomy of many Vespidae species was described by Schulthess (1910, 1934). A revision of the Vespidae of Belgian Congo was reported by Bequaert (1918), based on the collection of the American museum. Notes on 'Diplopterous' wasps in

the collection of the Indian museum were published by Dover *et al*. (1925 a, b). Soika *et al*. (1973, 1981, and 1994) made a vast study of the family Vespidae and reported taxonomy and discription of several new species of wasps.

The taxonomy of Vespidae was reviewed by Vecht (1957, 1962). He provided description of Indo-Australian and Indo-Malayan wasps of Vespidae. The distributional pattern of Indian Vespidae with reference to altitude was reported by Gupta and Das (1977).

A taxonomic review of Vespidae, a catalogue of the families Stenogastrinae and Vespidae from Indian subregion and also a monograph *'The social wasps of India and the adjacent countries*' was published by Das (1979); Das and Gupta (1989).

Notes on the phylogenetic relationship and natural classification of the Vespoidea were published by Carpenter (1982). In 1987, he described the phylogenetic relationship and classification of the Vespinae, commented on the evolutionary genetics of social wasps, and reviewed the subspecies in Eumeninae genus, *Zeta* Saussure.

He also published nomenclatural notes and synonymic checklists on different subfamilies of Vespidae and has made extensive studies on phylogeny and evolution of Vespidae.

Archer (1980, 1998) reported taxonomy and description of Vespidae wasps from various areas. Taxonomic, biochemical and ecological studies of Vespidae and description of many new species was provided by Yamane, (1976); Yamane and Yamane (1979). Kojima and Kojima (1988) and Kojima (2000) have worked on Vespidae systematics and provided several taxonomic notes and discriptions on genus, subgenus and many species of the family Vespidae, including the phylogeny and ecological parameters affecting the Vespidae wasps.

Two genus of Vespidae, *Discoelius* Laterile and *Anterhynchium* Saussure were reviewed by Kim (2005). Taxonomic notes on the genus Pseudodynerus (sub family Eumeninae) was provided by Hermes *et al*. (2005).

Taxonomic study of 3 new species of *Ropalidia* Guerin from Kerala was provided by Kojima *et al.* (2007). A study of the social wasps of subfamily Vespinae was made by Dubatolov and Milko (2004). A new Neotropical species of the wasp genus *Zethus* was described by Cooper (2004).

A Revision of the afrotropical species of the wasp genus *Jugurtia* was done by Gess (2004). The distribution of vespid wasps in Europe was explained by Fernandez (2004). Several taxonomic reviews and new species description along with biogeography of Vespidae wasps was provided by Saito and Kojima (2005); Saito *et al*. (2005). Dvorak (2006) conducted a study on oriental hornet, *Vespa orientalis* from

Mexico. Identification of atlas of Vespidae of northeastern nearctic region was done by Buck *et al*. (2008).

Bagriaçik and Samin (2011) provided a check list of Iranian Vespidae and studied on morphological variation within a colony of *Dolichovespula media*. Many taxonomic notes and catalogues for the species of Vespidae family from different areas were published by Gusenleitner and Madl (2009); Gusenleitner (2013).

Studies on taxonomy, biodiversity morphology, faunistics and ecology of Vespidae wasps were conducted by Abbassi *et al*. (2008, 2009). Revisions, new species descriptions and phylogenetic studies of Vespidae were performed by Silveira (2010, 2013).

Review on Vespidae species of India and new species description of many wasps were provided by Kumar and Sreenivasan (2010). Lijun and Hai-Tao (2008), provided keys to a genus and species of Vespidae and studied on the sociobiology of the wasp *Vespa fumida* (sub family Vespinae).

Several studies on Vespidae based on morphology, sociobiology and taxonomy were performed by Baracchi *et al*. (2010, 2013). Nugroho *et al*. (2010, 2012, 2014) published taxonomic notes, new species description, reviews and studied on geographical variation of melanisation patterns in hornets of Vespidae family.

1.1. Importance of Vespidae wasps in the ecosystem

Role of Vespidae wasps as predators of agricultural pests were explained in many studies. Prezoto and Machado (1999) conducted a study in Brazil, where the social wasp colony of *Polistes simillimus* (Vespidae, Polistinae) was transferred to maize plantings to control the attack of *Spodoptera frugiperda* caterpillars, which made a heavy loss in maize production in the area.

There was an increase of maize production after release of the wasps in the plantations. The pest populations such as Lepidopteran caterpillars, bugs, small flies and larvae are kept in control by vespid wasps like *Mischocyttarus flavitarsis*, *Polybias evicea* and *Polybias cuttellaris* (Lutz and Brian, 2013). The social wasps usually prey upon a large variety of insects of suitable size. These are seized and partially dismembered.

Some Vespinae species like *Vespula germanica* and *Vespula vulgaris* are quite serious predators of other Hymenopterans. The predatory activity of different species of wasps under Vespinae sub family viz. *Vespula germanica*, *Vespa tropica*, and *Vespula vulgaris* on honeybees was explained by Sakagami and Fukushima (1957). Many hymenopterans were predated by wasps, including winged ants, bees and spiders (Sakagami and Fukushima, 1957); O'Donnell *et al.*, 2013).

The predatory activity of two wasp species *Calymmochilus dispar* and *Gelisapterus* was explained by Korenko *et al*. (2013), where its larvae feed on the

juveniles of an ant eating spider *Zodarion styliferum*. Some species like *Polistes fuscatus, Parachartergus fraternus* and *Polybia sericea,* play a minor role as predators of caterpillars which feed on various crops like rice, wheat and cabbage (Stamp, 2001; Chilcutt and David, 1993; O'Donnell *et al.*, 2013).

A study by Gould and Jeanne (1984) which introduced *Polistes* wasps to a cabbage field, severly attacked by a Lepidopteran pest, *Pieris rapae,* showed significant progress in the quality and weight of cabbage in plots when exposed to foraging *Polistes* species, compared with cabbage in the unexposed control plots. It showed the significant reduction in numbers of the pest species, due to the predation of wasps. These studies points to the application of Vespidae wasps in the field of integrated pest management (IPM).

The vespids can be considered as good pollinators of plants. The role of social wasps in the pollination of plants in Brazil was extensively studied by Hermis and Kohler (2006). They observed that the wasps which are coming under subfamily Polistinae plays a vital role in pollination of some specific plant families like Asteraceae.

The importance of two vespid wasps *Vespa tropica* and a Polistinae species in the pollination process of hybrid variety sunflower plants in India was studied by Jadhav *et al.* (2011). In a nutshell, the fact highlighted in all these works is the significance of Vespidae wasps in our ecosystem.

2. Molecular studies

Determining the interrelationships among species and their mode and time of divergence for the major eukaryotic lineages exists as one of the most important and contentious issue in evolutionary biology. Later, the gene sequencing and analysis of phylogeny in ribosomal RNA (rRNA) considered as the first complete eukaryotic phylogenies, suggested a view that cellular complexity was obtained during the divergence of present unicellular eukaryote lineages (Hixson and Wesley, 1986). Later, newer changes in analytical methods and the availability of more and more genes for studies on phylogeny showed that the bigger part of the deep structure of primitive rRNA trees was an artifact.

The phylogenetic studies of multiple genes along with the discovery of crucial molecular phylogenetic characters divided eukaryotic diversity into 6 major hypothetical groups. But due to many contentious issues, species relationships among these groups became poorly studied (Roger and Laura, 2006).

DNA barcoding is a diagnostic tool identification of species, making use of a small consistent segment of DNA. An efficient DNA barcode maker would be very helpful for untying the poorly understood genetic variation of species in the natural environment.

DNA barcoding first became a matter of intrest of the scientific community in early 1990's. It was popularized by Paul Hebert and his co-workers in 2003 by

developing a BOLD system barcoding of DNA. They also published a paper entitled "Biological identifications through DNA barcodes". It became a novel technique for identification of species by analyzing a small portion of DNA from a consistent region of the genome. This DNA sequence can be used for identifying various species, in a way a supermarket scanner make use of the black stripes in the UPC barcode for the purpose of purchase identification of people (Hebert *et al.*, 2003).

The gene region commonly used for majority of animal groups, a 658 base-pair segment in the mitochondrial CO1 gene, observed as an excellent segment in the identification of species. It is of smaller in length, can be sequenced fast and is cheap, still long enough for identification of genetic variation in DNA (Arzanloua *et al*., 2013).

Molecular level studies depending on mitochondrial DNA began from the identification of mitochondrial DNA by Nass and Nass in chick embryo. They called them as 'intra mitochondrial fibers with DNA characteristics' (Nass and Nass, 1963). The occurence of mitochondrial DNA in insects was first reported by Wolstenholme (1966) as DNA and RNA containing bodies in Drosophila.

Later Minamori (1969) observed an extra chromosomal element 'Delta' in Drosophila where they described it as "Delta may be a self-reproducing particle or a symbiont, which is transmitted regularly from mothers to their progeny".

A description of mitochondrial DNA in Drosophila was made by Chase (1972). They observed three types of DNA, a bulk DNA and two satellite DNA's among which one is supposed to be of mitochondrial origin. Isolation and characterization of Drosophila mitochondrial DNA was performed by Polan *et al.* (1973). David (1976) studied the structural heterogeneity of Drosophila mitochondrial DNA and found each of them to possess an area which denatured at a particular temperature and are loaded with adenine and thymine.

2.1. DNA sequencing

Since the discovery of DNA sequencing by Sanger and Nicklen (1977) by chain termination method and Maxam and Gilbert (1977) by chemical degradation, the analysis and studies on the genetic diversity progressed on a large scale. The sequencing of the complete mitochondrial genome of human (Group, 1981), bovine (Anderson *et al.*, 1982) and mouse (Bibb *et al*., 1981) has been completed and much of the gene content of these DNA was identified. Clary *et al.* (1982) discribed the nucleotide sequences of some portions of the mtDNA molecule of *Drosophila yakuba* and studied the genes found within them.

They did the experiments by cloning the Drosophila DNA and then by sequencing using Sanger's chain termination method. Their results showed a number of variations between Drosophila and mammalian mtDNAs, and also a difference in

order of genes. Clary and Wolstehohne (1983) sequenced the genes for URF2, tRNAtrp, tRNAcys, tRNAtyr and (COI) in Drosophila.

Since most studies of animal mtDNA have used restriction analysis, it has been difficult to determine whether a high rate of evolution and a transition bias were characteristic of all animal mtDNAs (Palumbi and Allan, 1990). However, there has been a need for simple methods of sequencing mtDNA to examine the pattern of evolutionary substitution in other animal groups.

In 1983, Kary Mullis discovered a quick substitute to conventional cloning in the form of Polymerase Chain Reaction. Mullis developed oligo probes for a project at Cetus co-orporation to study a sickle cell anemia mutation. Mullis put forward an optional technique which was based on Sanger's DNA sequencing method. Understanding the difficulty in making the Sanger method specific to a single location in the genome, Mullis tailored the idea by using a second primer on the opposite strand. Repeated use of polymerase led to a chain reaction of replication for a specific segment of the genome.

The improvements were made by Mullis, allowing PCR to become a vital technique in biochemistry and molecular biology, which can be described as “highly original and significant, virtually dividing biology into the two epochs of before PCR and after PCR”. It was a breakthrough in the record of molecular techniques and

marked the commencement of revolution in the field of molecular biology. Saiki *et al*. (1985) reported this discovery in their paper.

Later Kocher *et al.* (1989) using a typical set of primers utilised the polymerase chain reaction for the amplification of homologous segments of mitochondrial DNA from more than 100 animal species. They described these three setsof primers that amplify homologous sequences from a wide array of animals. This innovation helped them to gather sequence data to evaluate the pattern of molecular evolution in a variety of animal species, including insects like Cicada and a spider Terandula.

While, the restriction endonuclease approach applied to whole mtDNA has a limited phylogenetic range, being useful mainly at or below the genus level (Harrison, 1989), these short sequences obtained by PCR was a versatile source of phylogenetic information, by which they made phylogenetic trees to give evidence concerning the phylogenetic relationships among these animals and their mode of evolution (Kocher, 1989).

The molecular level studies in Vespidae was first reported by Fang *et al.* (1988), where they explained the primary structures of 2 forms of antigen 5 protein by cDNA and protein sequencing of *Dolichovespula maculate*. Later gene sequence data of the mtDNA began to be used in more frequency for analysis of phylogenetic relationships within the animal taxa.

2.2. Mitochondrial genome sequencing

Mitochondria are the main energy generators in most eukaryotic cells. Focus of research was on biochemical, evolutionary and phylogenetic aspects of mitochondria (Chandra *et al.,* 2006). Unique sequences from mitochondrial DNA are being used to group species, study migratory pattern and also in diagnostics and forensics. With the progress in the complete genome sequencing of eukaryotes, mtDNA was inevitably sequenced and this has facilitated comparative studies.

The mitochondrial genome is a circular, double stranded DNA molecule, having low molecular weight, high nucleotide mutation and usually non recombining with other lineages, which is used easily in a laboratory compared to nuclear DNA. The mitochondrial genomes of eukaryotes unveil large size differences between animal, fungal and plant species.

The average size of animal mtDNA is approximately 16kb (Boore, 1999), but, plant mtDNAs varies from 200 to 2500kb (Galteir, 2011). The size of fungal mtDNA ranges from 19kb to 176kb (Hudspeth, 1995).

The structure of mtDNA genome contains 37 genes, needed for the functioning of mitochondria. Thirteen of these genes give commands for making enzymes concerned with oxidative phosphorylation. The rest gives instructions for making transfer RNA (tRNA) and ribosomal RNA (rRNA). 13 protein coding genes are NADH ubiquinone oxidoreductase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6),

Cytochrome c oxidase (CO1, CO2, CO3), Coenzyme Q – Cytochrome c reductse/Cytochrome b (CYB), ATP synthase (ATP6, ATP8). Among non-coding genes only 2 rRNAs (MT-RNR1 - 12S and MT-RNR2 - 16S) and 22 tRNAs genes (MT-TA, MT-TN, MT-TD, MT-TC, MT-TE, MT-TG, MT-TH, MT-TI, MT-TK, MT-TL1, MT-TL2, MT-TM, MT-TF, MT-TP, MT-TQ, MT-TR, MT-TSI, MT-TS2, MT-TT, MT-TV, MT-TW and MT-TY) are found.

Due to its structural and environmental characteristics, mtDNA sequences are being used as molecular markers in the study of molecular evolution (Yagi *et al.*, 2001). They can be amplified quickly due to their increased copy number, and they often present some highly conserved primer-binding sites. Whereas, they express saturation of substitutions because of th increased rates of their evolution.

In insect molecular systematics, DNA barcoding and phylogeny studies, mtDNA are usually the gene candidate for sequencing. Mitochondrial DNA has acomparitively fast mutation rate, which produces a notable variation in sequences between species and little variation within species (Boore, 1999).

Complete mitochondrial genome organization of various animal species are available in public database of NCBI and partial gene organization of other taxas (Boore, 1999). The order Hymenoptera has been observed to posess abnormally high mitochondrial substitution rates and increased frequency of gene rearrangements compared with other insects (Dowton and Austin, 1995). The positioning of genes in

the mitochondrial genome, particularly genes that code for proteins and rRNAs, is commonly conserved in an animal phylum, but varies considerably among phyla (Boore *et al.,* 1995; Boore and Brown, 1998).

2.3. Complete mitochondrial genome sequencing of wasps

Nearly complete mitochondrial genome of 3 species of parasitic wasps; *Nasonia vitripennis, N. giraulti* and *N. longicornis* were sequenced by Oliveira *et al.* (2006). In their study, extraordinary patterns of mitochondrial evolution were observed. The sequence exhibited 30 times faster nucleotide substitution rate, which is the greatest substitution rate in animal mitochondria relative to nuclear protein coding genes.

It was recommended in their study that the increased buildup of nucleotide replacements is because of some beneficial mutations required for compensating mild-lethal mutations. Further analyses of mtDNA sequences have discovered amazing things about evolutionary relationships of many groups of organisms. Mitochondrial DNA is an excellent genetic marker of gene flow in matrilineal inheritance.

2.4. Sequencing of cytochrome c oxidase genes

Cytochrome c oxidase I gene is one of the most significant protein-encoding genes of mtDNA and has been used in the analysis of molecular evolution and species

classification. The sequence differences at COI allow the discrimination of closely related species. COI is a trustable gene for insect molecular systematics (Hebert *et al.*, 2003).

Altschul *et al.* (1939) first identified the cytochrome c oxidase enzyme as a 'soluble cytochrome oxidase'. They wrote a letter to the editor of the '*Journal of Biochemistry'* published by the 'American Society for Biochemistry and Molecular Biology', mentioning about their discovery.

They worked with suitable strains of both brewer's and baker's yeasts, and were successful in obtaining a soluble form of cytochrome oxidase. They disproved the previous assumptions of considering cytochrome c oxidase (COI) as an insoluble enzyme by their experiment.

Slonimski and Tzagoloff (1976) explained methods for the genetic level examination of yeast cytoplasmic mutants (mil-mutants) which lacked cytochrome oxidase or coenzyme Q - cytochrome c reductase. Their methods allowed mutations in mtDNA to be mapped in relation to each other and with respect to drug-resistant markers. It was one of the earlier molecular level studies of this enzyme.

The first DNA sequencing of COI was done in humans by Barell *et al.* (1979). They analysed human mitochondrial DNA sequence of the COII gene and the gene sequence of the equivalent beef heart protein, wherein UGA is a tryptophan codon and not a termination codon. It was also suggested that AUA may be a methionine

and not an isoleucine codon. The incidence of five TGA stop codons in the translated sequence of mitochondrial DNA of the yeast, for COII gene was explained by Fox (1979). The length mutations in mtDNA of humans using direct sequencing of DNA which is amplified enzymatically, making use of a standard set of primers was explained by Lisa (1987).

COI gene is usually effective as a barcode sequence, giving about 95% and above resolution at species level. The standard sequence used for detection of sequence similarity is the amplified fragment of mitochondrial COI gene. Sequence similarities are then analysed and studied using numerical methods such as hierarchial clustering of genetic distances and statistical examination of the genetic distance thresholds (Hebert *et al.*, 2003).

COI gene is favoured for the identification of a large range of animal taxa, including butterflies (Hebert *et al.,* 2004; Hajibabaei *et al.,* 2006) gastropods (Fedosov *et al.,* 2014), Springtails (Hogg and Hebert, 2004), birds (Hebert *et al.,* 2004; Aliabadian *et al.,* 2013) and mayflies (Salles *et al.,* 2014).

Different areas of genes have been opted for species level biosystematics; however, DNA barcoding upholds a global standard in selection of barcode region, where COI has gained designation as the barcode region for animals because of its wide acceptance by scientific community. In addition, the pertinence and vigor of

COI in a typical high throughput barcoding analysis have been widely studied (Hajibabei *et al.,* 2006).

A 658bp portion of the gene has been opted so that a trustable sequence read is attained in a single sequence pass in conventional cycle sequencing platforms. It also proves to be an excellent barcode which is being utilised in the analysis of phylogenetic and evolutionary parameters of nearly complete Hymenopteran families like Vespidae, Sphecidae, Formicidae etc. (Francoso and Arias, 2013).

2.5. DNA barcoding

Folmer *et al.* (1994) described DNA primers for amplification of mitochondrial COI from different metazoan invertebrates. They designed 'universal' DNA primers for PCR amplification of a 710bp fragment of the mitochondrial COI from 11 invertebrate phyla.

It was revealed in their preliminary comparison that these primers produce revealing sequences for phylogenetic studies at the basic level and above. On this basis the concept of DNA barcoding began to develop. The limitations of morphology based identification systems and the declining pool of taxonomists signaled the necessity for a novel technique in taxon recognition (Hebert *et al.,* 2003).

Microgenomic identification systems, using small gene segment proved promising for analysis of biodiversity. This concept has already attained higher level

acceptance among those people working with smaller organisms like bacteria, viruses etc. whose identification by morphological charecters is not practical (Hamels *et al.,* 2001)f. There arise an increasing number of studies in which DNA-based identification systems have been used for higher organisms (Vincent *et al.,* 2000).

RFLP and RAPD using mtDNA were experimented for their capacity to distinguish 7 species of minute parasitic wasps of the genus *Trichogramma* in a study conducted by Vanlerberghe-Masutti (1994). Pair wise comparisons of the mtDNA restriction maps showed significant variation among the 7 species.

Hence, they can be considered as trustable species identification molecular markers. Genomic approaches to taxon diagnosis utilize diversity of DNA sequences for identification of species (Wilson *et al*., 1995). Later, Hebert *et al.* (2003) evaluated the robustness of COI as a taxonomic tool.

They first created a COI 'profile' for the seven most diverse animal phyla, by the study of representative species, they placed 96% of newly studied taxa to their proper phylum, and assigned 150 newly analyzed individuals to their proper species status, which was a 100% successful process. Around 10-25 families were examined including family Vespidae for each of the four most diverse orders (Coleoptera, Diptera, Hymenoptera and Lepidoptera).

2.5.1. Mini barcodes

While PCR amplification and sequencing of a 658bp fragment is consistent in freshly collected specimen, it is difficult to get a complete barcode in older museum specimens and other preserved specimens. A similar issue may avert effective DNA-based confirmation and testing in processed biological materials, like food products and nutraceuticals.

Here, smaller DNA sequences (100-200bp) or mini-barcodes is found to be effective in majority cases for species level identification. Furthermore, small DNA fragments can be utilized via high-throughput sequencing platforms providing an inexpensive and comprehensive means of large-scale species identification (Meusnier *et al*., 2008).

2.5.2. DNA barcoding in Vespidae

DNA barcoding provided useful informations to various researchers in identifying the predator - prey relationship of many organisms. Kasper *et al*. (2004) identified the protein foods of two wasp species viz. *Polistes humilis* and *Vespula germanica*.

Their method was particularly important since more number of prey items is chewed by these wasps to a level where it cannot be identified using usual

morphological methods. They used 16S rDNA gene for this purpose and also assessed the prey overlap of these wasps by using these DNA sequences.

The presence of a reproductive symbiont *Wolbachia* in social hymenoptera, from Italy and Northeastern U.S. was observed by Stahlhut *et al.* (2006). The results of their study using mtDNA analysis showed that those individuals which were infected from New York, Massachusetts, and Italy have the same strain of *Wolbachia* and also some mtDNA haplotypes comprises of both infected and uninfected individuals. They also described the possible implications of *Wolbachia* infection in this invasive social Hymenoptera.

A study on *Elasmus schmitti* and *Baryscapus elasmi*, which was recorded in southern Ukraine as expansive parasitoids of the paper wasps *Polistes dominulus* and *Polistes nimphus* nests were conducted by Gumovsky *et al.* (2007). DNA sequences of three genes (nuclear 28S D2 rDNA, mitochondrial CO1, and mitochondrial cytochrome b) were obtained for both parasitoid species.

The comparison of the 28Sf D2 sequences of *Elasmus schmitti* and *E. polistis* relative to other available *Elasmus* sequences preposed a single origin of parasitism on paper wasps in this genus.

Study of the insectivory of five carnivores in cool temperate deciduous forests was studied by Shinsuke *et al.* (2012). They assayed the dietary contents using DNA barcoding data and assigned each of them to a microhabitat according to the habitat

preference of insects consumed by them. Vespidae sequence was observed in the stomach and fecal content of martens and bears, which showed they use forest in three dimensions for feeding.

The DNA barcode data was analyzed by Wilson (2010) to study the mechanism underlying the displacement of two genera of native solitary Hymenoptera by a social continental invader *Vespula pensylvanica*. The Vespidae wasp *Provespa nocturna* was suggested as pollinator of epiphytic orchid *Coelogyne fimbriata* on the basis of its DNA sequence data by Nakase and Makoto (2013).

The identification of molecular operational taxonomic units (MOTUS) was made by Stahlhut *et al.* (2013) using DNA barcoding data for many Hymenoptera specimens collected from 2004 – 2010. MOTU is defined as 'a group of sequences that differ from one another by a maximum number of base pairs,' e.g., two or three nucleotides in a 500bp region of 18S rRNA (Blaxter *et al*., 2005).

Of the total resolved MOTUS for this collection, 75% were Ichneumonoids (Ichneumonidae + Braconidae) and 91% were parasitoids. Study implied the high diversity of potential host species throughout the range and information about the interspecific interactions in the area.

A new species of paper wasp genus *Polistes* in Europe was identified by Neumteyer *et al.* (2014), by analyzing morphology, along with nuclear and

mitochondrial DNA. Their analyses unambiguously revealed the presence of a cryptic species in Europe.

DNA sequence data of insects were also used in forensic entomology. In a study conducted by Turcinaviciene *et al.* (2014), species identification and genetic differentiation of European cavity nesting wasps was inferred from DNA barcode data, and within-species and between-species genetic distances were estimated to evaluate the differences of intraspecific and interspecific genetic diversity. Prey identification in the nests of potter wasp *Hypodynerus andeus* was determined using the DNA barcode data by Varga *et al.* (2014).

Cryptic diversity and host specificity in giant *Xenos,* a genus of the order Strepsiptera, which are parasitic to *Vespa* genus species was analyzed by Nakase and Makoto (2013). Molecular phylogenetic analyses based on CO1 (652bp) to investigate the cryptic diversity among 21 individuals of strepsipterans were performed.

The analyses, accompanied by morphological examination, revealed that these strepsipterans represent two distinct species, *X. Moutoni* du Buysson, 1903 and *X. Oxyodontes* sp. nov. which differed in their host-utilization pattern.

Geographic variation and melanisation pattern in a hornet species *Vespa velutina* was studied by Perrad *et al.* (2014) using molecular sequence data. It showed that the variation of coloration between populations was not related to their

geographic or climatic differences. Their observations suggested that the coloration patterns of hornets and their geographic variations are determined by genes with an influence of developmental constraints. Results also highlighted that *Vespa velutina* populations underwent several convergent evolutions of the coloration, possibly influenced by the inhibitory effect on aposematism and müllerian mimicry than by abiotic pressures on melanism.

DNA barcoding does not aim at determining classification but to identify an unknown sample in terms of a known classification. Most eukaryotic cells contain mitochondria and mtDNA has fast mutation rates, resulting in significant variation in mtDNA sequences between species and comparatively small variance within species as all mtDNA genes are maternally inherited, any occurrence of male-killing microorganisms, cytoplasmic incompatibility-including symbioses, hybridization and horizontal gene transfer, can lead to misleading results.

Barcoding is no replacement for comprehensive taxonomic analysis. For example, when an unknown specimen do not show any match to existing barcode library sequence, it cannot be designated as a new species.

Instead, such specimens are flagged for thorough taxonomic analysis. Viewing in the context of the traditional taxonomic framework, which is time consuming compared to DNA barcoding, this flagging of atypical specimens has much potential to assist species discovery (Hajibabaei *et al.*, 2006).

2.6. Molecular systematics

Systematics plays a fundamental role in describing, naming, classifying and determining relationships among the earth's biota (Prance, 1995). Molecular systematics aims to provide an overview of molecular methods currently used to analyze diversity within and among species.

Methodological advances such as cladistics and new sources of data such as nucleotide sequences (Wolfe and Liston, 1998) have greatly increased the vigour, credibility, and appeal of systematics over recent years (Davis *et al*., 2010). The resulting heightened interest in systematics has been predominantly associated with reconstructing phylogenetic relationships.

In molecular systematics, using conserved molecular sequences, it is possible to define and identify most species. Molecular biology has revolutionized the field of systematics.

Advances in molecular biology have had a profound impact on the field of insect taxonomy. Molecular systematics is the detection, description and explanation of molecular biological diversity, both within and among species. In molecular systematics, DNA barcoding can be used for routine identification of specimens. The comparison of DNA sequences among species represents a basis of phylogenetics, allowing biologists to explain the evolutionary relationships among species.

There is a fundamental synergy between studies of molecular systematics and molecular evolution. Molecular systematics uses genetic markers to make inferences about phylogeny and population processes which create a substantial comparative database for specific genes or proteins. For analyzing the molecular evolution, these datas are used to evaluate rates, processes, and constraints on molecular change through time (Gillespie, 1991).

In Molecular systematics, estimated molecular phylogenies can be used to detect intragenic recombination, exon shuffling, horizontal transfer (Robertson *et al.*, 1995), heterogeneous rates of evolution (Easteal, 1990) and to identify sequences subject to selection (Klein, 1993). These illustrate the growing interplay between molecular evolution and systematics in the analysis of DNA sequences.

2.6.1. Methods and gene candidates

The methods of molecular systematics has several steps including taxon sampling strategies, choice of appropriate markers, and analytical issues. Sequencing is generally the most appropriate method for studies at interspecific level and higher. DNA sequencing has become the dominant technique for generating comparative molecular data compared to RFLP, RAPD and SSCP.

Mitochondrial DNA is by far the most widely used DNA regions for insects as well as for animals in general for molecular systematics. The popularity of mtDNA markers accounts for its relative ease of isolation and amplification, even from poorly

preserved specimens. It is also amenable to straightforward analysis, individual typically being homoplasmic and part of a strictly dichotomous lineage of haplotypes. One of the main developments in the works on mtDNA is the documentation of paralogous nuclear copies of mitochondrial genes of pseudogenes. These may seriously confuse phylogenetic analysis (Hazkani–Covo *et al.,* 2010).

Nuclear rDNA genes are also widely used due to their extreme abundance in genome. The eukaryotic nuclear rDNA is tandemly organized with high copy numbers. Due to its different rates of evolution in different regions, the nucleotide sequences of nuclear rDNA have been used to infer broad spectrum phylogenetic relationships (Hwang and Won, 1999).

2.6.2. Molecular systematic studies in Vespidae

A detailed study in Vespidae molecular phylogeny was conducted by Schmitz (1998). The evolution of eusociality in them was analyzed in the study. The insect sequences were obtained from nuclear 28S rDNA and the mitochondrial 16S rDNA of two *Apis* species, nine social and three solitary wasp species of the family Vespidae. The study using parsimony, distance, and maximum-likelihood methods of both mitochondrial and nuclear DNA did not support the conventional phylogenetic position of Stenogastrinae.

In all phylogenetic reconstructions, the solitary Eumeninae were found to be a sister taxon to the cluster of Polistinae-Vespinae. The results of analysis put forward that sociality has independently evolved twice in the Vespidae.

Three new species in eastern Nearctic *Polistes* genus was revealed in a study by Buck *et al.* (2008) by the analysis of male genitalia, morphometric head measurements and DNA barcoding data. A case of misidentification of *Vespula alascensis* as *V. vulgaris* in North America was reported by Carpenter and Travis (2010).

A new species of *Vespula* and the first record of *Vespa crabro* from Guatemala, Central America was reported by Landolt *et al.* (2010) using the mtDNA sequences and morphological data. A revision of the nocturnal social wasp genus *Apoica* was done by Pickett and Carpenter (2010), based on a cladistic analysis of various characters of the nine species.

The paper wasp *Polistes formosanus* was found closely related to *P. japonicus* in a study by Saito *et al.* (2007). Morphological character analysis of specimens from Taiwan, the Nansei Islands and main islands of Japan showed that *P. formosanus* is a good species different from *P. japonicus*. Analysis using molecular phylogeny also supported these results. They could also explore the historical biogeography of the Nansei Island from their study.

2.7. Molecular phylogenetics

Monophyly of different organisms is calculated from the similarity in the biochemical and molecular mechanism. Molecular phylogenetics uses the structure and function of molecules with their pattern of divergence in time to infer these evolutionary relationships.

This branch of study emerged in the early 20th century but did not begin earnestly until the 1960s, with the advent of protein sequencing, DNA sequencing, PCR, and other similar techniques in molecular biology.

The foremost intention of molecular phylogenetic studies is to assess the order of evolutionary events and present them in evolutionary trees that graphically portray species interrelationships over time. This is a highly complicated task, further intricate for the fact that there is lack of an exact single method applicable for all phylogenetic issues.

But, for the past 30 years, as advanced computers became more accessible and computer algorithms became more sophisticated, researchers could now deal with the highly complicated stochastic and probabilistic issues that define evolution at the molecular level more efficiently (Hall, 2013).

But in recent past, this field has been further rejuvenated and redefined as whole genome sequencing became less expensive and handy for every organism. As

more and more of genomic data is becoming publically available and accessible, molecular phylogenetics continues to grow and find newer applications (Shokralla *et al*., 2014).

To build phylogenetic trees, statistical methods are used for tree topology and branch length determination. Many different methods for building trees exist and no single method performs well for all types of trees and datasets. The most common computational methods applied include distance-matrix methods, and discrete data methods, such as maximum parsimony and maximum likelihood (Hall, 2013).

There are several software packages, such as Paup **(**Phylogenetic Analysis Using Parsimony**)**, PAML (Phylogenetic Analysis by Maximum Likelihood) and PHYLIP (PHYLogeny Inference Package) that apply most popular methods (Tamura *et al*., 2013). Paup uses variety of techniques for phylogenetic inference, including maximum likelihood analysis for DNA data using variety models. It is available commercially. Paup also contains a set of exact and heuristic methods for searching optimal trees.

PAML is open-access programme for phylogenetic studies and evolutionary model comparison (Yang, 2007). PHYLIP is another large suite of open-access programs that estimates trees using numerous methods, including pair wise distance, maximum parsimony, and maximum likelihood.

The maximum likelihood softwares handle a few simple stochastic models and have good tree searching capabilities. PHYLIP is commonly considered good educational software for beginner phylogeneticists (Felsenstein, 1991).

2.7.1. The age of molecular phylogenetics

Over a long period, comparatively few authors attempted to resolve the Hymenopteran phylogeny (Rasnitsyn, 2002; Dowton and Austin, 1995, 2001; Carpenter and Wheeler, 1999; Heraty *et al.,* 2011; Pickett *et al.,* 2006, 2010).

In recent years, due to the interest of scientific community, there have been a notable increase in the publication of an extensive analysis based solely on characters like morphology, with the use of complete mitochondrial genomes (Dowton *et al.,* 2009; Heraty *et al.,* 2011; Lopez-Osorio *et al.,* 2014), a super tree approach by previously published trees (Davis *et al.,* 2010), a phylogenetic study using EST data and a taxon-rich four-gene study (Heraty *et al.,* 2011; Sharkey *et al.,* 2012).

In recent years, complete nuclear genomes of many hymenopterans have been sequenced. Most prominent in this context are *Nasonia vitripennis*, with its sibling species *N. Giraulti* and *N. Longicornis* (Werren *et al.,* 2010) and honey bee *Apis mellifera* (Weinstock *et al.,* 2006). These genomes made significant contribution to the quantity of data available for hymenopteran sequences. However, their limited number proves a barrier in augmenting phylogenetic analyses.

Protein-coding genes of mitochondria had a major role in phylogeny reconstruction, either singly or with other genes. The nuclear ribosomal 28S, and the mitochondrial cytochrome oxidase subunit 1 (COI) genes had a major role in phylogenetic studies of the order Hymenoptera (Quicke *et al.*, 2009).

They do possess their own limitations. The former seems hard to align due to the length variability, and for ambiguously aligned regions, its application is still under debate (Laurenne *et al.*, 2006).

COI evolves far more rapidly at its 3rd codon position, though the second and third positions do not vary easily. There has been much dispute over its efficacy for recovering phylogenies. The rate of its non-synonymous substitution is considerably slower compared to synonymous substitutions. 16s gene fragment is also used in phylogeny study, but for 16srRNA gene fragment, there is absence of an accepted cutoff value of sequence similarity for species definition (Palys *et al.*, 1997).

It is an urgent necessity to provide a wealth of phylogenetic and higher-level taxonomic information for analysis of species diversity and interrelationships. The supplementation of gene sequence data with morphological parameters has revolutionized the study of phylogeny in recent decades (Pagel, 1999).

The number of large-scale projects to determine branches of the Tree of Life is progressing. These involves selection of the target group and representative taxa, the attainment of sequence information, and phylogenetic trees construction by using

optimality criteria like Maximum Likelihood. But care must be taken in selection of loci and representing taxa for recovery of an accurate phylogenetic tree.

Earlier, gene selection was highly limited for universal targets and easy-to-sequence genes. However, recent advances have led to the sequencing of multiple loci, more often from various genomic compartments (nucleus, mitochondrion and chloroplast) to enhance resolution at different taxonomic levels (Hajibabaei *et al.*, 2006) and to avoid gene-specific biases.

With the progression of Whole-Genome Sequencing projects, some researchers began using entire genome for phylogenetic studies (Murphy *et al.*, 2004). DNA barcodes cannot provide adequate phylogenetic signal to determine evolutionary relationships, particularly at deeper levels (Hajibabaei *et al.*, 2006). It can support the construction of phylogenies by aiding the selection of taxa.

2.7.2. Molecular phylogenetic studies in Vespidae

2.7.2.1. Mitochondrial genes

RFLP pattern in Vespinae from Europe was studied by Schimitz and Moritz (1990) and they found the patterns more similar within genera than between them, distance trees were constructed which supported the hypothesis of monophyly of the genera *Vespula* and *Dolichovespula* by Carpenter (1987).

The importance and utility of using genetic distances by phenogram construction was explained by Carpenter (1990). He argued that the toplogical distances calculated by his data may be accurate even though evolutionary divergence study may not be interpretable. Davis *et al*. (1990) studied population structure and kinship in *Polistes* species by using six variable protein loci and one variable ribosomal DNA restriction site.

The genetic relationships of nine species of Polistine wasps of Japan were studied by Nozawa and Ito (1989). They were analysed by starch-gel electrophoresis of the enzymes.

Using Cladistic data from allozyme polymorphism of paper wasp social parasites and their hosts, Carpenter (1993) explained that the social parasites aren't most strongly associated to their hosts.

Evolution of parasitism in wasps was studied by Dowton (1995). It was explained that a elevated AT content and increased rate of mtDNA sequence divergence were found in parasitic wasps in comparison to non parasitic wasps, the increased rate of mtDNA sequence evolution probably arose during the early Jurassic, coincident with appearance of parasitic wasps fossils in the early records. Their results suggested a connecting link between the rate of sequence divergence and the parasitic lifestyle.

A work on Hymenoptera which was the first complete molecular study covering the complete order Hymenoptera was published by Carpenter *et al*. (2011). It is performed on about 7 kb DNA sequence from 4 different gene regions (18S, 28S, COI and EF-1α) for 116 species. It represented all super families and 23 outgroup taxa from 8 orders of Holometabola.

Their results agreed with the earlier hypotheses, that a single clade of parasitic Hymenoptera, the Vespinae arose from a basal grade of phytophagous families, from which the eusocial pollen-feeding, predatory and gall-forming forms evolved (Heraty *et al.,* 2011).

Considering other gene sequences together with mitochondrial COI sequence, and some morphological characters, Arevalo *et al.* (2004) constructed a phylogeny tree for Polistinae, by using 69 species. The results supported the new world subgenera; Polistini, as monophyletic, while the Old world subgenera being a paraphyletic group.

Phylogeny of aculeates Chrysidoidea and Vespoidea was studied by Brothers and Finnamore (1993).

The outcome of their comprehensive analyses of aculeate higher taxa was re-examined in the light of new results by other subsequent workers. This earlier work was reviewed by Brothers (1999) along with another superfamily Apoidea. A cladistic analysis depending on mt DNA and morphological parameters of 40 *Polistes* species

was presented by Pickett *et al*. (2006). The results suggested that some morphological signal comes out only by the analysis of DNA and morphological data together.

The close relationship of phytophagous Siricidae (horntails and wood wasp family) to the predominantly parasitic Apocrita than to the ectoparasitic Orussoidea (saw fly superfamily) was suggested in a study by Dowton *et al.* (1997). This explained lesser chance of single origin of parasitic lifestyle of wasps, unless the Siricidae have reverted to phytophagy recently.

Instead, parasitism evolved twice independently, once in the Orussoidea and then in the Apocrita. A concurrent analysis of morphology, 16S, 28S and COI genes in Apocrita was performed by Dowton and Austin (2001). Parasitic wasps showing transitions in evolutionary processes were analyzed in their study.

Molecular level data from 4 nuclear genes (long-wavelength rhodopsin, wingless and the D2–D3 regions of 28S ribosomal RNA and elongation factor-1α F2 copy) was collected by Pilgrim *et al.* (2008), for generating the foremost molecular level phylogenetic data of Vespoidea. They explained from their results the paraphyly of Vespoidea consequential of the nesting of Apoidea in a lineage containing Scoliidae, Formicidae, and 2 Bradynobaenidae subfamilies.

The study also revealed the paraphyly among 3 families viz. Bradynobaenidae, Mutillidae and Tiphiidae and a sister relationship among Rhopalosomatidae and Vespidae. They also identified Rhopalosomatidae + Vespidae

as a sister group for all other vespoids/apoids. A discussion of character evidence basing on the new phylogenetic data was made and they put forth a new classification of Aculeata which identifies eight superfamilies viz. Thynnoidea, Apoidea, Pompiloidea, Chrysidoidea, Formicoidea, Scolioidea, Tiphioidea, and Vespoidea.

The capacity of a series of phylogenetic approaches to pick up seven uncontroversial relationships, when lineages showed strikingly diverse rates of molecular evolution was also assessed by them.

Their analyses indicated that partitioned, Bayesian analysis of nucleotide data, excluding 3rd codon positions, recovered more of the uncontroversial relationships than any other approach. The results also proved that the analysis of complete mitochondrial genome sequences holds promise for the resolution of Hymenopteran superfamily relationships, even when lineages show markedly different rates of molecular evolution.

A phylogenetic study of *Epipona laterile*, a Neotropical social wasp species was performed by Andena *et al.* (2009). Their result indicated a single cladogram, and the species of *Epipona* genus were supported as a monophyletic group in the results. The phylogenomics which resolved evolutionary associations among bees, wasps and ants was explained by Johnson *et al.* (2013).

An important finding of their study says that the ants are more closely related to ectoparasitoid wasps, were ants and Apoidea were known earlier as a sister groups.

Vespid wasps were sister to the rest of aculeates except chrysidoids. So, all the species of eusocial Hymenoptera is being limited within 2 major groups, characterized by transfering of larval provisions and construction of nest, likely prerequisites for the evolution of eusociality. This phylogeny study provided newer horizons for exploring the evolution of social behavior, feeding, and nesting within the order Hymenoptera.

The phylogenetic data from hornets, considering 45 morphological characters together with data from 4 mitochondrial and 2 nuclear genes were studied by Perrad *et al.* (2013). The results supported many of the previously found species relations. The monophyly of *Vespa* and the reality of a main clade apart from *V. basalis* and *V. binghami* were established.

The molecular level information connected the previously unsolved *V. orientalis* to *V. affinis* + *V. mocsaryana*. A novel species *Euparagia unidendata* from the genus *Euparagia* was described by Carpenter and Lynn (2009) and the taxonomic status was confirmed by examining the phylogeny relations with other species of this genus.

2.7.2.2. Ribosomal genes

Ribosomal DNA is a classical tool in molecular phylogenetic reconstruction. The rDNA is ubiquitous and includes a mosaic of areas with different rate of evolution giving regions of rDNA for nearly all systematics related queries. The D2 area of nuclear 28S rDNA and the mitochondrial 16S rDNA (Cameron, 1993) has

verified to be exceptional tools using which it is possible to reconstruct phylogeny in wasps and bees.

Taxonomic levels where 16S rRNA is phylogenetically revealing were identified by Derr *et al.* (1992). The study was based on nucleotide sequence changes in a 573bp region of the mitochondrial 16S rRNA sequence taken as a representative for Hymenopteran taxa.

A phylogenetic study on the 23 species among the genus *Vespa,* was performed by Archer (1993) using the Polistinae and the rest ofspecies of the Vespinae taken as outgroups. Two species, *V. basalis* and *V. binghami*, were changed in his results, whereas the remaining 21 species were observed as a separate group. Here, some species groups were observed as having uncertain relationships like *V. crabro, V. affinis, V. tropica, and V. orientalis*.

Schmitz and Moritz (1994) sequenced the two variable domains near ribosomal RNA gene of *Vespa crabro*. The sequence was aligned to subsequent rDNA regions of *Melittobia digitata, Nasonia vitripennis,* and *Drosophila melanogaster*.

They studied the nucleotide composition together with the sequence match for different regions of the investigated sequences and presented the inferred secondary structure of *Vespa crabro*. Study demonstrated the feasbility of the selected 28S rDNA sequence for phylogenetic studies after applying the secondary structure in

sequence alignment of more distantly related organisms. They proposed 28S rRNA as a trustworthy gene fragment for further unraveling the phylogeny of wasps and that it may provide data for complete understanding of wasp evolution.

Vespidae phylogeny was reconstructed on the foundation of rDNA sequences of both the nuclear and mitochondrial genomes in a molecular systematic study by Schmitz (1998). Their analyzed sequences provided strong evidence that in Vespidae sociality has independently evolved twice and showed Vespidae being paraphyletic for Apidae.

This study was challenged by Brothers (1999) saying that their conclusions should be analysed with care since they did not include representatives of Euparagiinae or Masarinae. Furthermore, their results placed 2 species of Apoidea family inside Vespidae.

A concurrent study of these molecular data together with morphological charecters was made by Carpenter and Wheeler (1999). The consequential cladograms maintained the assumption off monophyly of Vespidae, as well as monophyly of social wasps, with the primitively social Stenogastrinae being more closely related to the highly social Polistinae+Vespinae than the solitary Eumeninae. A realignment of the earlier sequences was also made which supported his present result.

2.7.2.3. Complete mitochondrial gene sequence

The entire mitochondrial genome of *Abispa ephippium* (sub family Eumeninae) and most of the mitochondrial genome of *Polistes humilis* (sub family Polistinae) was sequenced by Cameron and Michael (2008). The arrangement of genes differed between the two genomes and also differed slightly from that inferred to be ancestral for the Hymenoptera. The mitochondrial genome organization for both vespids was found indifferent to all others previously reported.

A number of tRNA gene rearrangements were identified that represent potential synapomorphies for a subset of the Vespidae. Analysis of all available Hymenopteran mitochondrial genome sequences recovered an uncontroversial phylogeny, one consistent with analyses of other types of data.

The entire sequence of two new Hymenopteran mitochondrial genomes and a considerable portion of another 3 Hymenopterans were presented by Dowton *et al.* (2009). They analyzed them along with 9 other gene sequences and stated that the genetic divergence rate is two to three times more in some species of Hymenoptera, presenting this as a group having phylogenetic branches, both long and short.

Reconstruction of phylogenetic relationships of all the organisms using molecular data is one of the major aims of systematic biology today. The progression of sequencing technologies took phylogeny analysis to a higher level.

Phylogenetic studies have pervaded almost every branch of biology, and the growth of advanced methods and software packages which are becoming easily accessible to an experimental biologist, will make the future of molecular phylogenetics brighter (Yang and Bruce 2012).

The precise estimation of phylogeny proves to be the biggest challenge for systematists. Maximum likelihood (ML) is found to be an unfailing and proficient way to calculate phylogenies in a variety of restricted conditions where other methods fail to work (Felsenstein, 1978, 1981; Huelsenbeck, 1995).

ML is considered to be much defiant to changes in models and model parameters. Still, robust ML analyses for more amounts of data are computationally imperfect, while MP and the distance-based methods are less affected by large numbers of taxa (Hillis, 1996).

Recent advancement in Next Generation Sequencing helped researchers to think in terms of whole genome sequencing instead of single gene sequencing, which may leed to the replacement of molecular phylogenetics with molecular phylogenomics in the far future (Morisson, 2014).

MATERIALS AND METHODS

1. Insect collection

The specimens were collected alive from indoors and outdoors. Collection was done using a small insect net and transferred to a killing jar or tube with ethyl acetate. Later, the specimens were stored at -20 ^{0}C in a deep freezer.

1.1. Study area

Specimens were collected from various places of Kerala state which is the extreme South-Western state in India, possessing an area of about 38,863 km^2 and make up 1.18% of the total area of the country. The Kerala state lies in between the Lakshadweep Sea and the Western Ghats, between north latitudes 8°18' and 12°48' and east longitudes 74°52' and 77°22'.

1.2. Climate

Kerala has a wet and maritime tropical climate affected by the seasonal heavy rains of the south-west summer monsoon and north-east winter monsoon. The average monthly rainfall in Kerala ranges from 14mm in January - 687.2mm in July. The annual mean temperature is usually ranges from 28° to 32° C (82° to 90° F) on the plains but lowers to about 20° C (68° F) in the highlands.

2. Preparation of genomic DNA

Total genomic DNA was extracted from the thoracic muscles of experimental insects. The tissue was homogenized using a microhomogenizer. The genomic DNA

in the homogenate was extracted using Sigma Aldrich DNA extraction kit in accordance to the manufacturer's instructions. The quality of the DNA was verified by agarose gel electrophoresis. The total DNA was loaded onto 1% agarose gel and was run at a constant voltage of 100v for 30 minutes at a current of 45 mA. The gel was stained with EtBr, visualized under a UV transilluminator and photographed using a gel documentation system.

3. Polymerase Chain Reaction

About 2 nanogram of genomic DNA was used for amplifying the partial sequence of COI using the forward and reverse primers given in the table 1. PCR was carried out in a final reaction volume of 25 µl. Composition of reaction mixture for PCR is given in table 2 and the PCR protocol is given in table 3.

No	Species name	Primer name with sequence
1	*Vespa tropica*	FOM-F: 5'-GGTCAACAAATCATAAAGATATTGG-3' FOM-R: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al*., 2004)
2	*Rhynchium brunneum*	
3	*Delta pyriforme*	
4	*Delta conoideum*	
5	*Phimenes flavopictum*	
6	*Polistes strigosus*	C1-J-1751-F: 5'-GGATCACCTGATATAGCATTCCC-3' C1-N-2191-R: 5'-CCCGGTAAAATTAAAATATAAACTTC-3' (Jiggins, 2003)
7	*Ropalidia spatulata*	
8	*Ropalidia jacobsoni*	

Table 1. List of primers used in PCR amplification of partial coding sequence of COI of different wasps.

Components	Volume per reaction
Deionized water	16.8 µl
Taq buffer with MgCl2 (10 X)	2.5 µl
Forward Primer (2.5µM)	1 µl
Reverse Primer (2.5µM)	1 µl
dNTPs mix(10 mM each)	2.5 µl
Taq DNA Polymerase (5U/µl)	0.2 µl
Template DNA (2ng/µl)	1.0 µl
Final volume	25.0 µl

Table 2. Reaction mix for PCR.

Step	Process	Temperature	Time
1	Initial denaturation	95°C	3 minute
2	Denaturation	95°C	30 seconds
3	Annealing	50°C	45 seconds
4	Extension	72°C	1 minute
	Go to step 2 for 35 times		
5	Final elongation	72°C	3 minutes
6	End at 4°C		

Table 3. PCR cycles for CO1 gene amplification.

4. Agarose gel electrophoresis of PCR product

100bp DNA ladder was used as standard molecular weight DNA. 5µl of PCR product was loaded onto a 2% agarose gel stained with EtBr. Electrophoresis was done at constant voltage of 100V for a period 1 hr. The stained gel was visualized on a UV transilluminator and photographed using a gel documentation system.

5. Purification of PCR product

After ascertaining the PCR amplification of the corresponding COI fragment, the remaining portion of the PCR product was column purified using Mo Bio Ultraclean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California) as per the manufacturer's instructions. The purified PCR product was sequenced from both ends using the forward and reverse primers using Sanger's sequencing method at SciGenom Labs Pvt. Ltd., Cochin. The forward and reverse sequences obtained were

trimmed for the primer sequences, assembled by using ClustalW and the consensus was taken for the analysis.

6. Conceptual translation and phylogenetic analysis

The conceptual translation of the DNA sequences obtained was done using EMBL nucleotide sequence translation tool EMBOSS (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The nucleotide sequence and peptide sequence were searched for its similarity using BLAST programme of NCBI (www.ncbi.nlm.nih.gov/). The phylogenetic tree was plotted using Neighbor-Joining (N-J) method in traditional straight format by comparing with other sequences in the database.

RESULTS AND DISCUSSION

1. *Vespa tropica*

Vespa tropica (**Figures 1-4**) is commonly called as greater banded hornet. Their stings are more painful to humans than typical wasp stings because hornet venom contains a large amount of acetylcholine. The nest of *Vespa tropica* is usually seen underground, tree hollow or similar enclosed spaces, which is made of chewed barks of trees.

1.1. History of nomenclature

Spex tropica. Linnaeus, 1758. *Systema Naturae,* 10th ed., 1: 571.

Vespa cineta (Linnaeus). Fabricius, 1775. *Systema Entomologiae,* p.362 .

Vespa tenebrionis (Linnaeus). Christ, 1791. *Naturg. d. Insect,* p.216.

Vespa unifasciata (Linnaeus). Oliver, 1791. *Encycl. Method. Insect,* 6: 677.

Vespa tropica (Linnaeus). Schulz, 1912. *Berlin. Ent. Zeitschr,* 57: 57.

Vespa tropica tropica (Linnaeus). Bequaert, 1936. *Treubia,* 15: 328.

Vespa tropica tropica (Linnaeus). Van der Vecht, 1957. *Zool. Verh,* 34: 5.

Vespa (V.) tropica tropica (Linnaeus). Van der Vecht, 1959. *Medel,* 36: 226.

(Lambert, 2002)

Fig.1. *Vespa tropica* Voucher CUKAK1 lateral view (scale in cm).

Fig.2. *Vespa tropica* Voucher CUKAK1 upper view (scale in cm).

Fig.3. *Vespa tropica* Voucher CUKAK1 front view.

Fig.4. *Vespa tropica* Voucher CUKAK1 wing pattern.

1.2. Diagnosis

The species collected was identical to those described by Lambert (2002). The body was black with reddish brown and yellow markings. Reddish brown marking as follows: head, antenna, pronotum dorsally, anteriorly mesoscutum with two reddish line, scutellum and legs partly reddish black. Yellow marking as follows: second tergite and sternite almost entirely, sometimes apex of first tergite narrowly yellow; wings yellowish brown and veins dark brown at base and yellowish at apex.

1.3. Systematic position

Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Hymenoptera; Apocrita; Aculeata; Vespoidea; Vespidae; Vespinae; *Vespa*; *tropica.*

1.4. Distribution

The greater banded hornet is a tropical species of hornet found in Southeast Asia, from Afghanistan to New Guinea. In India it is reported from West Bengal, Sikkim, Bihar, Himachal Pradesh, Hariyana, Karnataka, Tamilnadu and Kerala (Lambert, 2002)

1.5. Collection

Vespa tropica, used in the present study was collected from Thrissur District of Kerala at 10° 26' 16" North, 76° 12' 36" East.

1.6. PCR amplification of cytochrome c oxidase subunit I gene of *Vespa tropica* Voucher CUKAK1

The gel pictures showing the genomic DNA and PCR product of COI gene of *Vespa tropica* Voucher CUKAK1 are given in **Figures 5 and 6**. The forward and reverse sequence chromatograms, partial COI gene sequence, BLASTn result, conceptual translation product and BLASTp result are presented in **Figures 7-10, 12** and **13** respectively. The sequence obtained is deposited in GenBank (GenBank Accession Number: KM455116).

The COI gene sequence from *V. tropica* is 91% similar to that of *Vespa ducalis* (GenBank Accession Number: KF933084.1). The COI sequence of *V. tropica* can be used for accurate taxonomic identification of the species. The nucleotide BLAST against the nucleotide redundant database showed that the COI gene sequence obtained is novel.

The conceptual translation of COI of *V. tropica* yielded a peptide of 219 amino acids. The peptide blast of COI from *V. tropica* showed 91% similarity with *V. ducalis* (GenBank Accession Number: AHC97222.1). The results of the BLASTp

indicated that the peptide of the cytochrome oxidase subunit I (COI) gene of *V. tropica* collected from Thrissur is novel. The mtDNA sequence of different wasps confirms the species differences of *V. tropica* from others.

1.7. Molecular phylogeny of *Vespa tropica* Voucher CUKAK1

The phylogenetic trees of DNA and peptide sequences were plotted using Neighbour-Joining method and are exhibited in **Figures 11 and 14** respectively. The DNA and peptide phylogenetic trees showed that *V. tropica* is more closely related to *V. ducalis,* which arose from a common ancestor. Perrad *et al.* (2013) also reported the phylogenetic relationship of *V. tropica* with *V. ducalis* by analyzing different wasps under the genus *Vespa*. Their study was based on 45 morphological charecters and 6 genes excluding COI sequence of *Vespa tropica*.

V. tropica showed more close relation to the species of the genus *Vespa* like *V. ducalis, V. bicolor, V. simillima, V. vivax, V. analis, V. velutina, V. mandariana* and *V. soror* than to species of other genera of Vespinae subfamily like *Vespula* and *Dolichovespula.* According to the DNA phylogeny tree, the most distantly related species to *V. tropica* is *V. basalis*.

From a single ancestor, evolutionary divergence occurred into two directions viz. *V. basalis* and the common ancestor of *Dolichovespula+Vespula* cluster in one and a cluster of *Vespa* genus species in the other. *V. tropica* and *V. ducalis* have a

single origin, similarly *V. ducalis, V. bicolor, V. simillima, V. vivax, V. analis, V. velutina, V. mandariana* and *V. soror* arose from a common ancestor. Among the species of *Vespa* genus, *V. bicolor*, *V. simillima* and *V. vivax*; *V. analis* and *V. velutina*; *V. mandariana* and *V. soror* is more closely related to each other by a common ancestor.

The phylogeny tree based on conceptual peptide sequences also supported the close relationship of *V. tropica* to *V. ducalis* having a common ancestor. They show ed more closer relation to the species of the genus *Vespa* viz. *V.analis, V. orientalis, V. basalis, V. velutina, V. affinis, V. mandarinia japonica, V. mandarinia, V. soror, V. bicolor* and *V. vivax* which arose from a common ancestor.

The species of the genus *Vespula* and *Dolichovespula* are found more closely related to each other having a single origin. The wasps of the subfamily Eumeninae and Polistinae are distanly related to *V. tropica* of the subfamily Vespinae. According to conceptual peptide tree, the most distantly related species to *V. tropica* is found to be *Discoelius zonalis,* a wasp of the subfamily Eumeninae. The present results indicated the significance of an identification system based on COI gene sequence. The result highlighted the significant sequence diversity of the COI gene of *V. tropica* from its allied species, thus enabling an easy discrimination of species.

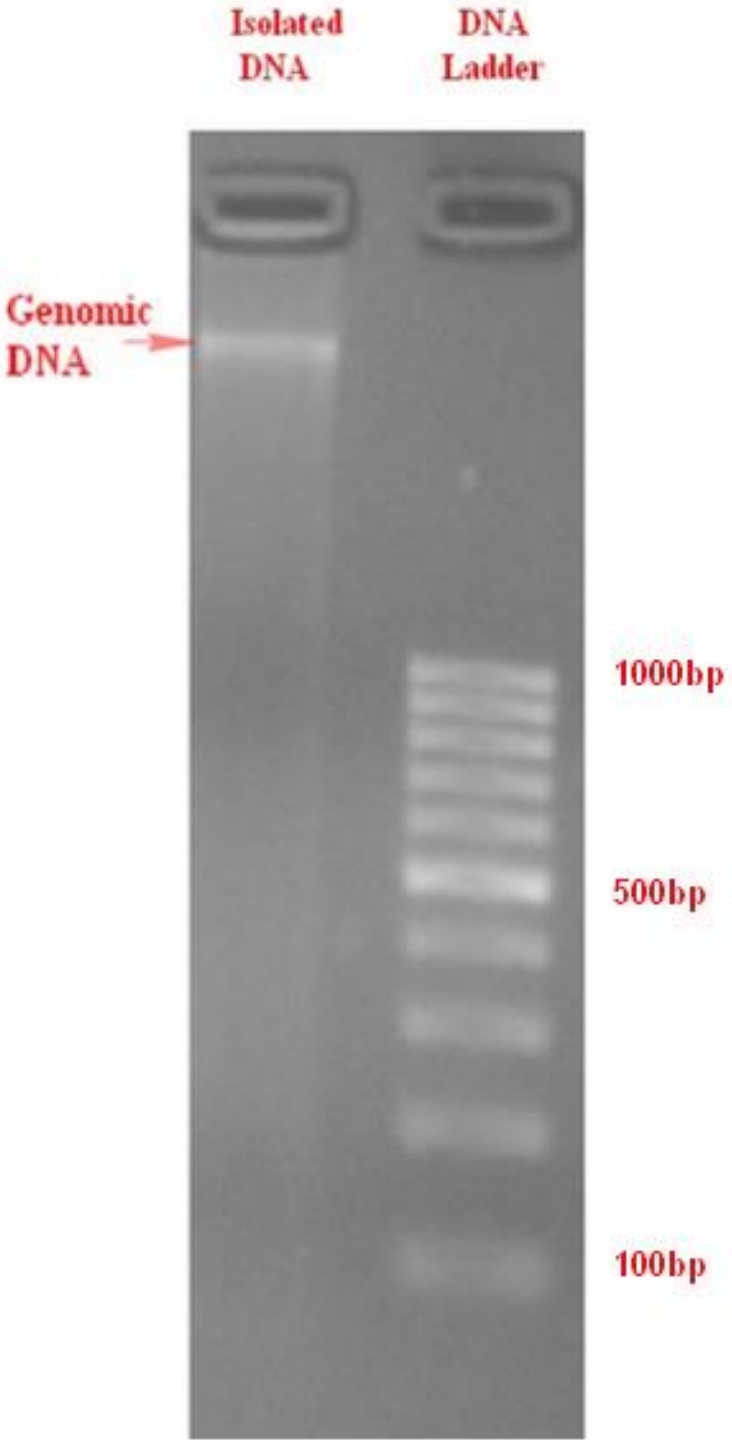

Fig.5. Gel picture of genomic DNA isolated from *Vespa tropica* Voucher CUKAK1.

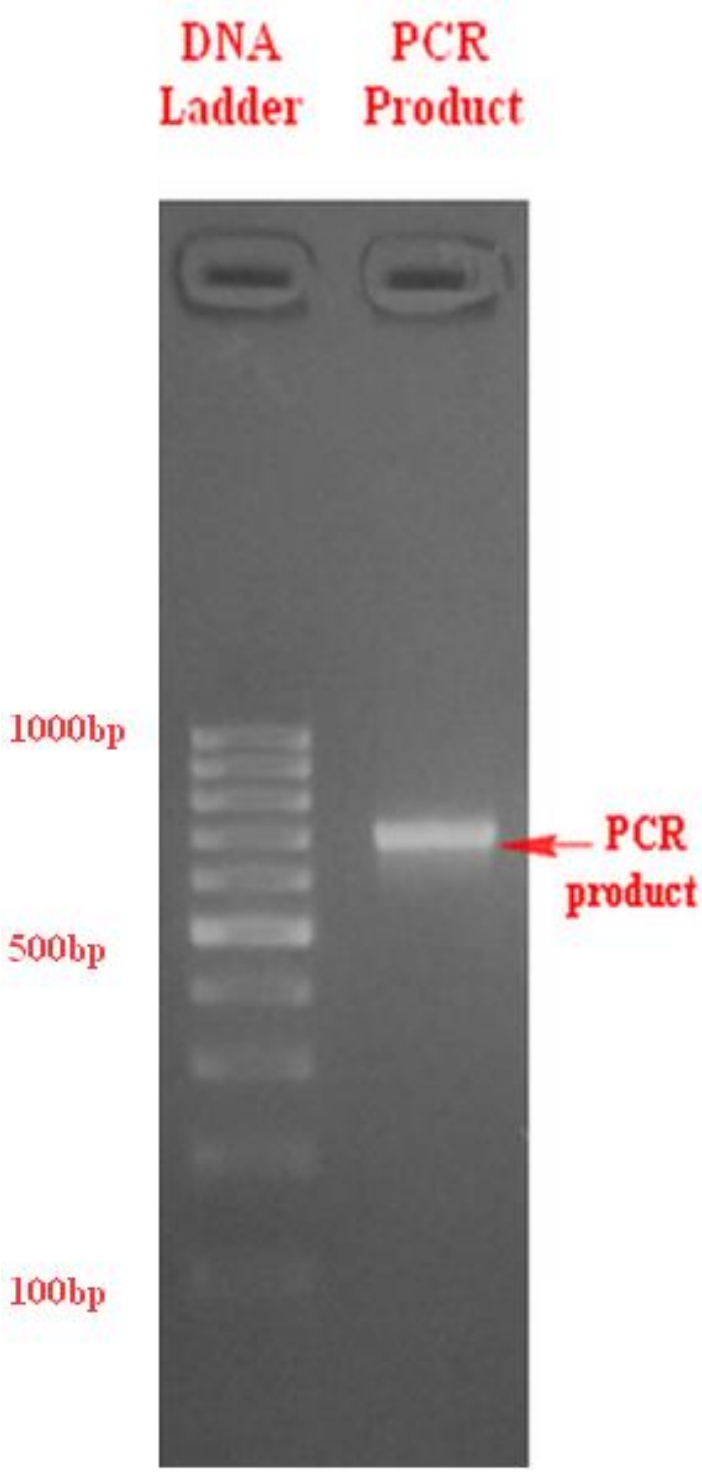

Fig.6. Gel picture showing the PCR product of partial COI gene of *Vespa tropica* Voucher CUKAK1.

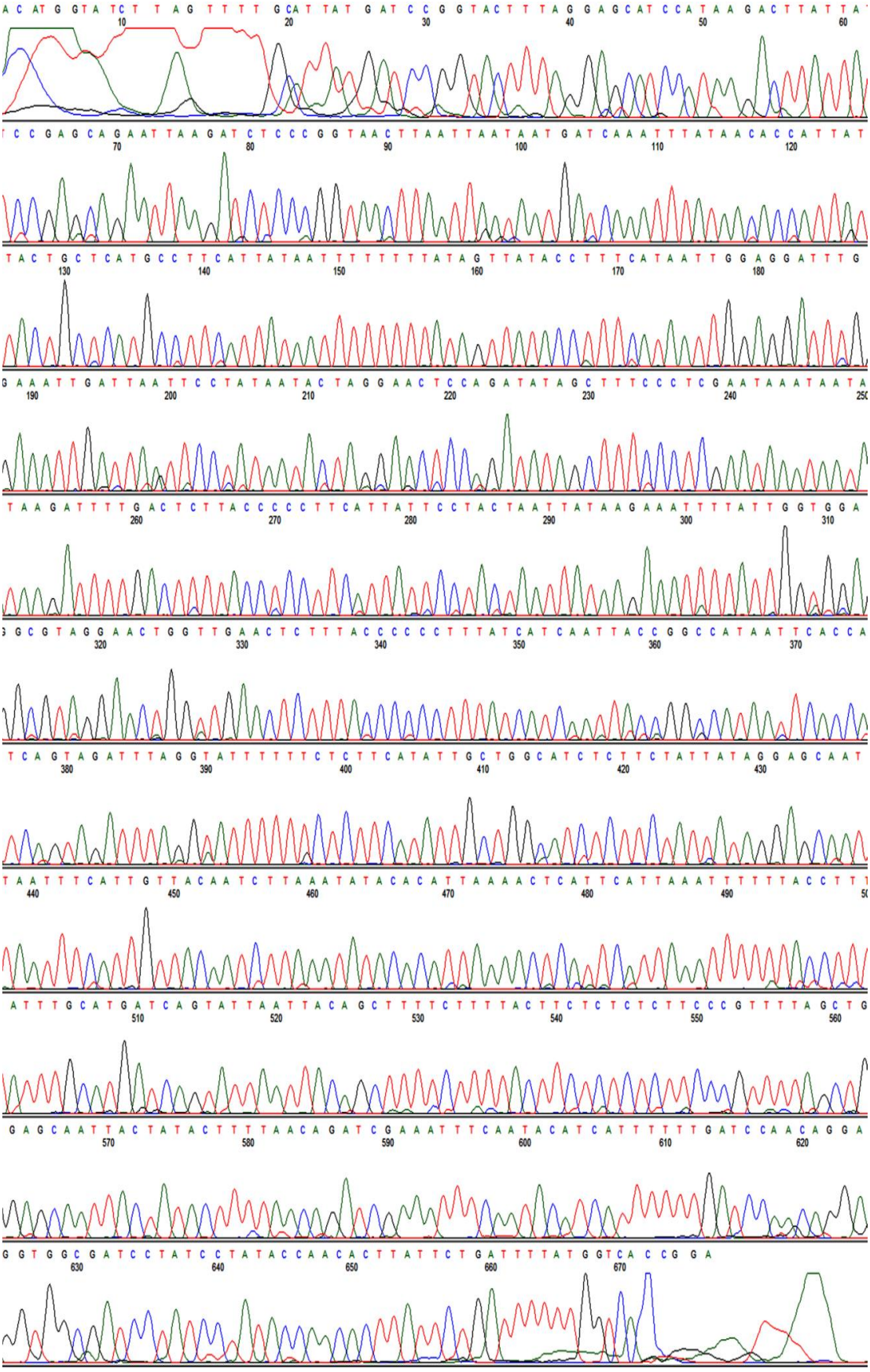

Fig.7. Sequencing chromatogram (forward sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *V. tropica* Voucher CUKAK1 (GenBank Accession Number: KM455116).

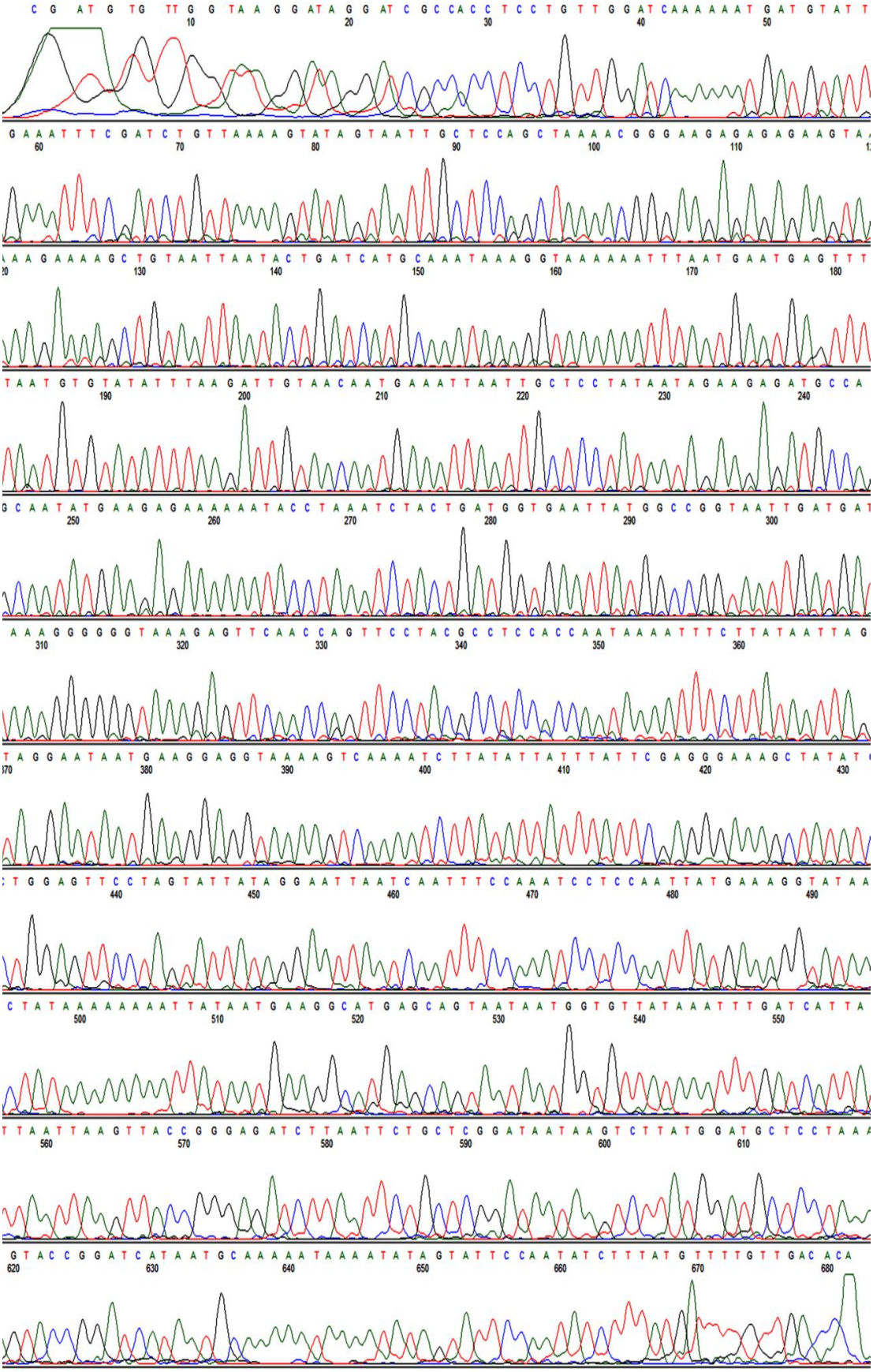

Fig.8. Sequencing chromatogram (reverse sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *V. tropica* Voucher CUKAK1 (GenBank Accession Number: KM455116).

>*V. tropica* voucher CUKAK1 mitochondrial cytochrome oxidase subunit I (COI) partial nucleotide sequence, 658 bases

AATACTATATTTTATTTTTGCATTATGATCCGGTACTTTAGGAGCATCC
ATAAGACTTATTATCCGAGCAGAATTAAGATCTCCCGGTAACTTAATTA
ATAATGATCAAATTTATAACACCATTATTACTGCTCATGCCTTCATTAT
AATTTTTTTTATAGTTATACCTTTCATAATTGGAGGATTTGGAAATTGA
TTAATTCCTATAATACTAGGAACTCCAGATATAGCTTTCCCTCGAATAA
ATAATATAAGATTTTGACTTTTACCTCCTTCATTATTCCTACTAATTAT
AAGAAATTTTATTGGTGGAGGCGTAGGAACTGGTTGAACTCTTTACCCC
CCTTTATCATCAATTACCGGCCATAATTCACCATCAGTAGATTTAGGTA
TTTTTTCTCTTCATATTGCTGGCATCTCTTCTATTATAGGAGCAATTAA
TTTCATTGTTACAATCTTAAATATACACATTAAAACTCATTCATTAAAT
TTTTTACCTTTATTTGCATGATCAGTATTAATTACAGCTTTTCTTTTAC
TTCTCTCTCTTCCCGTTTTAGCTGGAGCAATTACTATACTTTTAACAGA
TCGAAATTTCAATACATCATTTTTTGATCCAACAGGAGGTGGCGATCCT
ATCCTATACCAACACTTATTC

Fig.9. Partial coding sequence of *V. tropica* Voucher CUKAK1 cytochrome oxidase subunit I (COI) gene (GenBank Accession Number: KM455116).

Score	Expect	Identities	Gaps	Strand
865 bits (468)	0.0	578/633 (91%)	0/633 (0%)	Plus/Plus

```
Query       TGATCCGGTACTTTAGGAGCATCCATAAGACTTATTATCCGAGCAGAATTAAGATCTCCC  85
            ||||| || ||||||||||||||| |||||||||| |||||||||||||||||||||||||
Subject     TGATCTGGAACTTTAGGAGCATCTATAAGACTTATCATCCGAGCAGAATTAAGATCTCCC  60

Query       GGTAACTTAATTAATAATGATCAAATTTATAACACCATTATTACTGCTCATGCCTTCATT  145
            ||||| |||||||||||||||||||||||| || |||||||||| ||||| ||||||
Subject     GGTAATTTAATTAATAATGATCAAATTTATAATACTATTATTACTGCCCATGCTTTCATT  120

Query       ATAAtttttttttATAGTTATACCTTTCATAATTGGAGGATTTGGAAATTGATTAATTCCT  205
            |||||||||||||||| ||||| |||||||||||||||||||||||| ||||||||
Subject     ATAATTTTTTTTATAGTAATACCATTCATAATTGGAGGATTTGGAAATTGGTTAATTCCA  180

Query       ATAATACTAGGAACTCCAGATATAGCTTTCCCTCGAATAAATAATATAAGATTTTGACTT  265
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     ATAATACTAGGAACTCCAGATATAGCTTTCCCTCGAATAAATAATATAAGATTTTGACTT  240

Query       TTACCTCCTTCATTATTCCTACTAATTATAAGAAATTTTATTGGTGGAGGCGTAGGAACT  325
            |||||||| |||||||| ||| |||||| ||||||||||||||| ||||| |||||||||
Subject     TTACCTCCATCATTATTTCTATTAATTACAAGAAATTTTATTGGAGGAGGTGTAGGAACT  300

Query       GGTTGAACTCTTTACCCCCCTTTATCATCAATTACCGGCCATAATTCACCATCAGTAGAT  385
            ||||||||| | || |||||| |||||||||||| || ||||||||||||||||||||||
Subject     GGTTGAACTTTATATCCCCCTCTATCATCAATTACTGGTCATAATTCACCATCAGTAGAT  360

Query       TTAGGTATTTTTTCTCTTCATATTGCTGGCATCTCTTCTATTATAGGAGCAATTAATTTC  445
            |||||||||||||| ||||| |||||||| |||||||| ||||||||||||||||||||
Subject     TTAGGTATTTTTTCCCTTCACATTGCTGGAATCTCTTCCATTATAGGAGCAATTAATTTT  420

Query       ATTGTTACAATCTTAAATATACACATTAAAACTCATTCATTAAATTTTTTACCTTTATTT  505
            || || |||||| ||||||||||  |||||||||| ||||||||||||||||||||||||
Subject     ATCGTAACAATCCTAAATATACATGTTAAAACTCACTCATTAAATTTTTTACCTTTATTT  480

Query       GCATGATCAGTATTAATTACAGCTTTTCTTTTACTTCTCTCTCTTCCCGTTTTAGCTGGA  565
            ||||||||||  |||||||||||| |||||||||| ||||| || |||||||| |||
Subject     GCATGATCAGTGCTAATTACAGCTTTCCTTTTACTTCTTTCTCTACCTGTTTTAGCCGGA  540

Query       GCAATTACTATACTTTTAACAGATCGAAATTTCAATACATCATTTTTTGATCCAACAGGA  625
            ||||| || ||||||||||||||||||||||| |||||||||||||| || |||||||||
Subject     GCAATCACCATACTTTTAACAGATCGAAATTTTAATACATCATTTTTCGACCCAACAGGA  600

Query       GGTGGCGATCCTATCCTATACCAACACTTATTC  658
            || || || || || || || ||||||||||||
Subject     GGAGGTGACCCCATTCTTTATCAACACTTATTC  633
```

Fig.10. Nearest sequence match from the BLASTn result of *V. tropica* Voucher CUKAK1 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455116). Query = *V. tropica* Voucher CUKAK1; Subject = *Vespa ducalis* (GenBank Accession Number: KF933084.1). Note that the nearest match is only 91% similar to the sequence in database depicting that sequence obtained is novel.

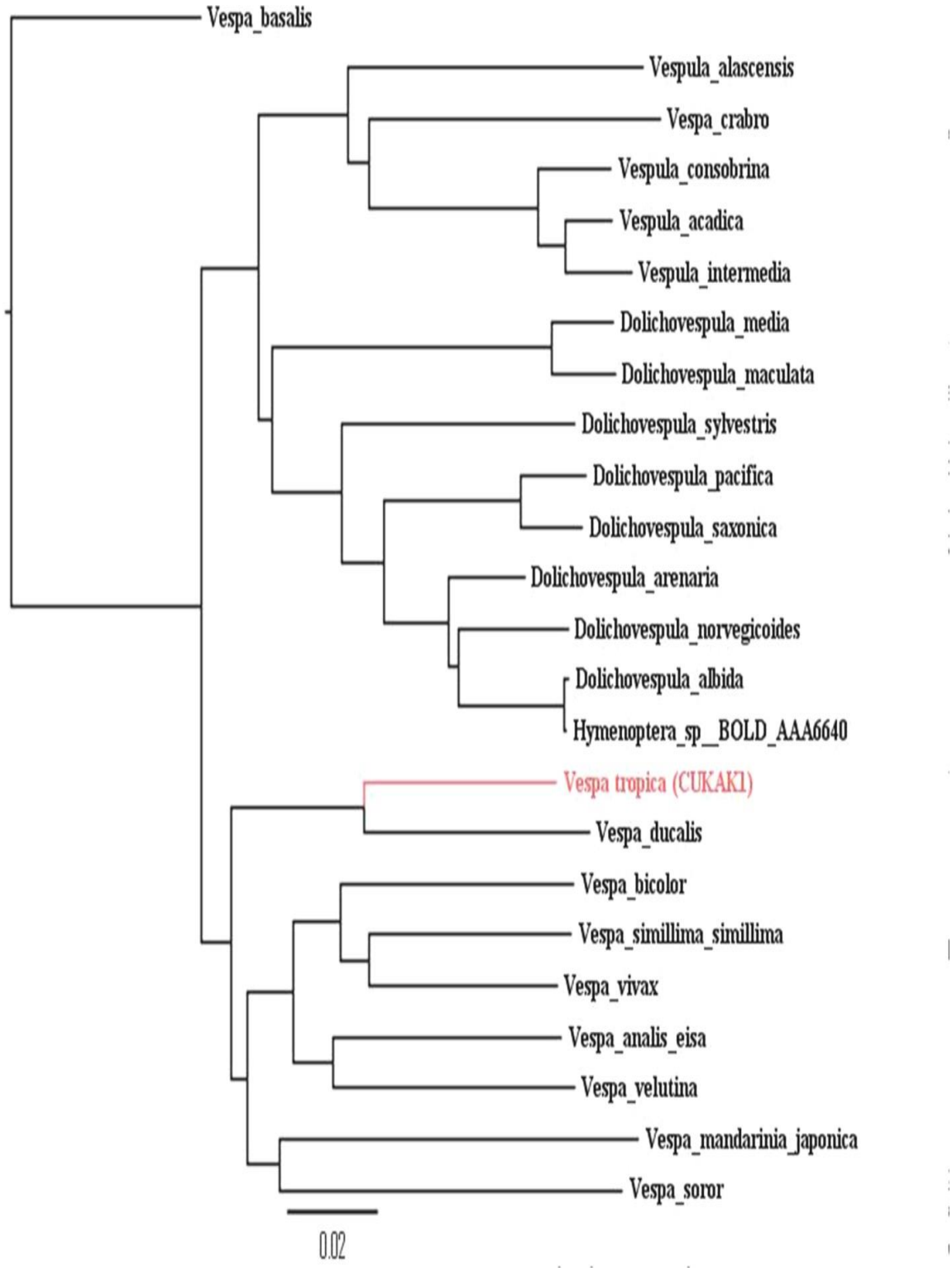

Fig.11. N-J tree plotted for *V. tropica* Voucher CUKAK1 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455116).

>*V. tropica* Voucher CUKAK1

MLYFIFALWSGTLGASMSLIIRAELSSPGNLINNDQIYNTIITAHAFIMIFFMVMPFMI
GGFGNWLIPMMLGTPDMAFPRMNNMSFWLLPPSLFLLIMSNFIGGGVGTGWTLYPPLSS
ITGHNSPSVDLGIFSLHIAGISSIMGAINFIVTILNMHIKTHSLNFLPLFAWSVLITAF
LLLLSLPVLAGAITMLLTDRNFNTSFFDPTGGGDPILYQHLF

Fig.12. The conceptual translation product of the DNA sequence of mitochondrial cytochrome oxidase subunit I (COI) gene of *V. tropica* Voucher CUKAK1.

Score	Expect	Method	Identities	Positives	Gaps
409 bits (1050)	1e^{-138}	Compositional matrix adjusts.	209/211 (99%)	210/211 (99%)	0/211 (0%)

```
Query       WSGTLGASMSLIIRAELSSPGNLINNDQIYNTIITAHAFIMIFFMVMPFMIGGFGNWLIP  68
            WSGTLGASMSLIIRAELSSPGNLINNDQIYNTIITAHAFIMIFFMVMPFMIGGFGNWLIP
Subject     WSGTLGASMSLIIRAELSSPGNLINNDQIYNTIITAHAFIMIFFMVMPFMIGGFGNWLIP  60

Query       MMLGTPDMAFPRMNNMSFWLLPPSLFLLIMSNFIGGGVGTGWTLYPPLSSITGHNSPSVD  128
            MMLGTPDMAFPRMNNMSFWLLPPSLFLLI SNFIGGGVGTGWTLYPPLSSITGHNSPSVD
Subject     MMLGTPDMAFPRMNNMSFWLLPPSLFLLITSNFIGGGVGTGWTLYPPLSSITGHNSPSVD  120

Query       LGIFSLHIAGISSIMGAINFIVTILNMHIKTHSLNFLPLFAWSVLITAFLLLLSLPVLAG  188
            LGIFSLHIAGISSIMGAINFIVTILNMH+KTHSLNFLPLFAWSVLITAFLLLLSLPVLAG
Subject     LGIFSLHIAGISSIMGAINFIVTILNMHVKTHSLNFLPLFAWSVLITAFLLLLSLPVLAG  180

Query       AITMLLTDRNFNTSFFDPTGGGDPILYQHLF  219
            AITMLLTDRNFNTSFFDPTGGGDPILYQHLF
Subject     AITMLLTDRNFNTSFFDPTGGGDPILYQHLF  211
```

Fig.13. Nearest peptide sequence match from the BLASTp result of *V. tropica* Voucher CUKAK1 cytochrome oxidase subunit I (COI) conceptual translation product. Query = *V. tropica* Voucher CUKAK1; Subject = *Vespa ducalis* (GenBank Accession Number: AHC97222.1). Note that the nearest match is 99% similar to the sequence in database depicting that the sequence obtained is novel.

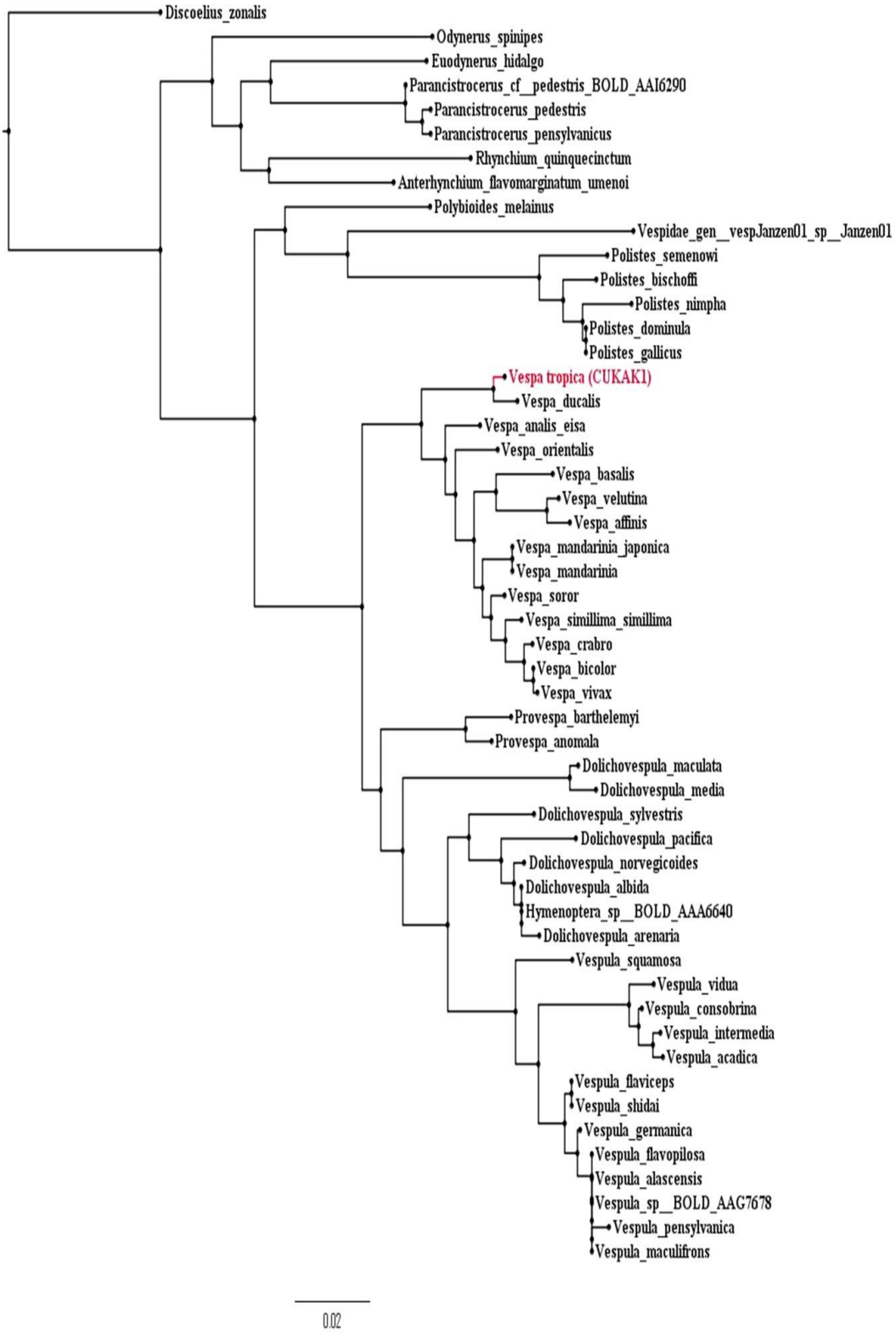

Fig.14. N-J tree plotted for conceptual translation product of *V. tropica* Voucher CUKAK1 cytochrome oxidase subunit I (COI) peptide sequence.

2. *Rhynchium brunneum*

Rhynchium brunneum (**Figures 15-18**) is a potter wasp or mud wasp which builds its nest using mud or soft clay in walls, ceilings or any other open spaces. It is known to be an important pollinator of plants.

2.1. History of nomenclature

Vespa brunnea. Fabricius, 1793. *Ent. Syst*, 2: 264.

Rhynchium brunneum (Fabricius). Bingham, 1897. *Fauna Brit. India, Hym*, 1: 355- 356.

(Srinivasan and Kumar, 2010).

2.2. Diagnosis

The species collected was identical to those described by Srinivasan and Kumar (2010). Body was reddish brown with black markings. Black marking as follows: a spot between antennae, a vertical line on frons, a large triangular spot on mesoscutum in front, a line along its apex, sutures on mesopleuron, basal half of legs, basal two-thirds of first, and basal half of second metasomal segment. Pale black fasica on base of third and fourth metasomal segments. Wings yellowish brown, deeper and darker towards base; veins yellow. Wings yellowish hyaline, deeper and darker towards base.

Fig.15. *Rhynchium brunneum* Voucher CUKAK2 lateral view (scale in cm).

Fig.16. *Rhynchium brunneum* Voucher CUKAK2 upper view (scale in cm).

Fig.17. *Rhynchium brunneum* Voucher CUKAK2 front view.

Fig.18. *Rhynchium brunneum* Voucher CUKAK2 wing pattern.

2.3. Systematic position

Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Hymenoptera; Apocrita; Aculeata; Vespoidea; Vespidae; Eumeninae; *Rhynchium*; *brunneum.*

2.4. Distribution

In India it is reported from Arunachal Pradesh, Delhi, Meghalaya, Sikkim, Uttarakhand, West Bengal and Kerala. It is also reported from Afghanistan, Indonesia, Iran, Malaysia, Myanmar, Nepal, Pakistan, Sri Lanka and Thailand (Srinivasan and Kumar, 2010).

2.5. Collection

Rhynchium brunneum, used in the present study was collected from Kasaragod District of Kerala at 12° 20' 0" North, 75° 6' 0" East.

2.6. PCR amplification of the cytochrome c oxidase subunit I gene of *Rhynchium brunneum* Voucher CUKAK2

The gel pictures showing genomic DNA and the PCR product of COI gene of *Rhynchium brunneum* Voucher CUKAK2 are shown in **Figures 19 and 20**. The PCR of

the COI gene fragment of *R. brunneum* from Kasaragod yielded a product of 606bp. The forward and reverse sequence chromatograms, partial COI sequence, BLASTn result, conceptual translation product, BLASTp result are presented in **Figures 21- 24, 26 and 27.** The sequence obtained was deposited in the GenBank (GenBank Accession Number: KM455117).

The hypervariable region of COI gene of *R. brunneum* is 99% similar to that of *Rhynchium quinquecinctum* COI gene (GenBank Accession Number: AB969818.1).

The COI sequence of *R. brunneum* in the present study can be used for its accurate taxonomic identification. The nucleotide BLAST against the nucleotide redundant database showed that the cytochrome oxidase gene sequence obtained is novel.

The conceptual translation of partial COI gene of *R. brunneum* yielded a peptide of 202 amino acids. The peptide blast of COI from *R. brunneum* showed 100% similarity to that of *R. quinquecinctum* (GenBank Accession Number: BAP05525.1). This may be due to the degeneracy of genetic code, where more than one codon codes for a single amino acid, thus generating a similar peptide sequence, eventhough the nucleotide sequence showed variation. The mtDNA sequence of different wasps confirms the species differences of *R. brunneum* from others.

2.7. Molecular phylogeny of *Rhynchium brunneum* Voucher CUKAK2

The phylogenetic trees of DNA and peptide are plotted using Neighbour-Joining method and are exhibited in **Figures 25 and 28**.

The DNA and peptide phylogenetic trees showed that *R. brunneum* is more closely related to *R. quinquecinctum,* of the same genus and subfamily. The DNA tree also depicted the close relationship of *R. brunneum* to species of the subfamily Eumeninae, under the genera *Ancistrocerus, Parancistrocerus, Euodynerus, Symmorphus, Leptochilus, Anterhynchium, Ancistroceroides, Tachyancistrocerus*, *Eumenes* and a Hymenopteran species.

R. brunneum and *R. quinquecinctum* have been originated from a common ancestor. They share common ancestry with other species of the genera *Ancistrocerus, Parancistrocerus, Euodynerus, Symmorphus, Leptochilus Anterhynchium, Ancistroceroides, Tachyancistrocerus, Eumenes* and a Hymenopteran species coming under the subfamily Eumeninae. Among these genera, *Ancistrocerus; Parancistrocerus* and *Euodynerus; Symmorphus; Leptochilus* and Hymenoptera species; *Anterhynchium* and *Ancistroceroides; Euodynerus; Tachyancistrocerus* and *Eumenes* is found to have a single origin.

The species of the subfamily Eumeninae shared a common ancestry with species of the subfamily Vespinae viz. *Vespula germanica, Vespula vulgaris, Vespula flavopilosa* and a Polistinae species, *Polistes hellenicus.* The DNA tree also depicted the distant relationsip of species of the subfamily Eumeninae to Polistinae species. The most distantly related species to *R. brunneum* is *Apoica flavissima* and *Apoica albimacula* of the subfamily Polistinae.

The phylogeny tree based on conceptual peptide also showed the close relationship of *R. brunneum* to *R. quinquecinctum.* They are closely related to the species of the subfamily Eumeninae, under the genera *Anterhynchium, Orancistrocerus, Euodynerus, Ancistrocerus, Parancistrocerus, Taeniogonalus, Leptochilus, Parazumia, Odynerus* and *Symmorphus.* The peptide tree showed the relationship of these species to the wasps *Monobia quadridens, Discoelius zonalis* and *Discoelius dufourii* of the subfamily Eumeninae. *R. brunneum* is distantly related to the species of the subfamily Vespinae, under the genera *Vespa, Provespa, Vespula* and *Dolichovespula*. It is most distantly realted to *Polistes metricus* of Polistinae subfamily. The species of the subfamily Eumeninae and Vespinae have been originated from a common ancestor. The present results indicated the significance of an identification system based on COI gene sequence. The result highlighted the significant sequence diversity of the COI gene of *R. brunneum* from its allied species, thus enabling an easy discrimination of species.

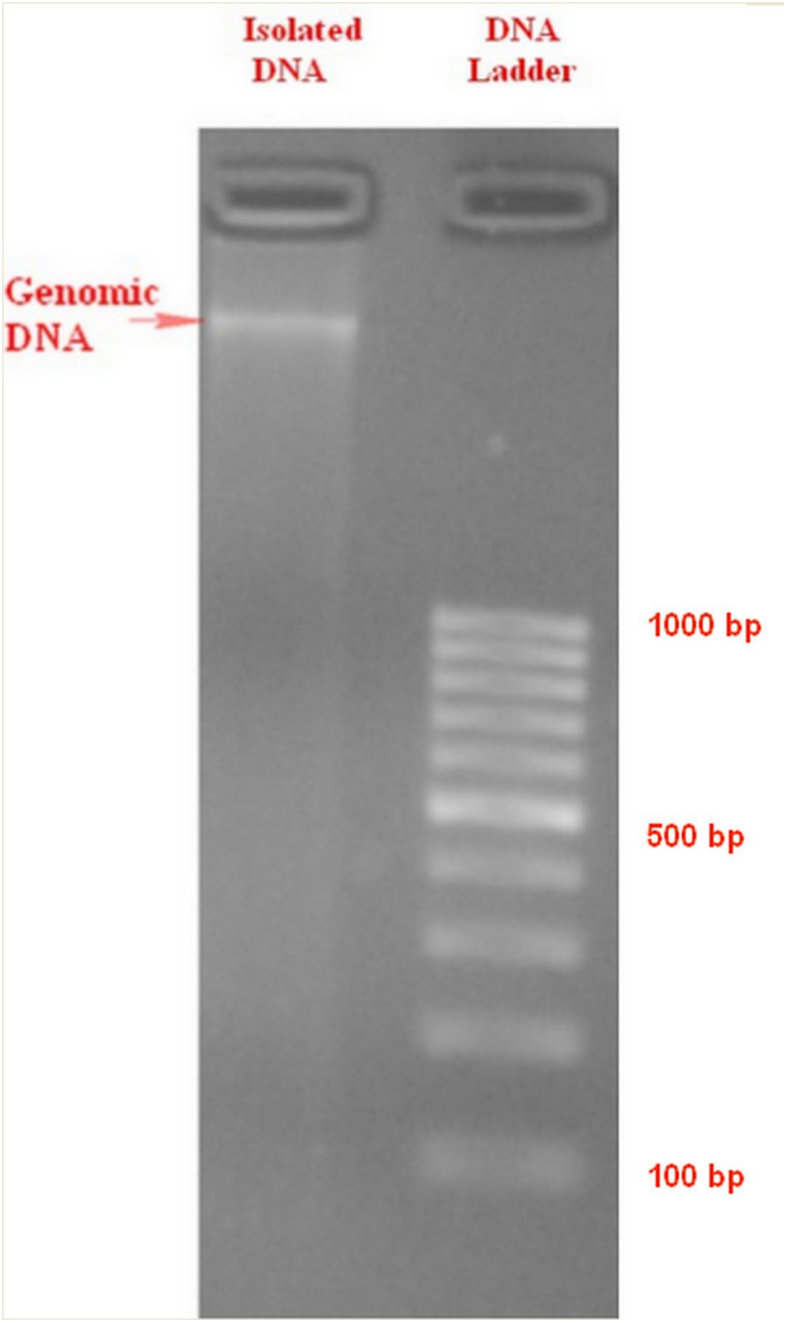

Fig.19. Gel picture of total DNA isolated from *Rhynchium brunneum* Voucher CUKAK2.

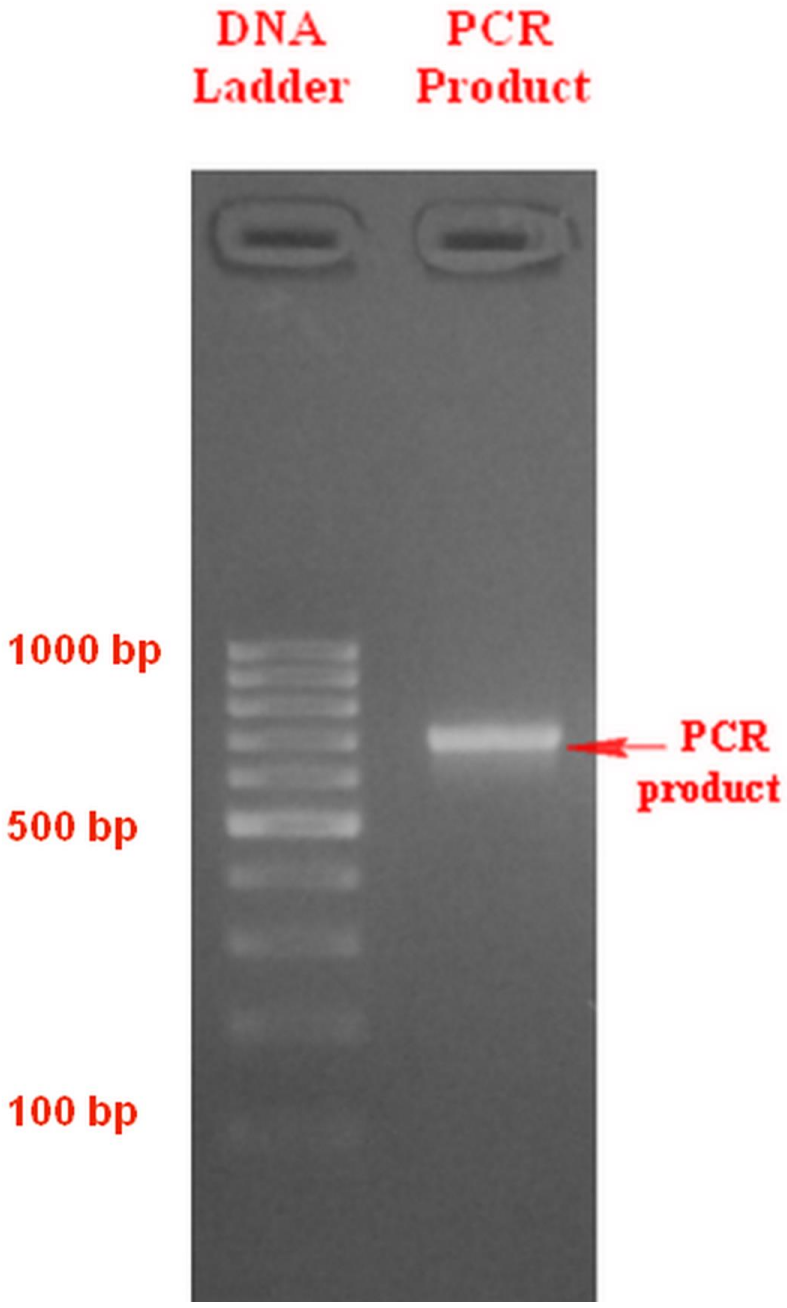

Fig.20. Gel picture showing the PCR product of partial COI gene of *Rhynchium brunneum* Voucher CUKAK2.

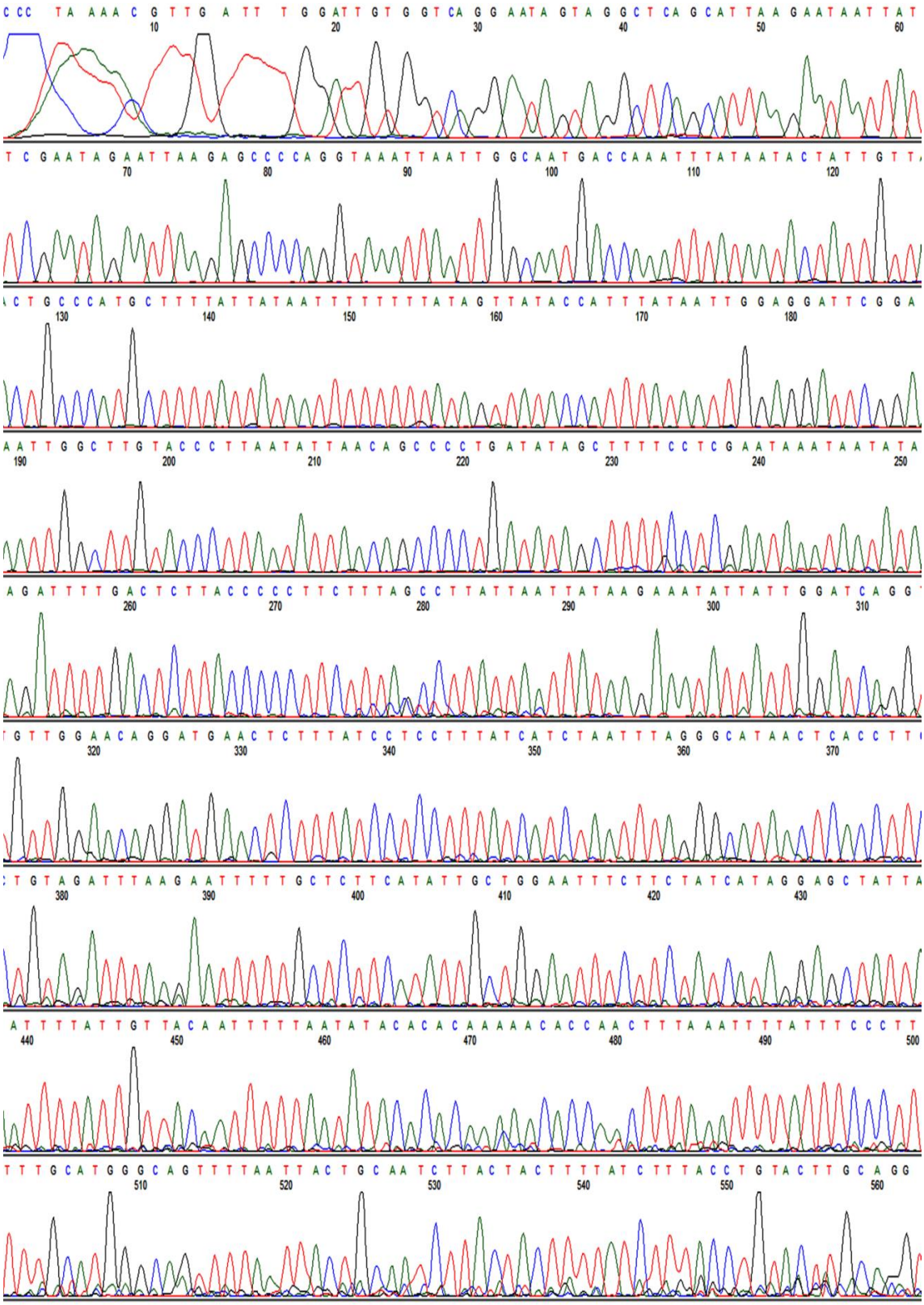

Fig.21. Sequencing chromatogram (forward sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *R. brunneum* Voucher CUKAK2 (GenBank Accession Number: KM455117).

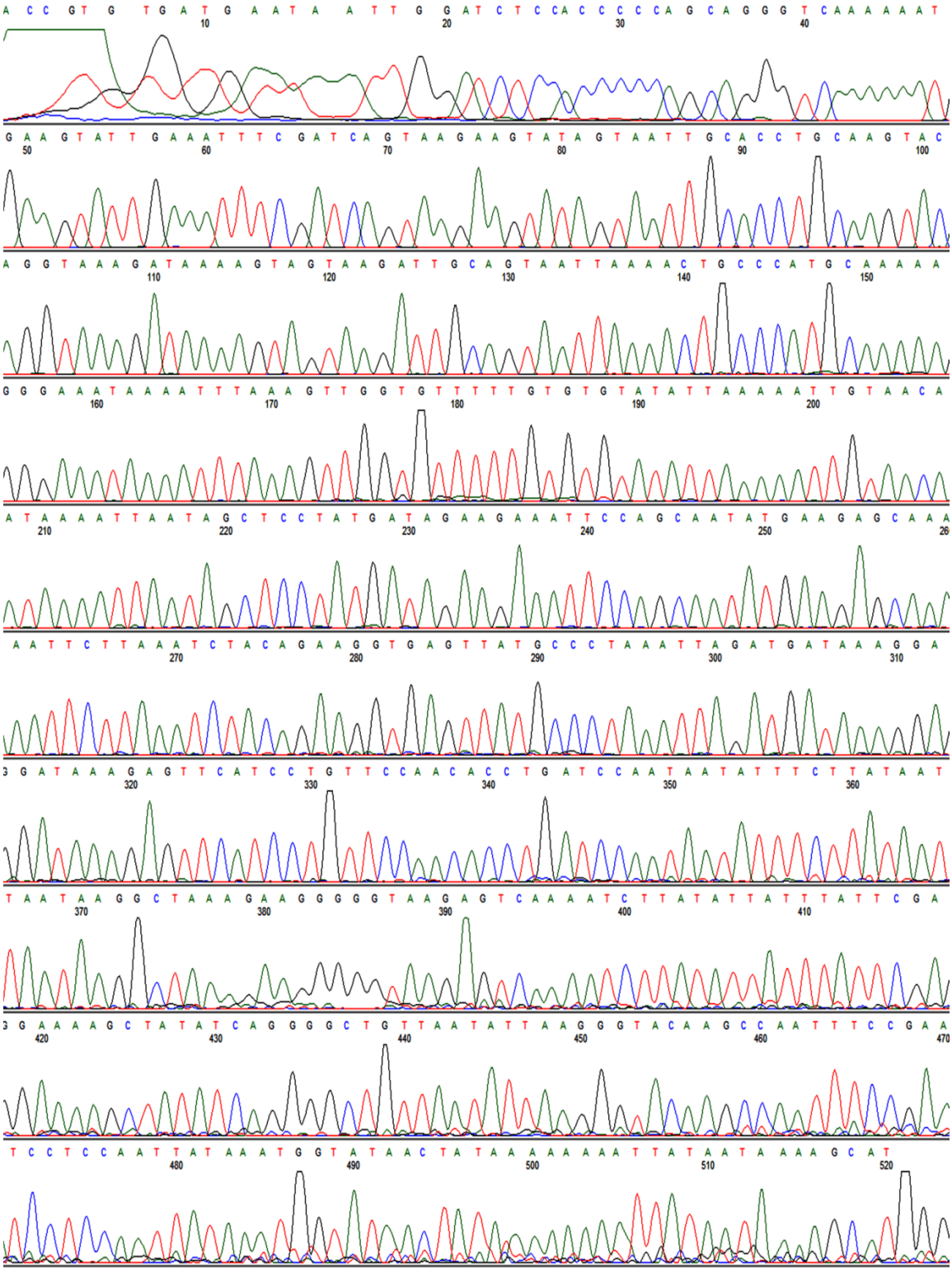

Fig.22. Sequencing chromatogram (reverse sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *R. brunneum* Voucher CUKAK2 (GenBank Accession Number: KM455117).

>*R. brunneum* Voucher CUKAK2 mitochondrial cytochrome oxidase subunit I (COI) partial nucleotide sequence, 606 bases

GGAATAGTAGGCTCAGCATTAAGAATAATTATTCGAATAGAATTAAGAGCC
CCAGGTAAATTAATTGGCAATGACCAAATTTATAATACTATTGTTACTGCC
CATGCTTTTATTATAATTTTTTTTATAGTTATACCATTTATAATTGGAGGA
TTCGGAAATTGGCTTGTACCCTTAATATTAACAGCCCCTGATATAGCTTTT
CCTCGAATAAATAATATAAGATTTTGACTCTTACCCCCTTCTTTAGCCTTA
TTAATTATAAGAAATATTATTGGATCAGGTGTTGGAACAGGATGAACTCTT
TATCCTCCTTTATCATCTAATTTAGGGCATAACTCACCTTCTGTAGATTTA
AGAATTTTTGCTCTTCATATTGCTGGAATTTCTTCTATCATAGGAGCTATT
AATTTTATTGTTACAATTTTTAATATACACACAAAAACACCAACTTTAAAT
TTTATTTCCCTTTTTGCATGGGCAGTTTTAATTACTGCAATCTTACTACTT
TTATCTTTACCTGTACTTGCAGGTGCAATTACTATACTTCTTACTGATCGA
AATTTCAATACTTCATTTTTTGACCCTGCTGGGGGTGGAGATCCA

Fig.23. Partial coding sequence of *R. brunneum* Voucher CUKAK2 cytochrome oxidase subunit I (COI) gene (GenBank Accession Number: KM455117).

Score	Expect	Identities	Gaps	Strand
1075 bits (582)	0.0	598/606 (99%)	0/606 (0%)	Plus/Plus

```
Query       GGAATAGTAGGCTCAGCATTAAGAATAATTATTCGAATAGAATTAAGAGCCCCAGGTAAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     GGAATAGTAGGCTCAGCATTAAGAATAATTATTCGAATAGAATTAAGAGCCCCAGGTAAA  91

Query       TTAATTGGCAATGACCAAATTTATAATACTATTGTTACTGCCCATGCTTTTATTATAAtt  120
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Subject     TTAATTGGCAATGACCAAATTTATAACACTATTGTTACTGCCCATGCTTTTATTATAATT  151

Query       ttttttATAGTTATACCATTTATAATTGGAGGATTCGGAAATTGGCTTGTACCCTTAATA  180
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Subject     TTTTTTATAGTTATACCATTTATAATTGGAGGATTCGGAAATTGACTTGTACCCTTAATA  211

Query       TTAACAGCCCCTGATATAGCTTTTCCTCGAATAAATAATATAAGATTTTGACTCTTACCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     TTAACAGCCCCTGATATAGCTTTTCCTCGAATAAATAATATAAGATTTTGACTCTTACCC  271

Query       CCTTCTTTAGCCTTATTAATTATAAGAAATATTATTGGATCAGGTGTTGGAACAGGATGA  300
            |||||||||||||||||||||||||||||||||||| |||||||||||| |||||||||||
Subject     CCTTCTTTAGCCTTATTAATTATAAGAAATATTATTGGGTCAGGTGTTGGGACAGGATGA  331

Query       ACTCTTTATCCTCCTTTATCATCTAATTTAGGGCATAACTCACCTTCTGTAGATTTAAGA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     ACTCTTTATCCTCCTTTATCATCTAATTTAGGGCATAACTCACCTTCTGTAGATTTAAGA  391

Query       ATTTTTGCTCTTCATATTGCTGGAATTTCTTCTATCATAGGAGCTATTAATTTTATTGTT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     ATTTTTGCTCTTCATATTGCTGGAATTTCTTCTATCATAGGAGCTATTAATTTTATTGTC  451

Query       ACAATTTTTAATATACACACAAAAACACCAACTTTAAATTTTATTTCCCTTTTTGCATGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     ACAATTTTTAATATACACACAAAAACACCAACTTTAAATTTTATTTCCCTTTTTGCATGG  511

Query       GCAGTTTTAATTACTGCAATCTTACTACTTTTATCTTTACCTGTACTTGCAGGTGCAATT  540
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Subject     GCAGTTTTAATTACTGCAATCTTATTACTTTTATCTTTACCTGTACTTGCAGGTGCAATT  571

Query       ACTATACTTCTTACTGATCGAAATTTCAATACTTCATTTTTTGACCCTGCTGGGGGTGGA  600
            |||||||||||||||| |||||||||||||||||||||||||||||||||||| |||
Subject     ACTATACTTCTTACTGACCGAAATTTCAATACTTCATTTTTTGACCCTGCTGGGGGAGGA  631

Query       GATCCA  606
            ||||||
Subject     GATCCA  637
```

Fig.24. Nearest sequence match from the BLAST result of *R. brunneum* Voucher CUKAK2 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455117). Query = *R. brunneum* Voucher CUKAK2; Subject = *R. quinquecinctum* (GenBank Accession Number: AB969818.1). Note that the nearest match is 99% similar to the sequence in the database depicting that the nucleotide sequence obtained is novel.

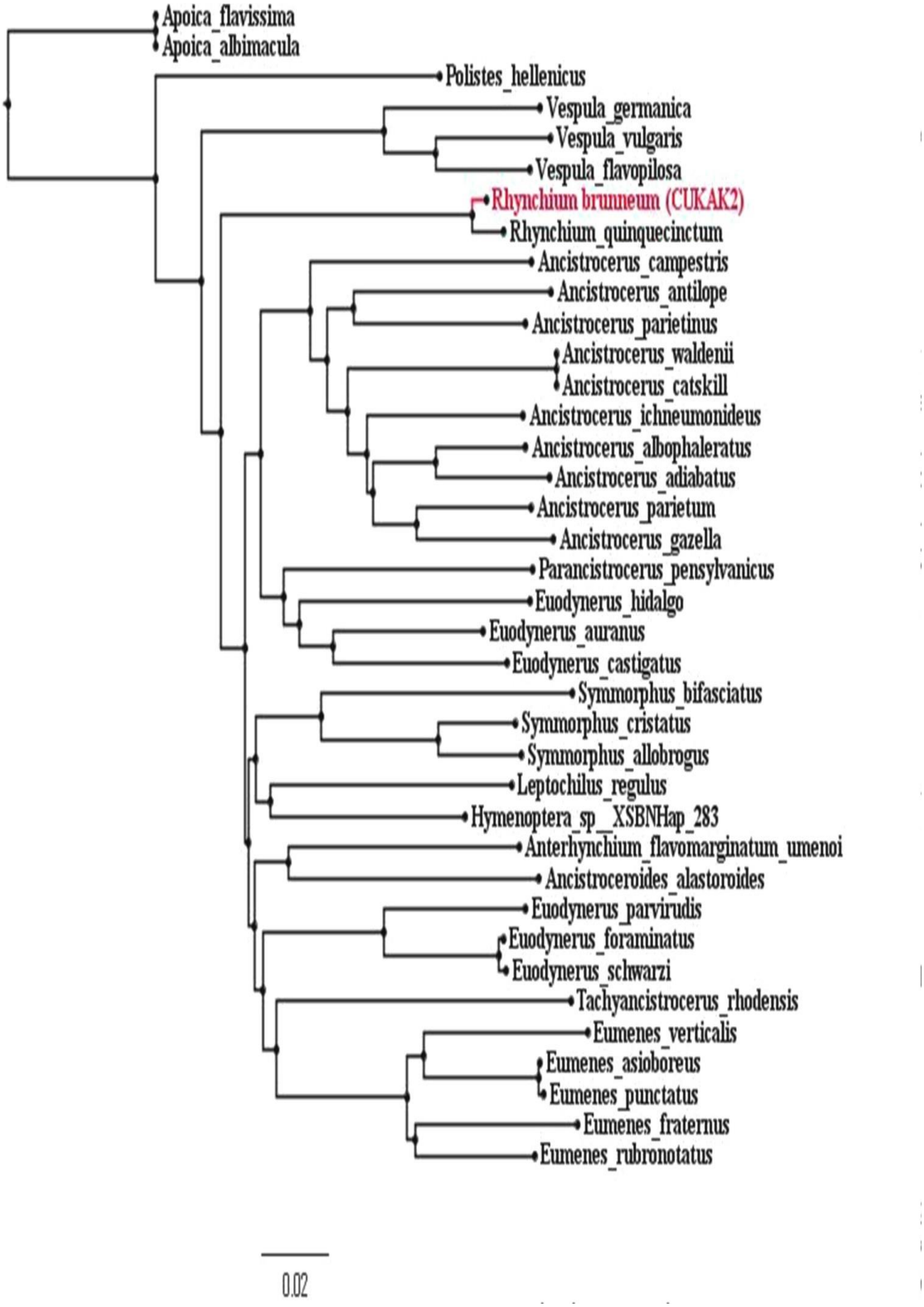

Fig.25. N-J tree plotted for *R. brunneum* Voucher CUKAK2 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455117).

> *R. brunneum* Voucher CUKAK2

GMVGSALSMIIRMELSAPGKLIGNDQIYNTIVTAHAFIMIFFMVMPFMIGGFGNWLV
PLMLTAPDMAFPRMNNMSFWLLPPSLALLIMSNIIGSGVGTGWTLYPPLSSNLGHNS
PSVDLSIFALHIAGISSIMGAINFIVTIFNMHTKTPTLNFISLFAWAVLITAILLLL
SLPVLAGAITMLLTDRNFNTSFFDPAGGGDP

Fig.26. The conceptual translation product of the DNA sequence of mitochondrial cytochrome oxidase subunit I (COI) gene of *R. brunneum* Voucher CUKAK2.

Score	Expect	Method	Identities	Positives	Gaps
391 bits (1005)	$7e^{-136}$	Compositional matrix adjusts.	202/202 (100%)	202/202 (100%)	0/202 (0%)

```
Query       GMVGSALSMIIRMELSAPGKLIGNDQIYNTIVTAHAFIMIFFMVMPFMIGGFGNWLVPLM  60
            GMVGSALSMIIRMELSAPGKLIGNDQIYNTIVTAHAFIMIFFMVMPFMIGGFGNWLVPLM
Subject     GMVGSALSMIIRMELSAPGKLIGNDQIYNTIVTAHAFIMIFFMVMPFMIGGFGNWLVPLM  70

Query       LTAPDMAFPRMNNMSFWLLPPSLALLIMSNIIGSGVGTGWTLYPPLSSNLGHNSPSVDLS  120
            LTAPDMAFPRMNNMSFWLLPPSLALLIMSNIIGSGVGTGWTLYPPLSSNLGHNSPSVDLS
Subject     LTAPDMAFPRMNNMSFWLLPPSLALLIMSNIIGSGVGTGWTLYPPLSSNLGHNSPSVDLS  130

Query       IFALHIAGISSIMGAINFIVTIFNMHTKTPTLNFISLFAWAVLITAILLLLSLPVLAGAI  180
            IFALHIAGISSIMGAINFIVTIFNMHTKTPTLNFISLFAWAVLITAILLLLSLPVLAGAI
Subject     IFALHIAGISSIMGAINFIVTIFNMHTKTPTLNFISLFAWAVLITAILLLLSLPVLAGAI  190

Query       TMLLTDRNFNTSFFDPAGGGDP  202
            TMLLTDRNFNTSFFDPAGGGDP
Subject     TMLLTDRNFNTSFFDPAGGGDP  212
```

Fig.27. Nearest peptide sequence match from the BLAST result of *R. brunneum* Voucher CUKAK2 cytochrome oxidase subunit I (COI) conceptual translation product. Query = *R. brunneum* Voucher CUKAK2; Subject = *R. quinquecinctum* (GenBank Accession Number: BAP05525.1). Note that the nearest match is 100% similar to the peptide sequence in the database.

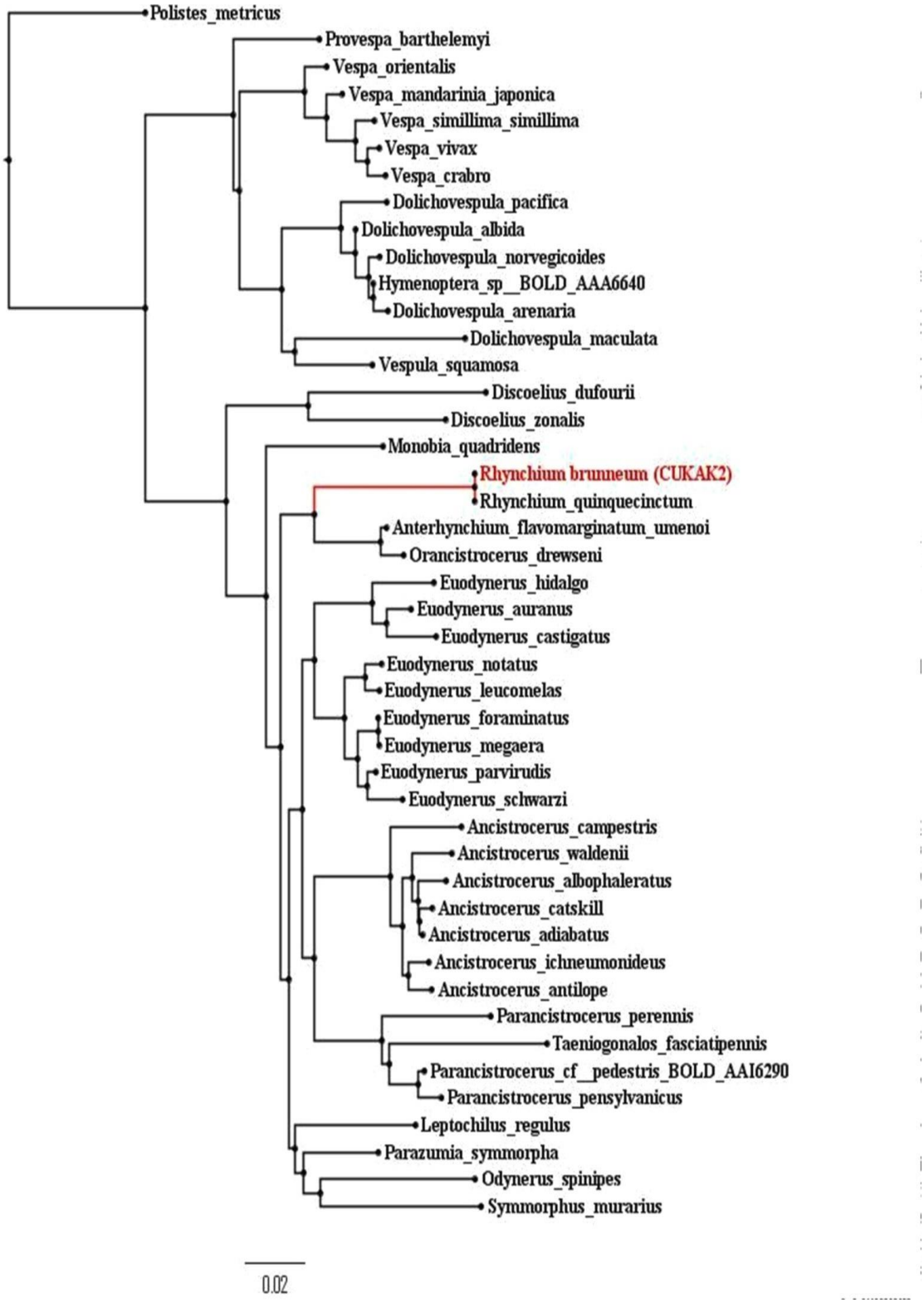

Fig.28. N-J tree plotted for conceptual translation product of *R. brunneum* Voucher CUKAK2 cytochrome oxidase subunit I (COI) peptide sequence.

3. *Delta pyriforme*

Delta pyriforme (**Figures 29-32**) is commonly called the Indian potter wasp, building nest with mud or soft clay in walls, ceilings and other open spaces. They also play their roles as pollinators of plants and in pest management by preying upon small insects.

3.1. History of nomenclature

Vespa petiolate. Fabricius, 1775. *Ent. Syst,* 2: 278.

Eumenes petiolate (Fabricius). Bingham, 1897. *Fauna Brit. India, Hym*, 1: 341, 342.

Delta pyriforme pyriforme (Fabricius). Krombein, 1991. *Smithsonian Contrib. Zool,* 515: 8.

Delta pyriforme pyriforme (Fabricius). Gusenleitner, 2006. *Linzer Biol. Beitr*, 38: 694.

(Srinivasan and Kumar, 2010)

3.2. Diagnosis

The species collected was identical to those described by Srinivasan and Kumar (2010). Head yellow, a broad black band between the eyes on the vertex; occiput black; antenna reddish-brown; pronotum entirely and mesoscutum anteriorly yellow, the later posteriorly black; scutellum and postscutellum reddish-brown; propleuron black; mesopleuron, metapleuron and legs reddish-brown variegated with black; propodeum

Fig.29. *Delta pyriforme* Voucher CUKAK3 lateral view (scale in cm).

Fig.30. *Delta pyriforme* Voucher CUKAK3 upper view (scale in cm).

Fig.31. *Delta pyriforme* Voucher CUKAK3 front view.

Fig.32. *Delta pyriforme* Voucher CUKAK3 wing pattern.

reddish-brown with a narrow medial vertical black line; the sutures between the scutellum, postscutellum and propodeum black; petiole and basal third of the second gastral segment reddish-brown, the former black at base and with a subapical black band, the middle of the later black, its posterior third and the remaining abdominal segments yellow. Frons and vertex of the head and thorax with close shallow punctures, petiole smooth, the rest of the abdomen with the surface finely longitudinally aciculate; clypeus pyriforme, its apex truncate; petiole broadening towards the apex and slightly flattened. Wings deep flavohyaline.

3.3. Systematic position

Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Hymenoptera; Apocrita; Aculeata; Vespoidea; Vespidae; Eumeninae; *Delta*; *pyriforme.*

3.4. Distribution

In India it is reported from Arunachal Pradesh, Gujarat, Karnataka, Kerala, Orissa, Rajasthan, Sikkim, Tamil Nadu and West Bengal. It is also distributed in Bhutan, Cambodia, China, Hawaii, Hong Kong, Indonesia, Malaysia, Moluccas, Myanmar, Nepal, New Guinea, Sri Lanka, Taiwan, Thailand and Vietnam (Srinivasan and Kumar, 2010).

3.5. Collection

Delta pyriforme, used in the present study was collected from Malappuram District of Kerala at 11° 3' 0" North, 75° 52' 0" East.

3.6. PCR amplification of cytochrome c oxidase subunit I gene of *Delta pyriforme* Voucher CUKAK3

The gel pictures showing the genomic DNA and PCR product of COI gene of *Delta pyriforme* Voucher CUKAK3 are given in **Figures 33 and 34.** The PCR of the COI gene fragment of *D. pyriforme* from Malappuram yielded a product of 658bp. The forward and reverse sequence chromatograms, partial COI sequence, BLASTn result, conceptual translation product and BLASTp result are given in **Figures 35-38, 40 and 41** respectively**.** The sequence obtained is deposited in GenBank (GenBank Accession Number: KM455118). The hypervariable region of DNA of *D. pyriforme* is 99% similar to that of *Eumenes sp.* COI gene (GenBank Accession Number: HM996890.1).

The COI sequence of *D. pyriforme* in the present study can be used for its accurate taxonomic identification. The nucleotide BLAST against the nucleotide redundant database showed that the cytochrome oxidase gene sequence obtained is novel.

The conceptual translation of partial COI gene of *D. pyriforme* yielded a peptide of 202 amino acids .The peptide blast of COI from *D. pyriforme* showed 100% similarity

to that of *Eumenes sp.* (GenBank Accession Number: ADP21898.1). This may be due to the degeneracy of genetic code, where more than one codon codes for a single amino acid, thus generating a similar peptide sequence, eventhough the nucleotide sequence showed variation. The mtDNA sequence of different wasps confirms the species differences of *D. pyriforme* from others.

3.7. Molecular phylogeny of *Delta pyriforme* Voucher CUKAK3

The phylogenetic trees of DNA and peptide are plotted using Neighbour-Joining method and are exhibited in **Figures 39 and 42.** The DNA and peptide phylogenetic trees showed that *D. pyriforme* is more closely related to *Eumenes sp.* LK 2010 (GenBank Accession Number: HM996890.1) having a common ancestor. The *Eumenes sp.* LK 2010 is potter wasp collected from Kerala. It was the first report of COI sequence of family Vespidae from Kerala and India as well. *D. pyriforme* and *Eumenes sp*. is closely related to *D. esuriens* of the genus *Delta* under the subfamily Eumeninae. The DNA tree depicted the relationship of *D. pyriforme* to the species *Zeta mendozanum, Katamenes dimidiatus*, and species of the genus *Eumenes* viz. *Eumenes pedunculatus, Eumenes crucifera, Eumenes fraternus, Eumenes asiboreus* and *Eumenes punctatus* coming under the subfamily Eumeninae. The DNA tree also showed the relationship of *D. pyriforme* with the species of subfamily Vespinae viz. *Dolichovespula sylvestris, Dolichovespula*

albida, a Hymenoptera sp, *Dolichovespula arenaria, Dolichovespula norvegicoides* and a species of subfamily Polistinae, *Polistes formosanus*. The species of the genus *Eumenes* and *Dolichovespula* is having a common ancestor. They are closely related to the species of the genus *Euodynerus, Ancistroceroides,* and *Leptochilus* of the subfamily Eumeninae. The most distantly related species to *D. pyriforme* from the DNA tree is *Parancistrocerus pedestris.*

The phylogeny tree based on conceptual peptide also showed the close relationship of *D. pyriforme* to *Eumenes sp*. LK 2010 having a common ancestor. They are closely related to species of the genus *Eumenes* viz. *Eumenes verticalis, Eumenes fraternus, Eumenes pedunculatus* and *Eumenes crucifera,* having a single origin. The peptide tree also showed the relationship pattern of *D. pyriforme* to other species of the subfamily Eumeninae, Polistinae and Vespinae. The most distanly related species to *D. pyriforme* according to the conceptual peptide phylogeny tree is found to be *Katamenes dimidiatus,* of the subfamily Eumeninae.

The present results indicated the significance of an identification system based on COI gene sequence. The result highlighted the significant sequence diversity of the COI gene of *D. pyriforme* from its allied species, thus enabling an easy discrimination of species.

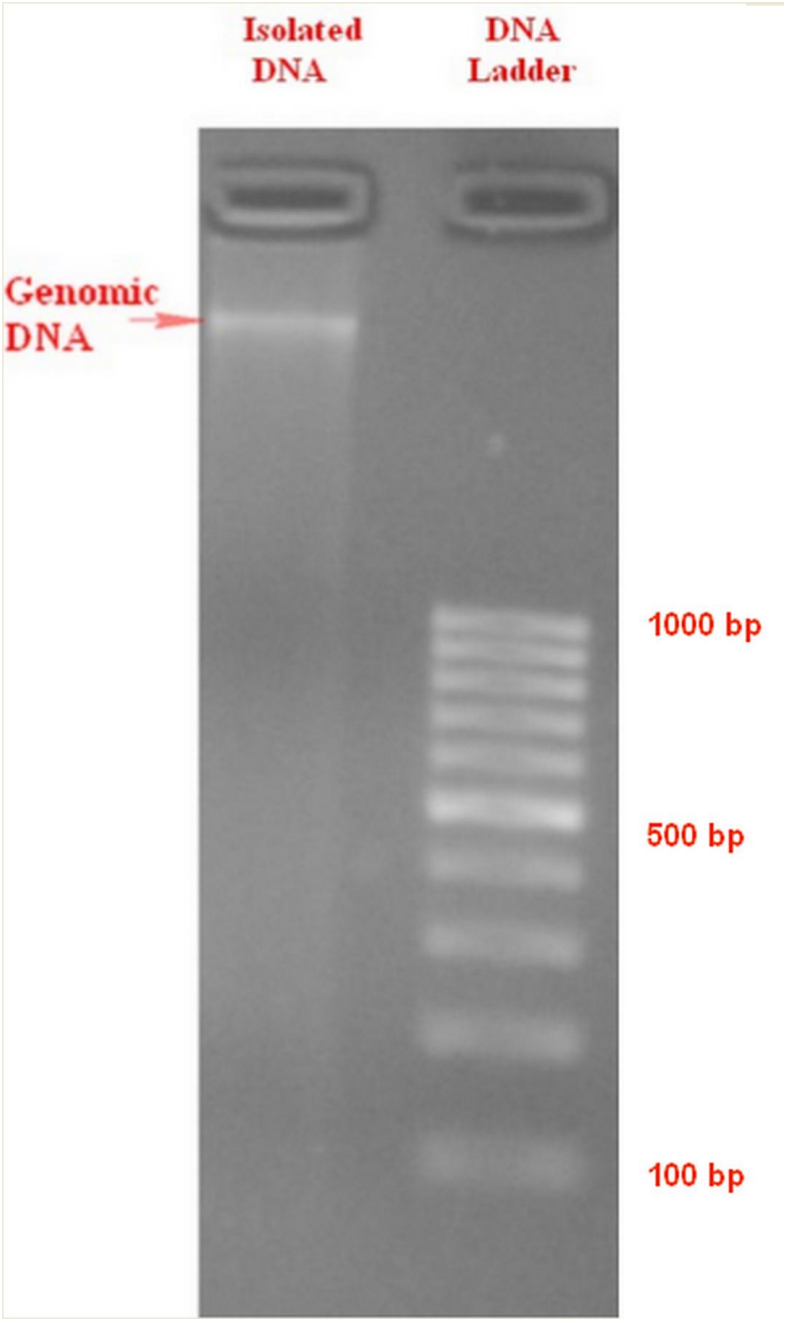

Fig.33. Gel picture of total DNA isolated from *Delta pyriforme* Voucher CUKAK3.

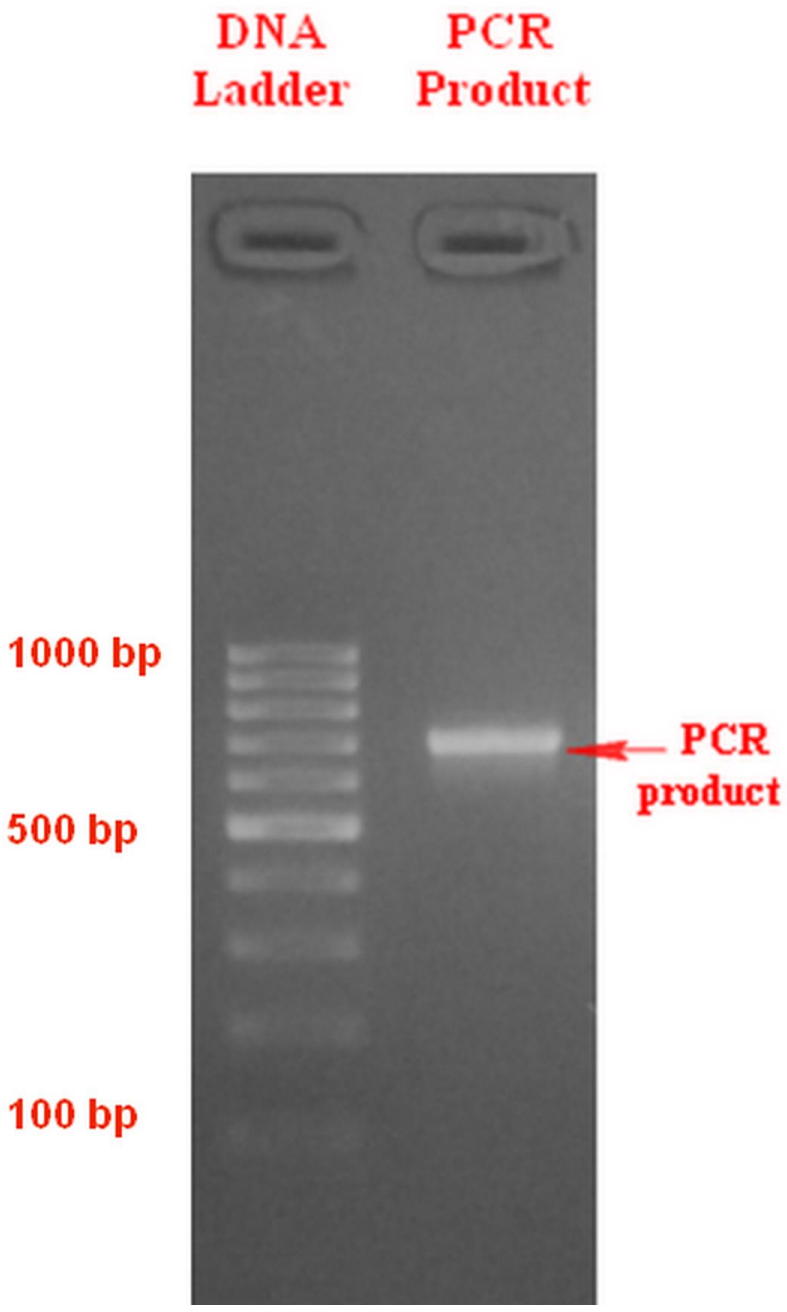

Fig.34. Gel picture showing the PCR product of partial COI gene of *Delta pyriforme* Voucher CUKAK3.

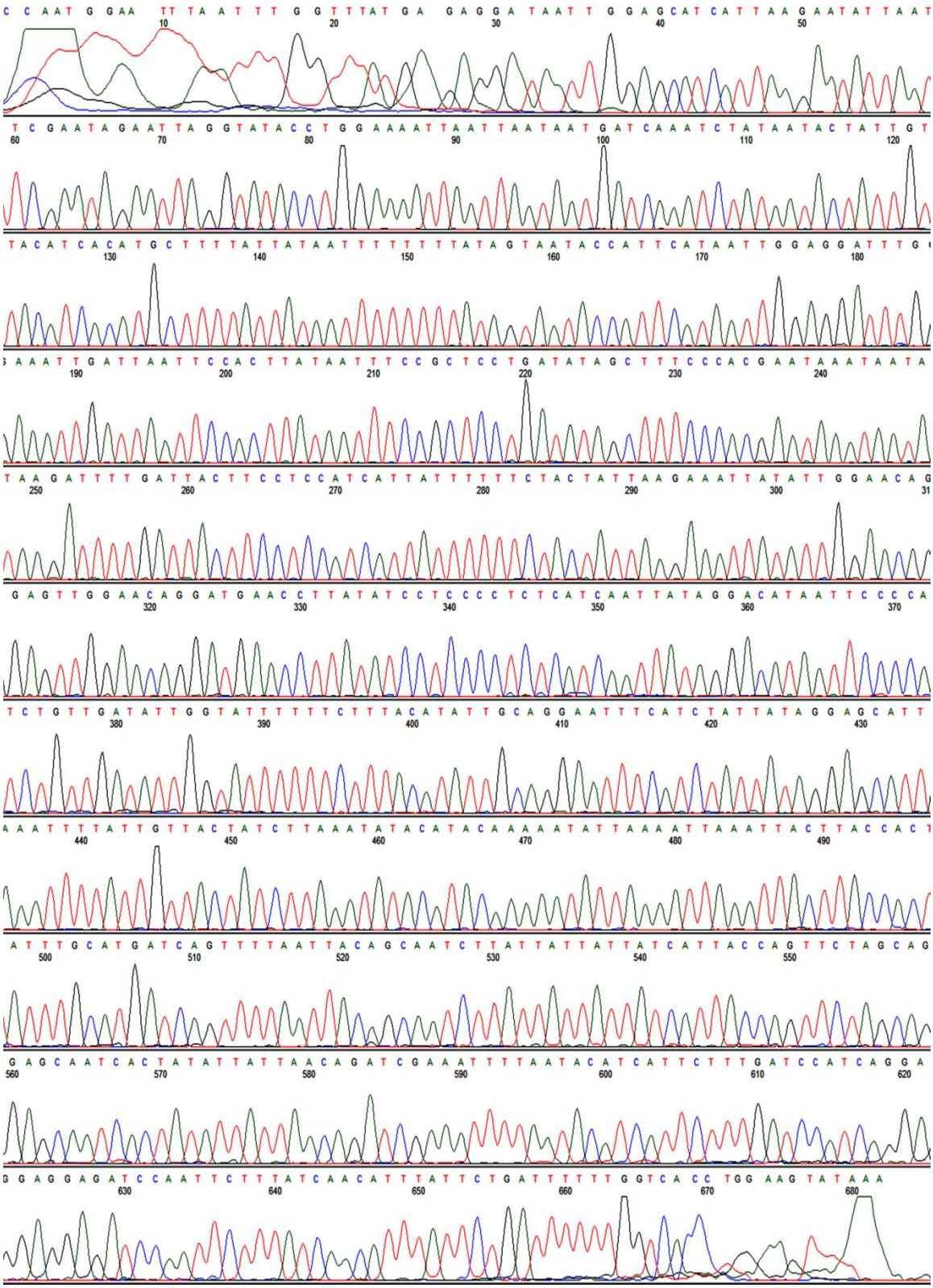

Fig.35. Sequencing chromatogram (forward sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *D. pyriforme* Voucher CUKAK3 (GenBank Accession Number: KM455118).

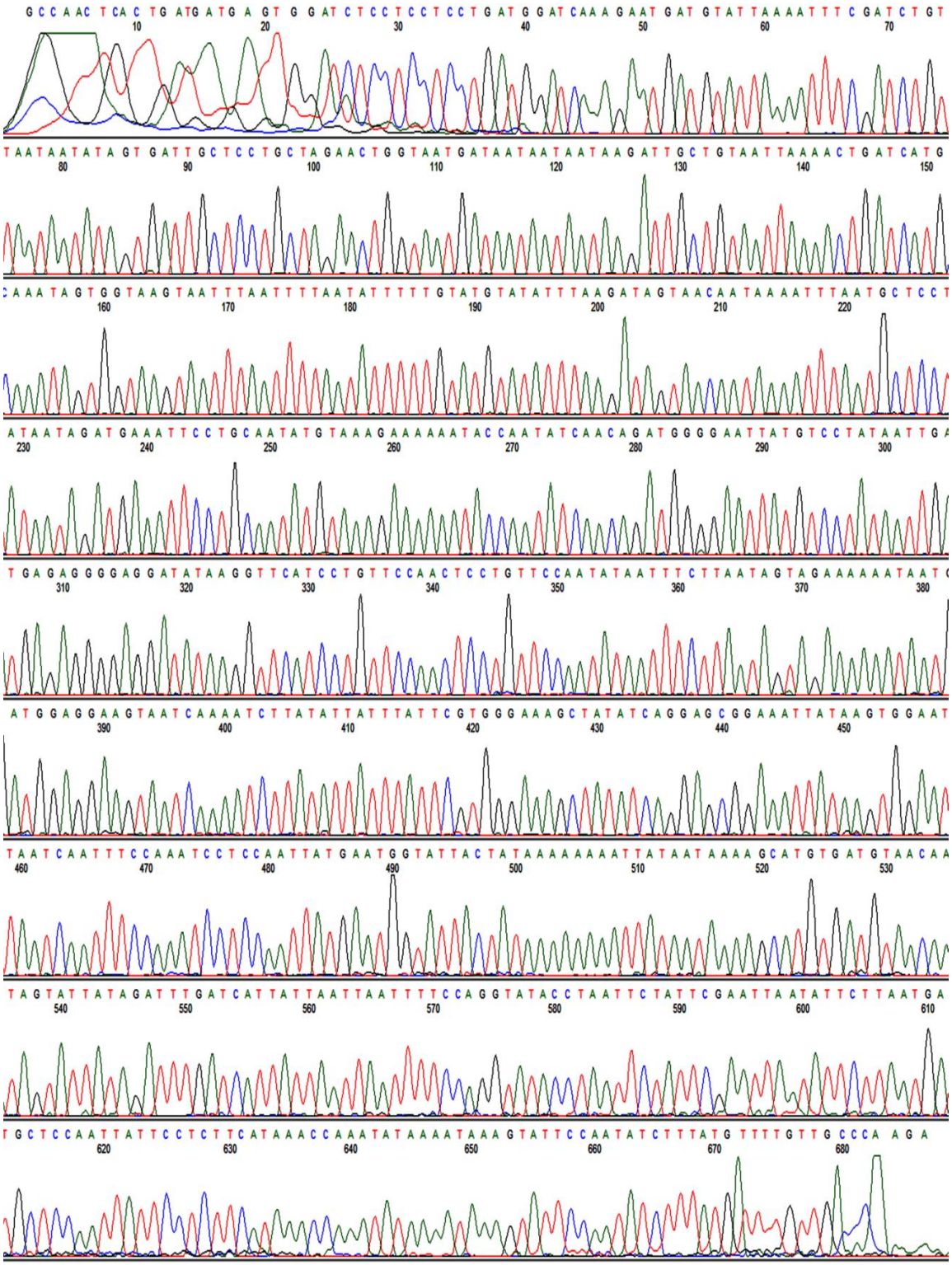

Fig.36. Sequencing chromatogram (reverse sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *D. pyriforme* Voucher CUKAK3 (GenBank Accession Number: KM455118).

>*D. pyriforme* Voucher CUKAK3 mitochondrial cytochrome oxidase subunit I (COI) partial nucleotide sequence, 658 bases

AATACTTTATTTTATATTTGGTTTATGAAGAGGAATAATTGGAGCATCATT
AAGAATATTAATTCGAATAGAATTAGGTATACCTGGAAAATTAATTAATAA
TGATCAAATCTATAATACTATTGTTACATCACATGCTTTTATTATAATTTT
TTTTATAGTAATACCATTCATAATTGGAGGATTTGGAAATTGATTAATTCC
ACTTATAATTTCCGCTCCTGATATAGCTTTCCCACGAATAAATAATATAAG
ATTTTGATTACTTCCTCCATCATTATTTTTTCTACTATTAAGAAATTATAT
TGGAACAGGAGTTGGAACAGGATGAACCTTATATCCTCCCCTCTCATCAAT
TATAGGACATAATTCCCCATCTGTTGATATTGGTATTTTTTCTTTACATAT
TGCAGGAATTTCATCTATTATAGGAGCATTAAATTTTATTGTTACTATCTT
AAATATACATACAAAAATATTAAAATTAAATTACTTACCACTATTTGCATG
ATCAGTTTTAATTACAGCAATCTTATTATTATTATCATTACCAGTTCTAGC
AGGAGCAATCACTATATTATTAACAGATCGAAATTTTAATACATCATTCTT
TGATCCATCAGGAGGAGGAGATCCAATTCTTTATCAACATTTATTC

Fig.37. Partial coding sequence of *D. pyriforme* Voucher CUKAK3 cytochrome oxidase subunit I (COI) gene (GenBank Accession Number: KM455118).

Score	Expect	Identities	Gaps	Strand
1114 bits (603)	0.0	607/609 (99%)	0/609 (0%)	Plus/Plus

```
Query       AATACTTTATTTTATATTTGGTTTATGAAGAGGAATAATTGGAGCATCATTAAGAATATT  60
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Subject     AATACTTTATTTTATATTTGGTTTATGAAGAGGAATAATTGGAGCATCATTAAGAATATT  73

Query       AATTCGAATAGAATTAGGTATACCTGGAAAATTAATTAATAATGATCAAATCTATAATAC  120
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Subject     AATTCGAATAGAATTAGGTATACCTGGAAAATTAATTAATAATGATCAAATCTATAATAC  133

Query       TATTGTTACATCACATGCTTTTATTATAAtttttttttATAGTAATACCATTCATAATTGG  180
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Subject     TATTGTTACATCACATGCTTTTATTATAATTTTTTTTATAGTAATACCATTCATAATTGG  193

Query       AGGATTTGGAAATTGATTAATTCCACTTATAATTTCCGCTCCTGATATAGCTTTCCCACG  240
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     AGGATTCGGAAATTGATTAATTCCACTTATAATTTCCGCTCCTGATATAGCTTTCCCACG  253

Query       AATAAATAATATAAGATTTTGATTACTTCCTCCATCATTATTTTTTCTACTATTAAGAAA  300
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     AATAAATAACATAAGATTTTGATTACTTCCTCCATCATTATTTTTTCTACTATTAAGAAA  313

Query       TTATATTGGAACAGGAGTTGGAACAGGATGAACCTTATATCCTCCCCTCTCATCAATTAT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     TTATATTGGAACAGGAGTTGGAACAGGATGAACCTTATATCCTCCCCTCTCATCAATTAT  373

Query       AGGACATAATTCCCCATCTGTTGATATTGGTATTTTTTCTTTACATATTGCAGGAATTTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     AGGACATAATTCCCCATCTGTTGATATTGGTATTTTTTCTTTACATATTGCAGGAATTTC  433

Query       ATCTATTATAGGAGCATTAAATTTTATTGTTACTATCTTAAATATACATACAAAAATATT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     ATCTATTATAGGAGCATTAAATTTTATTGTTACTATCTTAAATATACATACAAAAATATT  493

Query       AAAATTAAATTACTTACCACTATTTGCATGATCAGTTTTAATTACAGCAATCTTATTATT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     AAAATTAAATTACTTACCACTATTTGCATGATCAGTTTTAATTACAGCAATCTTATTATT  553

Query       ATTATCATTACCAGTTCTAGCAGGAGCAATCACTATATTATTAACAGATCGAAATTTTAA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     ATTATCATTACCAGTTCTAGCAGGAGCAATCACTATATTATTAACAGATCGAAATTTTAA  613

Query       TACATCATT  609
            |||||||||
Subject     TACATCATT  622
```

Fig.38. Nearest sequence match from the BLAST result of *D. pyriforme* Voucher CUKAK3 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455118). Query = *D. pyriforme* Voucher CUKAK3; Subject = *Eumenes sp.* (GenBank Accession Number: HM996890.1). Note that the nearest match is 99% similar to the sequence in the database depicting that the nucleotide sequence obtained is novel.

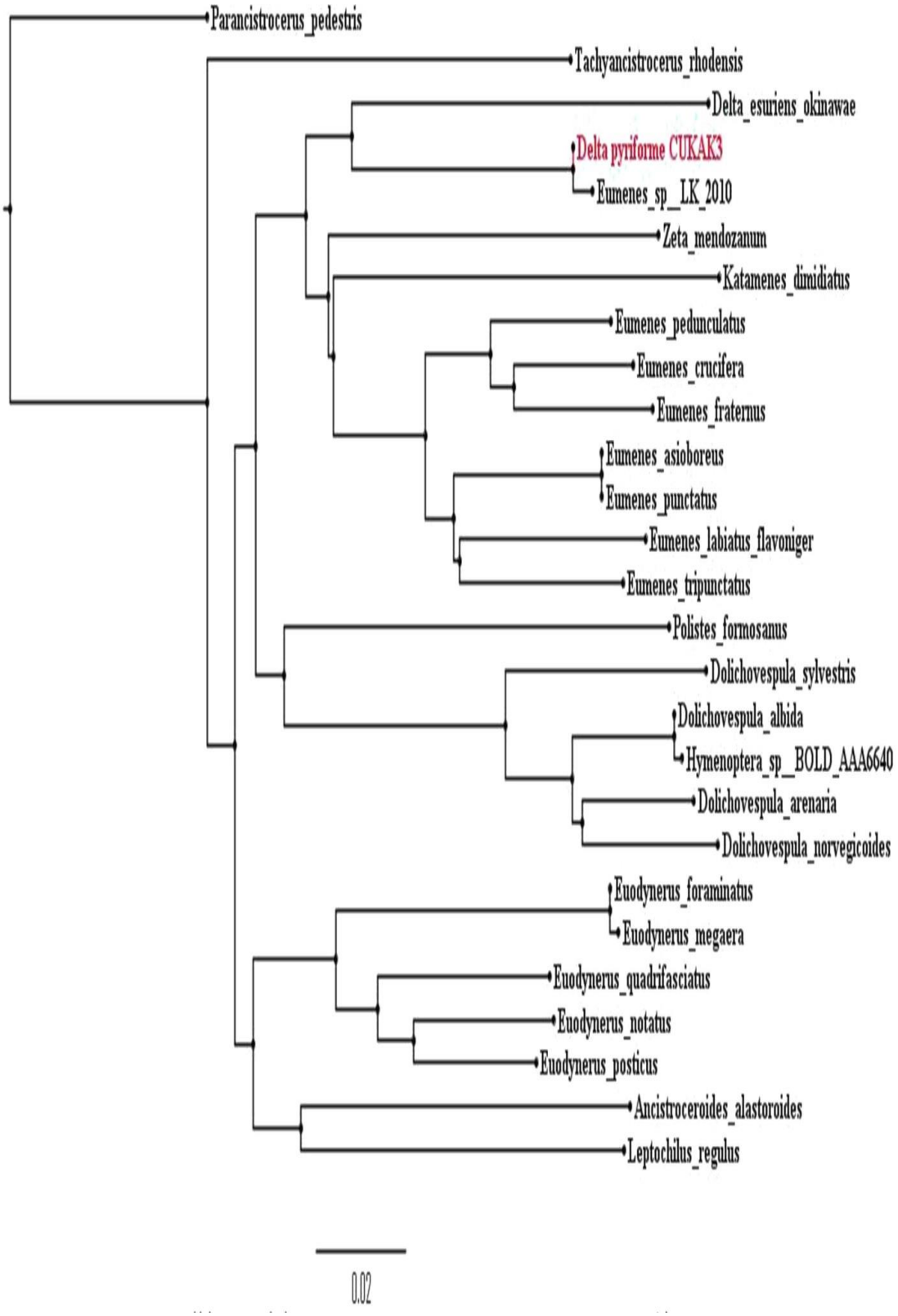

Fig.39. N-J tree plotted for *D. pyriforme* Voucher CUKAK3 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455118).

> *D. pyriformе* Voucher CUKAK3

MLYFMFGLWSGMIGASLSMLIRMELGMPGKLINNDQIYNTIVTSHAFIMIFFMVMPFMI
GGFGNWLIPLMISAPDMAFPRMNNMSFWLLPPSLFFLLLSNYIGTGVGTGWTLYPPLSS
IMGHNSPSVDIGIFSLHIAGISSIMGALNFIVTILNMHTKMLKLNYLPLFAWSVLITAI
LLLLSLPVLAGAITMLLTDRNFNTSFFDPSGGGDPILYQHLF

Fig.40. The conceptual translation product of the DNA sequence of mitochondrial cytochrome oxidase subunit I (COI) gene of *D. pyriformе* Voucher CUKAK3.

Score	Expect	Method	Identities	Positives	Gaps
389 bits (999)	$6e^{-135}$	Compositional matrix adjusts.	202/202 (100%)	202/202 (100%)	0/202 (0%)

```
Query       MLYFMFGLWSGMIGASLSMLIRMELGMPGKLINNDQIYNTIVTSHAFIMIFFMVMPFMIG  60
            MLYFMFGLWSGMIGASLSMLIRMELGMPGKLINNDQIYNTIVTSHAFIMIFFMVMPFMIG
Subject     MLYFMFGLWSGMIGASLSMLIRMELGMPGKLINNDQIYNTIVTSHAFIMIFFMVMPFMIG  64

Query       GFGNWLIPLMISAPDMAFPRMNNMSFWLLPPSLFFLLLSNYIGTGVGTGWTLYPPLSSIM  120
            GFGNWLIPLMISAPDMAFPRMNNMSFWLLPPSLFFLLLSNYIGTGVGTGWTLYPPLSSIM
Subject     GFGNWLIPLMISAPDMAFPRMNNMSFWLLPPSLFFLLLSNYIGTGVGTGWTLYPPLSSIM  124

Query       GHNSPSVDIGIFSLHIAGISSIMGALNFIVTILNMHTKMLKLNYLPLFAWSVLITAILLL  180
            GHNSPSVDIGIFSLHIAGISSIMGALNFIVTILNMHTKMLKLNYLPLFAWSVLITAILLL
Subject     GHNSPSVDIGIFSLHIAGISSIMGALNFIVTILNMHTKMLKLNYLPLFAWSVLITAILLL  184

Query       LSLPVLAGAITMLLTDRNFNTS  202
            LSLPVLAGAITMLLTDRNFNTS
Subject     LSLPVLAGAITMLLTDRNFNTS  206
```

Fig.41. Nearest peptide sequence match from the BLAST result of *D. pyriformе* Voucher CUKAK3 cytochrome oxidase subunit I (COI) conceptual translation product. Query = *D. pyriformе* Voucher CUKAK3; Subject = *Eumenes sp.* LK-2010 (GenBank Accession Number: ADP21897.1). Note that the nearest match is 100% similar to the peptide sequence in the database.

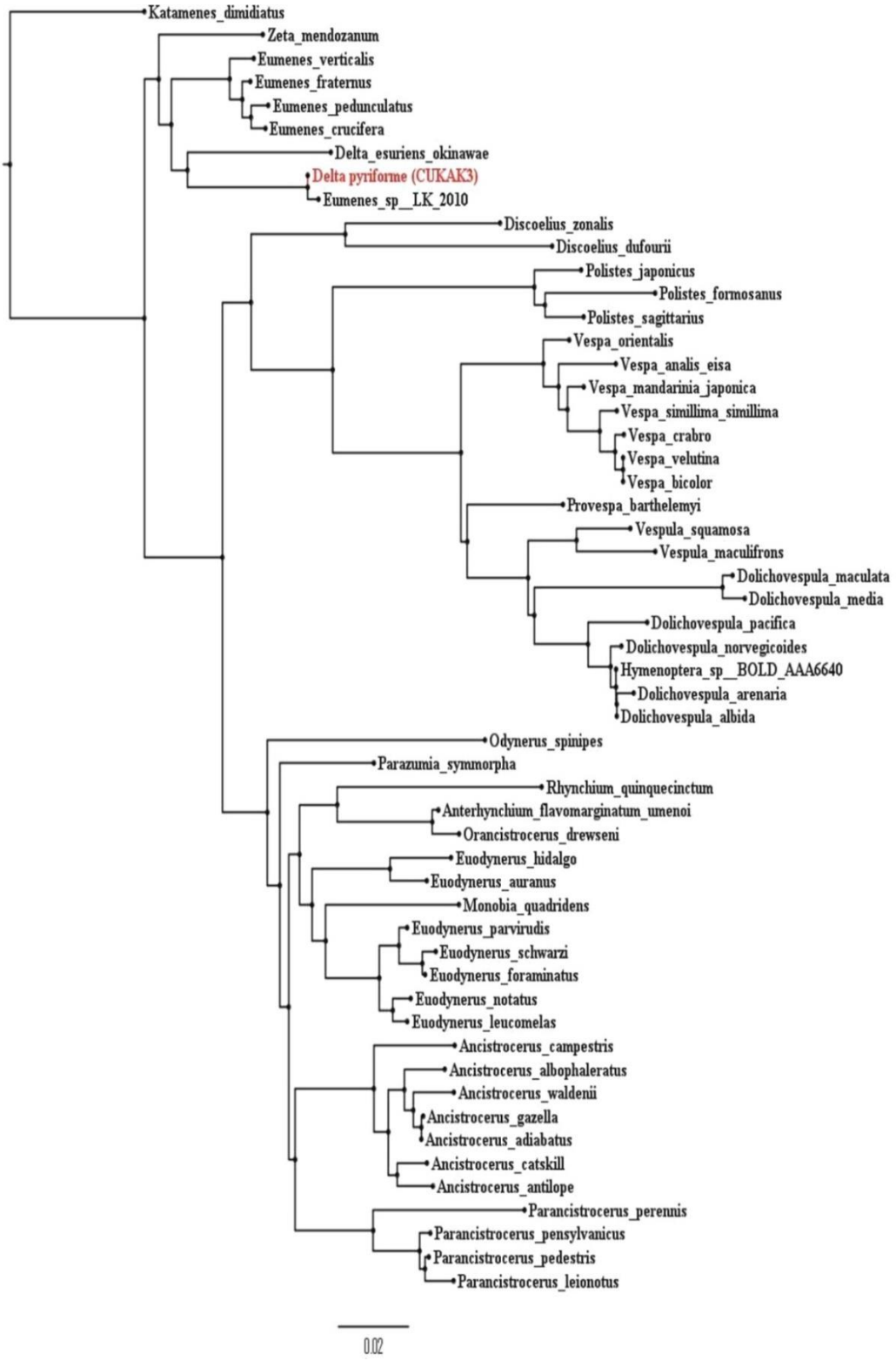

Fig.42. N-J tree plotted for conceptual translation product of *D. pyriforme* Voucher CUKAK3 cytochrome oxidase subunit I (COI) peptide sequence.

4. *Delta conoideum*

Delta conoideum (**Figures 43-46**) is a common potter wasp which builds its nest using mud or soft clay. It is also known to be a good pollinator of plants.

4.1. History of nomenclature

Vespa conica. Fabricius, 1787. *Mant. Ins*, 1-293.

Eumenes conica (Fabricius). Bingham, 1897. *Fauna Brit. India, Hym*, 1: 343, 344.

Vespa conoidea. Gmelin. 1790. *Linne. Syst. Nat*. Ed. B, Vol. 1.

Delta emarginatum conoideum (Gmelin). Krombein, 1991. *Smithsonian Contrib. Zool,* 515: 8.

Delta conoideum (Gmelin). Gusenleitner, 2006. *Linzer Biol. Beitr*, 38: 694.

(Srinivasan and Kumar, 2010)

4.2. Diagnosis

The species collected was identical to those described by Srinivasan and Kumar (2010). Head yellow except mandibles and antenna reddish yellow, a broad transverse band across the apex between the tops of the eyes black, black mark extends behind the vertex to occiput; thorax dark red with black patches on mesoscutum, metapleuron and

Fig.43. *Delta conoideum* Voucher CUKAK4 lateral view (scale in cm).

Fig.44. *Delta conoideum* Voucher CUKAK4 upper view (scale in cm).

Fig.45. *Delta conoideum* Voucher CUKAK4 front view.

Fig.46. *Delta conoideum* Voucher CUKAK4 wing pattern.

median area of propodeum, propleuron entirely black; legs pale reddish; gaster dark red with base of the second tergite and a short transverse medially interrupted band on its middle above black. Head above the antenna and thorax closely and lightly punctured; clypeus pyriforme, its apex truncate; gaster smooth and shining with the surface minutely aciculate. Forewing basally flavohyaline and apically fuscohyaline, with a slight purplish refringes.

4.3. Systematic position

Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Hymenoptera; Apocrita; Aculeata; Vespoidea; Vespidae; Eumeninae; *Delta*; *conoideum.*

4.4. Distribution

In India it is reported from Arunachal Pradesh, Orissa, Sikkim, Tamil Nadu, Tripura, Uttar Pradesh and Kerala. It is also distributed in Arabia, China, Malaysia, Myanmar, Pakistan, Sri Lanka and Thailand (Srinivasan and Kumar, 2010).

4.5. Collection

Delta conoideum, used in the present study was collected from Alappuzha District of Kerala at 9° 42' 0" North, 76° 20' 0" East.

4.6. PCR amplification of the cytochrome c oxidase I gene of *Delta conoideum* Voucher CUKAK4

The gel pictures showing genomic DNA and the PCR product of COI gene of *Delta conoideum* CUKAK4 are shown in **Figures 47 and 48**. The PCR of the COI gene fragment of *D. conoideum* from Alappuzha yielded a product of 621bp. The forward and reverse sequence chromatograms, partial COI sequence obtained, BLASTn result, conceptual translation product and BLASTp result are presented in **Figures 49-52, 54 and 55.** The sequence obtained was deposited in GenBank (GenBank Accession Number: KM455119).

The COI sequence of *D. conoideum* in the present study can be used for its accurate taxonomic identification. The nucleotide BLAST against the nucleotide redundant database showed that the cytochrome oxidase gene sequence obtained is novel.

The hypervariable region of DNA of *D. conoideum* is 90% similar to that of *Eumenes asioboreus* COI gene (GenBank Accession Number: KJ634035.1).

The conceptual translation of partial COI gene of *D. conoideum* yielded a peptide of 207 amino acids. The peptide blast of COI gene of *D. conoideum* showed 95% of similarity to that of cytochrome oxidase subunit I of *Eumenes verticalis* (ACE81461.1). The results of the BLASTp indicated that the peptide of the cytochrome oxidase subunit I

(COI) gene of *D. conoideum* collected from Alappuzha is novel. The mtDNA sequence of different wasps confirms the species differences of *D. conoideum* from others.

4.7. Molecular phylogeny of *Delta conoideum* Voucher CUKAK4

The phylogenetic trees of DNA and peptide are plotted using Neighbour-Joining method and are exhibited in **Figures 51 and 54.** The DNA tree showed that *D. conoideum* is more closely related to the wasps of the genus *Eumenes*, viz. *Eumenes pedunculatus, Eumenes coarctatus, Eumenes rubrofemoratus, Eumenes verticalis, Eumenes asiboreus, Eumenes punctatus, Eumenes tripunctatus, Eumenes labiatus flavoniger, Eumenes sp.* LK 2010 and a *Delta* genus species, *Delta esuriens* under the same subfamily Eumeninae. They share a common ancestry.

Among these species *Eumenes coarctatus* and *Eumenes rubrofemoratus*; *Eumenes asiboreus* and *Eumenes punctatus*; *Eumenes tripunctatus* and *Eumenes labiatus flavoniger*; *Delta esuriens* and *Eumenes sp.* LK 2010 arose from a common ancestor. The DNA tree also showed the relationship of species of *Eumenes* and *Delta* genus to a solitary wasp *Zeta mendoznaum* of the subfamily Eumeninae.

The DNA tree depicted the pattern of relationship of *D. conoideum* to other species of the genera *Ancistroceroides, Leptochilus, Anterhynchium, Symmorphus, Euodynerus, Ancistrocerus, Tachyancistrocerus, Discoelius* and *Parancistrocerus.* The

species of the subfamily Polistinae viz. *Polistes formosanus* and *Polistes bicolor*; *Ropalidia sp* KMP 2004 and *Ropalidia romandi cabeti* is found to have a common ancestor. The most distantly related species to *D. conoideus* according to DNA phylogeny tree is the Vespinae wasp *Vespula flavopilosa*, which have a sister relationship with the common ancestor of whole cluster of Eumeninae and Polistinae species.

The conceptual peptide phylogenetic tree depicted the close relationship of *D. conoideus* to the species *Delta esuriens* of the genus *Delta*, having a common ancestor. They are also closely related to the species of the genus *Eumene*s viz. *Eumenes punctatus* and *Eumenes verticalis*; *Eumenes sp.* LK 2010 and *Zeta mendozanum* by a common ancestor. This whole cluster containing the species of Eumeninae genus has a sister relationship with the wasp, *Katamenes dimidiatus* by a single origin.

The cluster containing species of the genus *Eumenes* and *Zeta mendozanum* possess a common ancestor, which diverged into another clade which includes two clusters viz. the cluster of species of the genus *Discoelius, Odynerus, Parazumia, Parancistrocerus, Euodynerus, Rhynchium, Anterhynchium, Orancistrocerus* and *Ancistrocerus* of Eumeninae subfamily; the cluster of species of the genus *Polistes* of Polistinae subfamily; *Provespa, Vespa* and *Dolichovespula* of Vespinae subfamily.

The present results indicated the significance of an identification system based on COI gene sequence. The result highlighted the significant sequence diversity of the COI gene of *D. conoideum* from its allied species, thus enabling an easy discrimination of species.

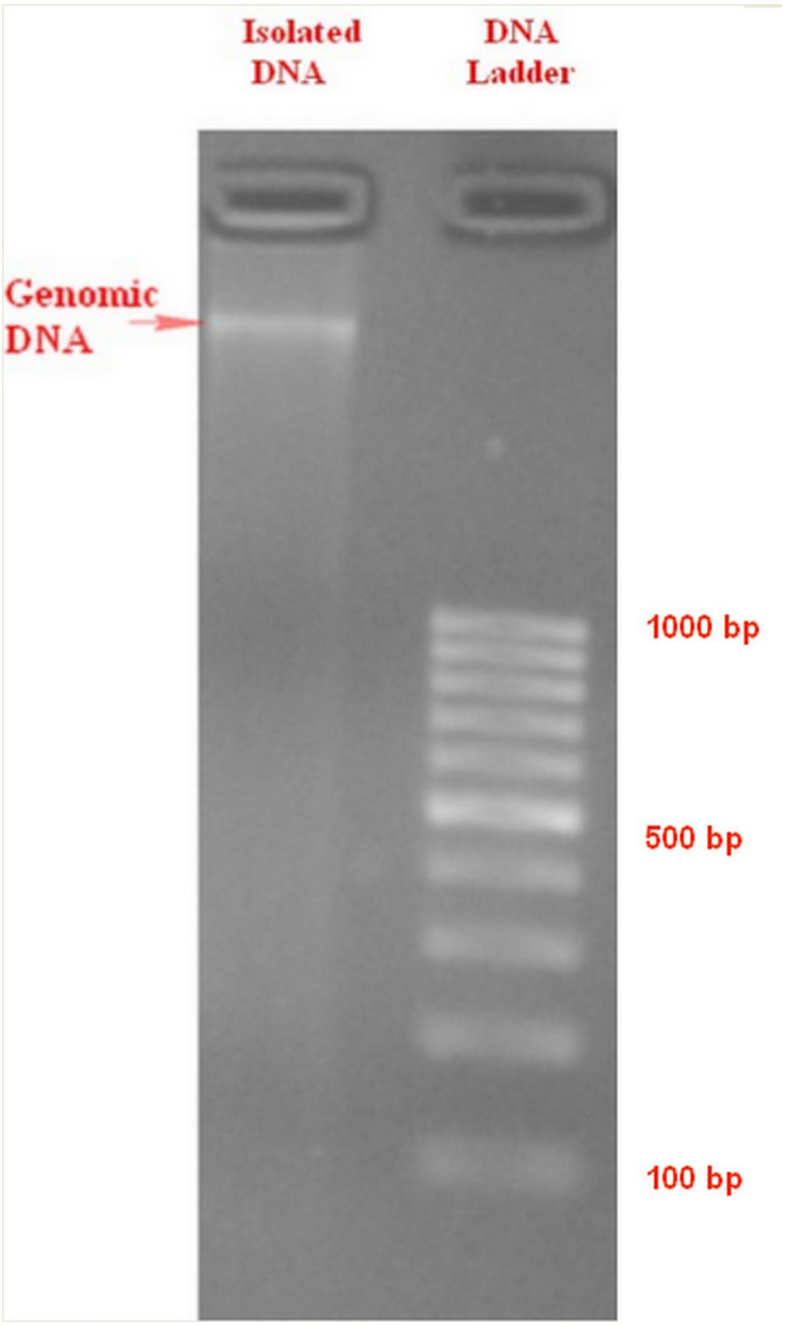

Fig.47. Gel picture of total DNA isolated from *Delta conoideum* Voucher CUKAK4.

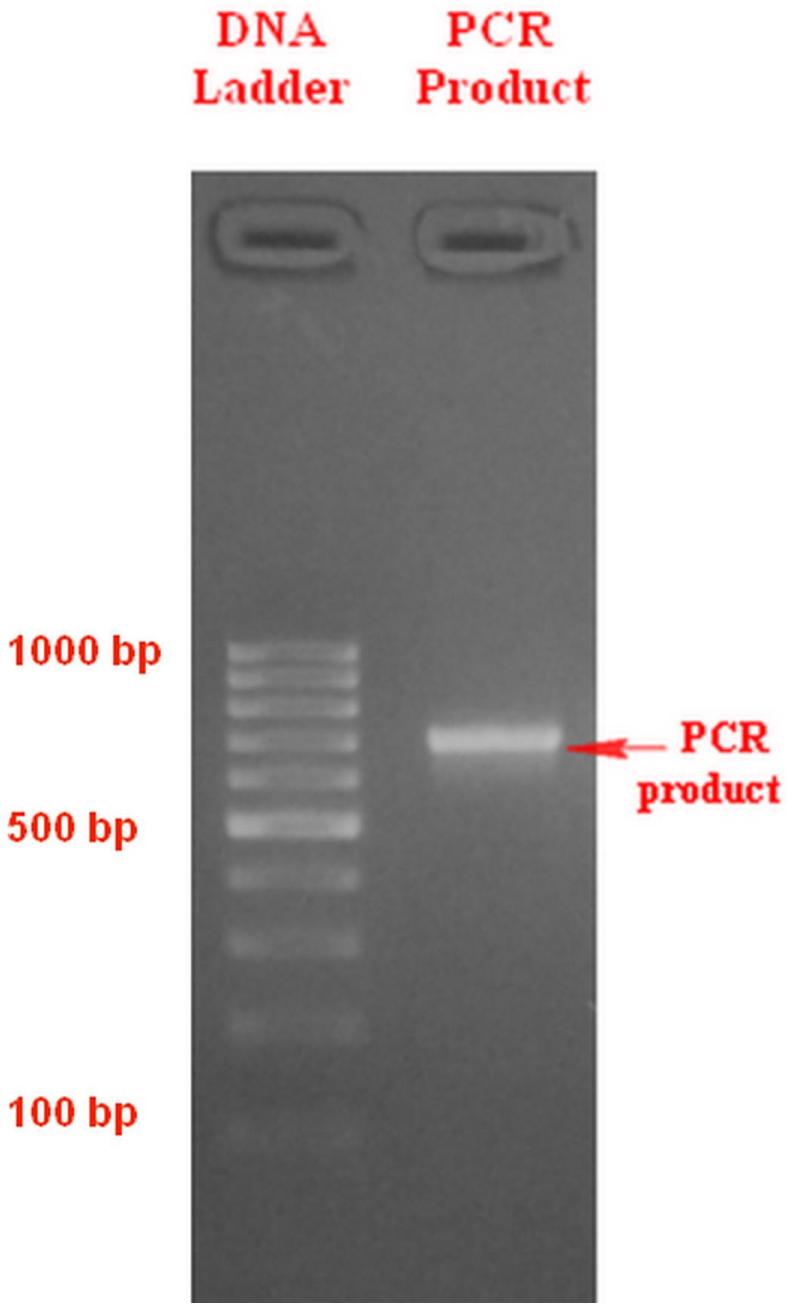

Fig.48. Gel picture showing the PCR product of partial COI gene of *Delta conoideum* Voucher CUKAK4.

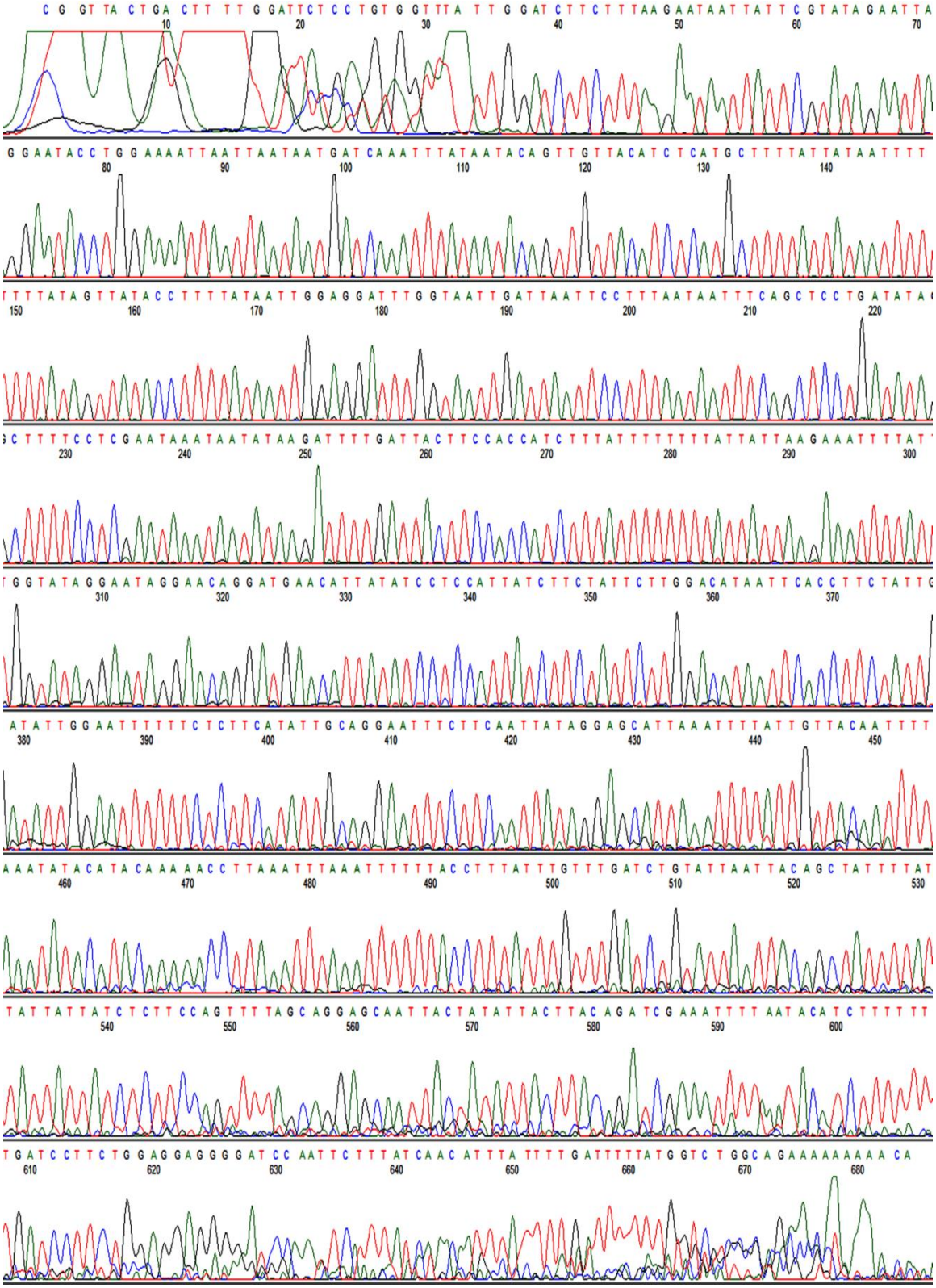

Fig.49. Sequencing chromatogram (forward sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *D. conoideum* Voucher CUKAK4 (GenBank Accession Number: KM455119).

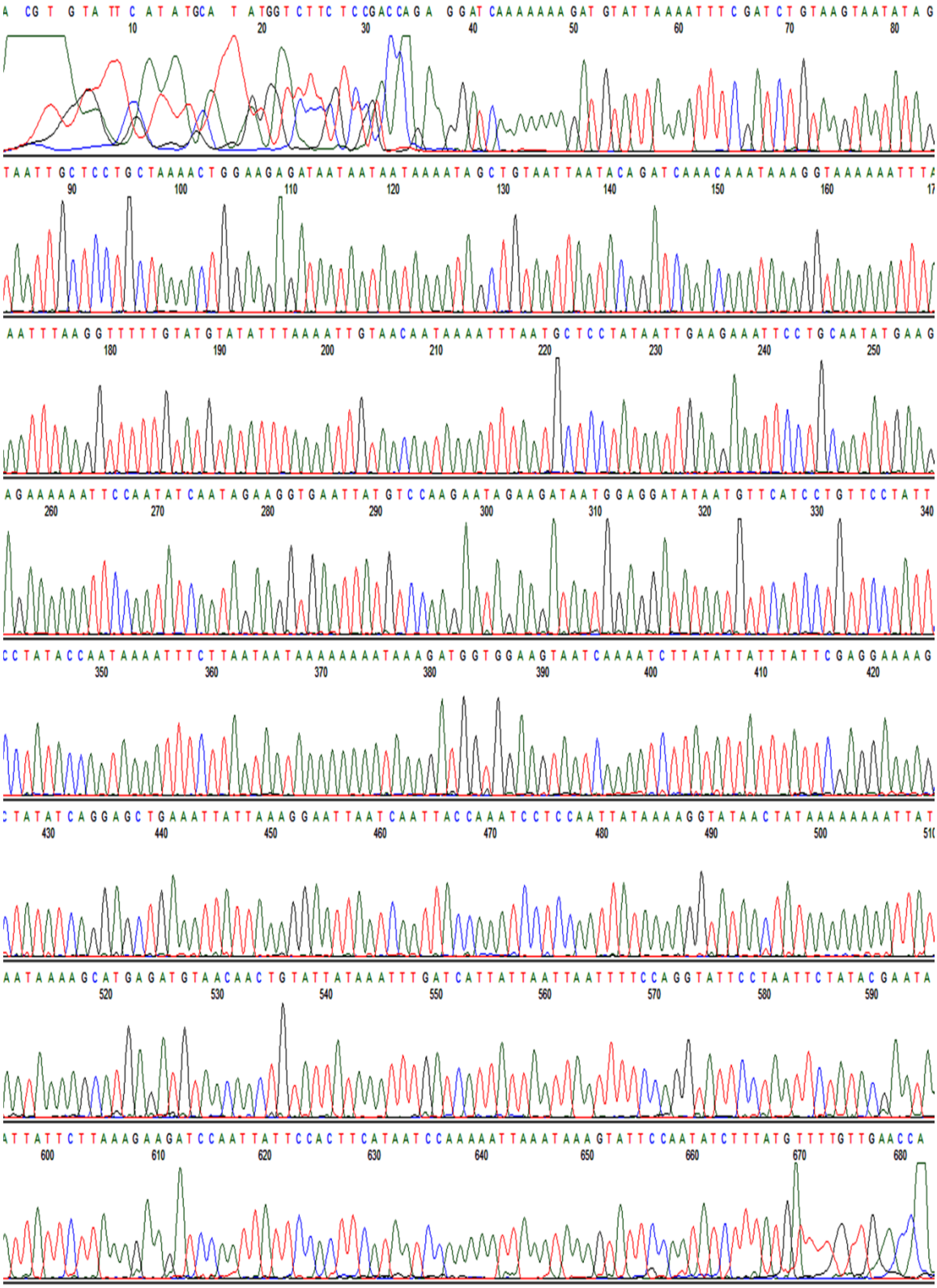

Fig.50. Sequencing chromatogram (reverse sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *D. conoideum* Voucher CUKAK4 (GenBank Accession Number: KM455119).

>*D. conoideum* Voucher CUKAK4 mitochondrial cytochrome oxidase subunit I (COI) partial nucleotide sequence, 621 bases

AATACTTTATTTAATTTTTGGATTATGAAGTGGAATAATTGGATCTTCTTT
AAGAATAATTATTCGTATAGAATTAGGAATACCTGGAAAATTAATTAATAA
TGATCAAATTTATAATACAGTTGTTACATCTCATGCTTTTATTATAATTTT
TTTTATAGTTATACCTTTTATAATTGGAGGATTTGGTAATTGATTAATTCC
TTTAATAATTTCAGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAG
ATTTTGATTACTTCCACCATCTTTATTTTTTTTATTATTAAGAAATTTTAT
TGGTATAGGAATAGGAACAGGATGAACATTATATCCTCCATTATCTTCTAT
TCTTGGACATAATTCACCTTCTATTGATATTGGAATTTTTTCTCTTCATAT
TGCAGGAATTTCTTCAATTATAGGAGCATTAAATTTTATTGTTACAATTTT
AAATATACATACAAAAACCTTAAATTTAAATTTTTTACCTTTATTTGTTTG
ATCTGTATTAATTACAGCTATTTTATTATTATTATCTCTTCCAGTTTTAGC
AGGAGCAATTACTATATTACTTACAGATCGAAATTTTAATACATCTTTTTT
TGATCCTTC

Fig.51. Partial coding sequence of *D. conoideum* Voucher CUKAK4 cytochrome oxidase subunit I (COI) gene (GenBank Accession Number: KM455119).

Score	Expect	Identities	Gaps	Strand
771 bits (417)	0.0	543/606 (90%)	0/606 (0%)	Plus/Plus

```
Query       AATACTTTATTTAATTTTTGGATTATGAAGTGGAATAATTGGATCTTCTTTAAGAATAAT  60
            |||| | ||||| |||||||| |||||||| |||||||||||||||| ||||||||||
Subject     AATATTATATTTTATTTTTGGTTTATGAAGAGGAATAATTGGATCTTCATTAAGAATAAT  60

Query       TATTCGTATAGAATTAGGAATACCTGGAAAATTAATTAATAATGATCAAATTTATAATAC  120
            ||||||  |||||||||||||||| ||||||||||  |||||| ||||||||||||||
Subject     TATTCGATTAGAATTAGGAATACCAGGAAAATTAATTGGTAATGACCAAATTTATAATAC  120

Query       AGTTGTTACATCTCATGCTTTTATTATAAttttttttATAGTTATACCTTTTATAATTGG  180
              |||||||||||||||||||||||||||||| |||||||||||||| ||||||||||
Subject     TATTGTTACATCTCATGCTTTTATTATAATTTTCTTTATAGTTATACCATTTATAATTGG  180

Query       AGGATTTGGTAATTGATTAATTCCTTTAATAATTTCAGCTCCTGATATAGCTTTTCCTCG  240
             |||||||| ||||||||| |||||||||||||||| |||||||||||||||| |||||
Subject     TGGATTTGGAAATTGATTAGTTCCTTTAATAATTTCTGCTCCTGATATAGCTTTCCCTCG  240

Query       AATAAATAATATAAGATTTTGATTACTTCCACCATCTTTAttttttttATTATTAAGAAA  300
            |||||||||||||| |||||||||| || || ||||||||| ||||||||||||||||
Subject     AATAAATAATATAAGTTTTTGATTACTACCCCCTTCTTTATTTCTTTTATTATTAAGAAA  300

Query       TTTTATTGGTATAGGAATAGGAACAGGATGAACATTATATCCTCCATTATCTTCTATTCT  360
            |||||||| |  ||  | |||||||||||||| | |||||||| |||||||| |||
Subject     TTTTATTGGAAATGGTGTTGGAACAGGATGAACACTTTATCCTCCTTTATCTTCAATTAC  360

Query       TGGACATAATTCACCTTCTATTGATATTGGAATTTTTTCTCTTCATATTGCAGGAATTTC  420
             |||||||||| |||||| | ||||||||||||||||||||||||||||||||||||||
Subject     AGGACATAATTCTCCTTCTGTAGATATTGGAATTTTTTCTCTTCATATTGCAGGAATTTC  420

Query       TTCAATTATAGGAGCattaaattttattgttacaattttaaatatacatacaaaaaccttt  480
            ||| ||||||||||||||||||||||||||||||| |||||||||| ||||||   ||
Subject     TTCTATTATAGGAGCATTAAATTTTATTGTTACAATTATAAATATACACACAAAATTTTT  480

Query       aaatttaaatttttttacctttatttgtttgatctgtattaattacagctattttattatt  540
            |||||||||||| ||||||||||  |||||| || |||||||| ||| ||||| ||||
Subject     AAATTTAAATTTCTTACCTTTATTTACTTGATCAGTTTTAATTACTGCTTTTTTACTATT  540

Query       attatCTCTTCCAGTTTTAGCAGGAGCAATTACTATATTACTTACAGATCGAAATTTTAA  600
            ||||||| | |||||||||||||||| ||||| |||||| | || ||||||||||||||
Subject     ATTATCTTTACCAGTTTTAGCAGGAGCTATTACAATATTATTAACTGATCGAAATTTTAA  600

Query       TACATC  606
            ||||||
Subject     TACATC  606
```

Fig.52. Nearest sequence match from the BLAST result of *D. conoideum* Voucher CUKAK4 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455119). Query = *D. conoideum* Voucher CUKAK4; Subject = *Eumenes asioboreus* (GenBank Accession Number: KJ634035.1). Note that the nearest match is 90% similar to the sequence in the database depicting that the sequence obtained is novel.

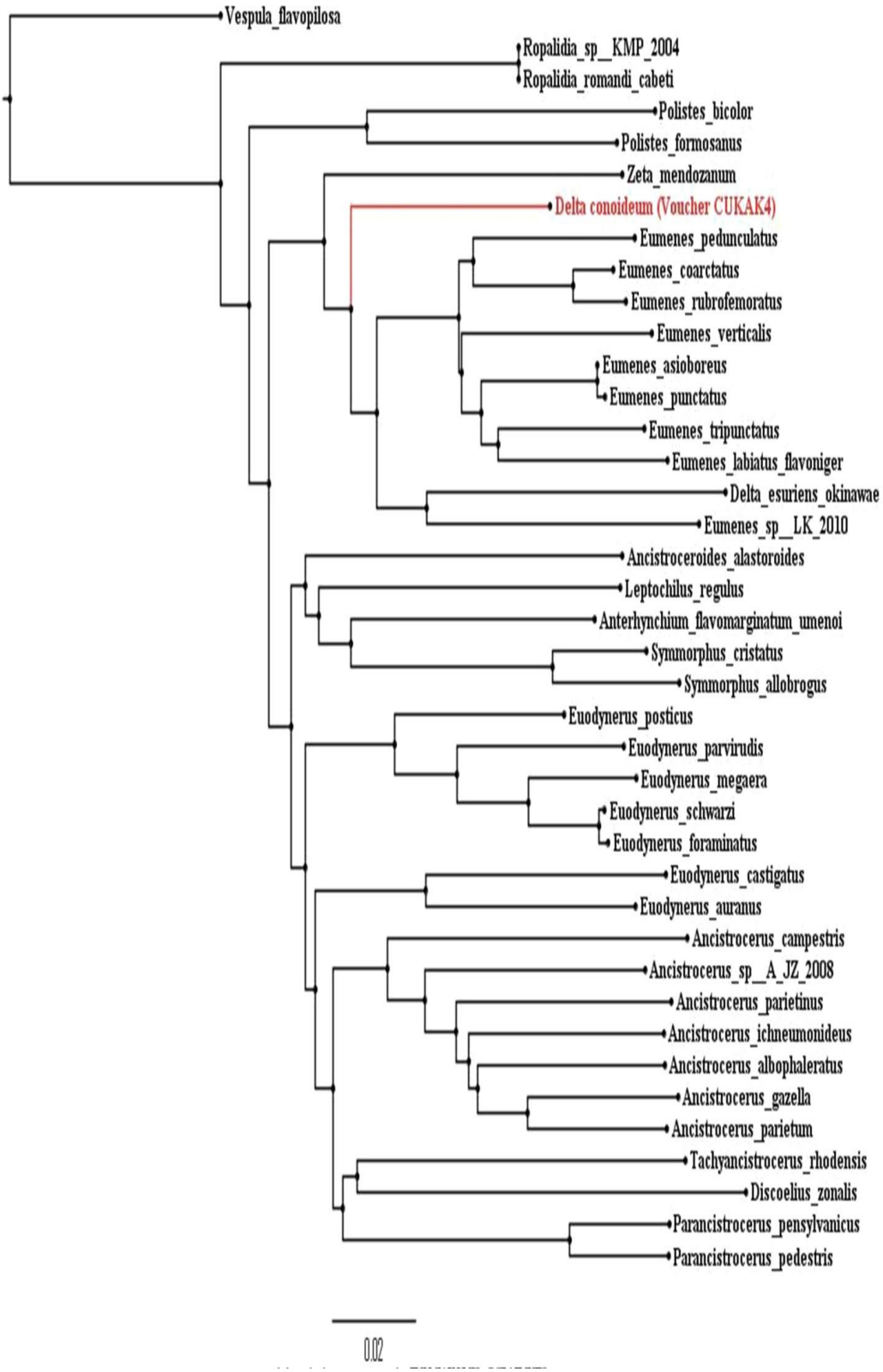

Fig.53. N-J tree plotted for *D. conoideum* Voucher CUKAK4 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455119).

> *D. conoideum* Voucher CUKAK4

MLYLIFGLWSGMIGSSLSMIIRMELGMPGKLINNDQIYNTVVTSHAFIMIFFMVMPF
MIGGFGNWLIPLMISAPDMAFPRMNNMSFWLLPPSLFFLLLSNFIGMGMGTGWTLYP
PLSSILGHNSPSIDIGIFSLHIAGISSIMGALNFIVTILNMHTKTLNLNFLPLFVWS
VLITAILLLLSLPVLAGAITMLLTDRNFNTSFFDPS

Fig.54. The conceptual translation product of the DNA sequence of mitochondrial cytochrome oxidase subunit I (COI) gene of *D. conoideum* Voucher CUKAK4.

Score	Expect	Method	Identities	Positives	Gaps
320 bits (820)	1e-107	Compositional matrix adjusts.	196/207 (95%)	201/207 (97%)	0/207 (0%)

```
Query       MLYLIFGLWSGMIGSSLSMIIRMELGMPGKLINNDQIYNTVVTSHAFIMIFFMVMPFMIG  60
            MLY IFGLWSGMIGSSLSMIIR+ELGMPGKLINNDQIYNT+VTSHAFIMIFFMVMPFMIG
Subject     MLYFIFGLWSGMIGSSLSMIIRLELGMPGKLINNDQIYNTIVTSHAFIMIFFMVMPFMIG  60

Query       GFGNWLIPLMISAPDMAFPRMNNMSFWLLPPSLFFLLLSNFIGMGMGTGWTLYPPLSSIL  120
            GFGNWL+PLMISAPDMAFPRMNNMSFWLLPPSLF LLLSNFIG.G+GTGWTLYPPLSSIL
Subject     GFGNWLVPLMISAPDMAFPRMNNMSFWLLPPSLFLLLLSNFIGNGVGTGWTLYPPLSSIL  120

Query       GHNSPSIDIGIFSLHIAGISSIMGALNFIVTILNMHTKTLNLNFLPLFVWSVLITAILLL  180
            GHNSPS+DIGIFSLHIAGISSIMGALNFIVTILNMHTK LNLNFLPLFIWSVLITAILLL
Subject     GHNSPSVDIGIFSLHIAGISSIMGALNFIVTILNMHTKLLNLNFLPLFAWSVLITAFLLL  180

Query       LSLPVLAGAITMLLTDRNFNTSFFDPS  207
            LSLPVLAGAITMLLTDRNFNTSFFDPS
Subject     LSLPVLAGAITMLLTDRNFNTSFFDPS  207
```

Fig.55. Nearest peptide sequence match from the BLAST result of *D. conoideum* Voucher CUKAK4 cytochrome oxidase subunit I (COI) conceptual translation product. Query = *Delta conoideum* Voucher CUKAK4; Subject = *Eumenes verticalis* (GenBank Accession Number: ACE81461.1). Note that the nearest match is only 95% similar to the peptide sequence in the database depicting that the sequence obtained is novel.

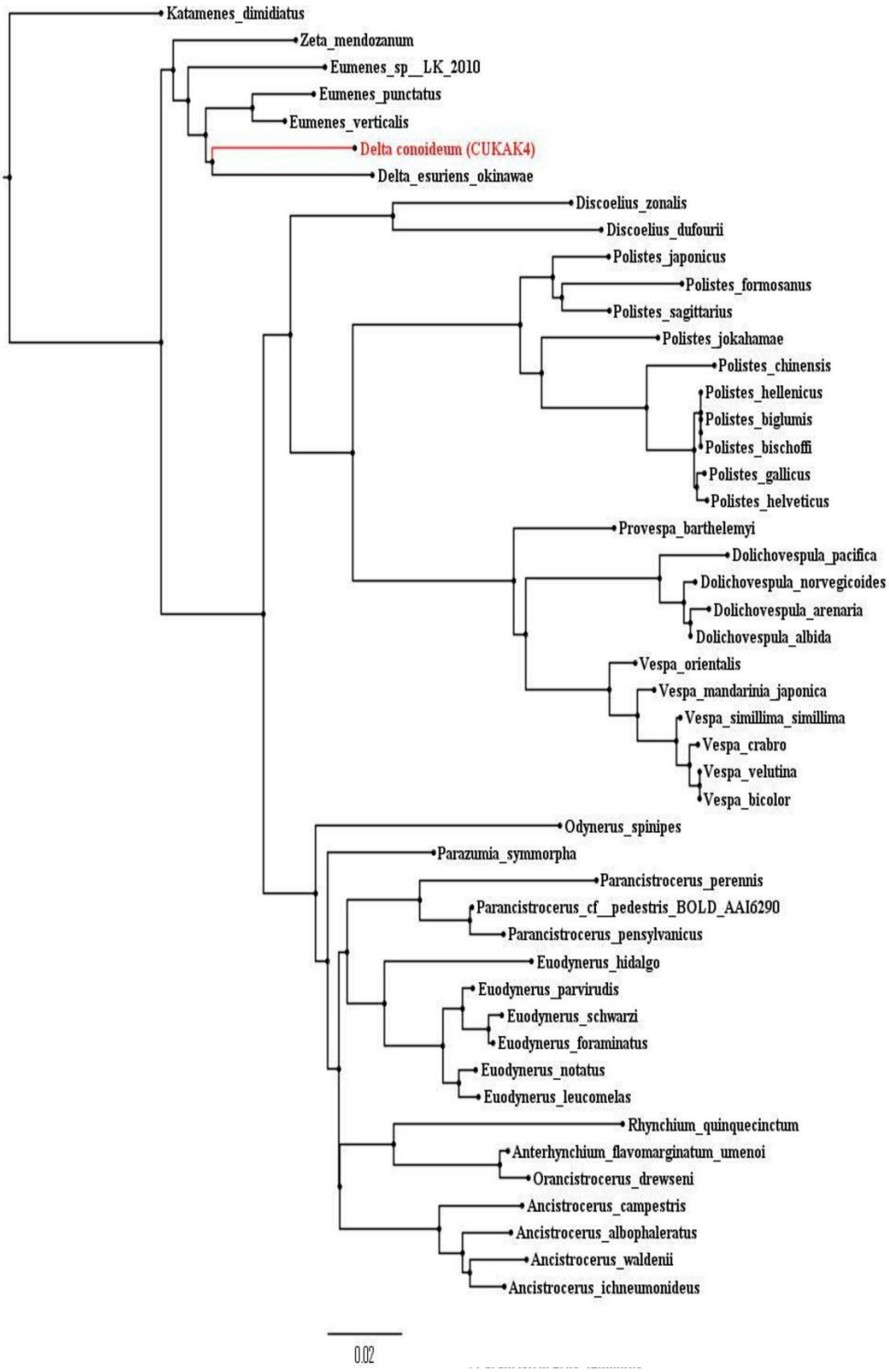

Fig.56. N-J tree plotted for conceptual translation product of *D. conoideum* Voucher CUKAK4 cytochrome oxidase subunit I (COI) peptide sequence

5. *Phimenes flavopictum*

Phimenes flavopictum (**Figures 57-60**) is a predatory wasp having yellow markings on its black body, giving it coloring that resembles that of a yellow and black striped tiger, hence it is commonly called 'tiger striped potter wasp'. It is also known for its flower visiting and pollination activity. They play important roles as predators of pest insects in gardens and farms.

5.1. History of nomenclature

Eumenes arcuata continentalis. Zimmermann, 1931. *Zietschr*. *Morph. Oek. Tiere*, 22: 203.

Eumenes flavopictus continentalis (Zimmermann). Van der Vecht, 1959. *Zool. Verh. L eiden*, 41: 36.

Phimenes flavopictum continentale (Zimmermann). Gusenleitner, 2006. Linzer *Boil. Beitr*, 38: 694.

(Srinivasan and Kumar, 2010)

5.2. Diagnosis

The species collected was identical to those described by Srinivasan and Kumar (2010). The body was black with following yellow markings: Clypeus, interantennal space, inner orbit, ocular sinus, a line behind the eyes, pronotum in front, two curved spots and two parallel longitudinal lines on mesoscutum, a broad outer border to the tegula, a spot on each side of the scutellum, a broad line on the posterior margin of postscutellum, a broad vertical mark on mesopleuron, sides of dorsum of propodeum

Fig.57. *Phimenes flavopictum* Voucher CUKAK5 lateral view (scale in cm).

Fig.58. *Phimenes flavopictum* Voucher CUKAK5 upper view (scale in cm).

Fig.59. *Phimenes flavopictum* Voucher CUKAK5 front view.

Fig.60. *Phimenes flavopictum* Voucher CUKAK5 wing pattern.

(with median Maltese cross-shaped black mark), two small lateral spots at the base of the petiole, two about the middle and a subapical band of the same above, two large pyriforme spots near the base of second gastral segment, two minute lateral spots on second gastral sternites, subapical interrupted bands on the posterior margins of second and the following segments. Legs black, variegated with yellow. Clypeus smooth, pyriforme, the apex sharply truncated; frons, vertex and thorax with fine shallow punctures, petiole and gaster smooth. Wings fuscohyaline.

5.3. Systematic position

Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Hymenoptera; Apocrita; Aculeata; Vespoidea; Vespidae; Eumeninae; *Phimenes*; *flavopictum.*

5.4. Distribution

In India it is reported from Arunachal Pradesh, Karnataka, Kerala, Meghalaya, Sikkim, Uttarakhand and West Bengal. It is also distributed in China, Hong Kong, Indonesia (Bangka, Sumatra, Sunda), Malaysia, Myanmar, Singapore and Thailand (Srinivasan and Kumar, 2010).

5.5. Collection

Phimenes flavopictum, used in the present study was collected from Kottayam District of Kerala 9° 28' 0" North, 76° 33' 0" East.

5.6. PCR amplification of cytochrome c oxidase subunit I gene of *Phimenes flavopictum* Voucher CUKAK5

The gel pictures showing genomic DNA and the PCR product of COI gene of *Phimenes flavopictum* are shown in **Figures 61 and 62**. The PCR of the COI gene fragment of *P. flavopictum* from Kottayam yielded a product of 658bp. The forward and reverse sequence chromatograms, COI sequence obtained, BLASTn result, conceptual translation product and BLASTp result are presented in **Figures 63- 66, 68 and 69.** The sequence obtained is deposited in GenBank (GenBank Accession Number: KM455120).

The COI sequence of *P. flavopictum* in the present study can be used for its accurate taxonomic identification. The nucleotide BLAST against the nucleotide redundant database showed that the cytochrome oxidase gene sequence obtained is novel. The hypervariable region of DNA of *P. flavopictum* is 91% similar to that of *Eumenes rubronotatus* COI gene (GenBank Accession Number: KJ634030.1). The conceptual translation of partial COI gene of *P. flavopictum* yielded a peptide of 219 amino acids. The peptide blast of COI gene of *P. flavopictum* showed 95% of similarity to that of cytochrome oxidase subunit I of *Delta esuriens okinawae* (GenBank Accession Number: KJ634030.1). The results of the BLASTp indicated that the peptide of the cytochrome oxidase subunit I (COI) gene of *P. flavopictum* collected from Kottayam is novel. The

mtDNA sequence of different wasps confirms the species differences of *P. flavopictum* from others.

5.7. Molecular phylogeny of *Phimenes flavopictum* Voucher CUKAK5

The phylogenetic trees of DNA and peptide are plotted using Neighbour-Joining method and are exhibited in **Figures 67 and 70.** The DNA tree showed *Phimenes flavopictum* to be closer to *Delta esuriens okinawae,* having a single origin. They are closely related to the species of the genus *Eumenes* viz. *Eumenes verticalis, Eumenes crucifera, Eumenes pedunculatus, Eumenes coarctatus* and a Hymenoptera sp BOLD ACG0063 having a common ancestor. The wasp *Zeta mendozanum* diverged from the common ancestor of the cluster containing *Eumenes* species, *P. flavopictum*, *Delta esuriens* and the Hymenoptera sp.

The DNA tree also showed the relationship pattern of *P. flavopictum* to the species of the genus *Parancistrocerus* viz. *Parancistrocerus pensylvanicus, P. pedestris, P. leionotus; Anterhynchium flavomarginatum, Monobia quadridens*; species of the genus *Euodynerus* viz. *Euodynerus notatus, Euodynerus leucomelas, Euodynerus parvirudis, Euodynerus schwarzi, Euodynerus foraminatus;* species of the genus *Ancistrocerus* viz. *Ancistrocerus albophaleratus* and *A. waldenii.*

The peptide tree showed that *Phimenes flavopictum* is more closely related to *Katamenes dimidiatus* (GenBank Accession Number: GU596835.1) having a single origin. The peptide tree also showed the relationship of *P. flavopictum* to the species of the genus *Eumenes* viz. *Eumenes sp.* LK 2010, *Eumenes pedunculatus, Eumenes fraternus, Eumenes crucifera, Eumenes verticalis, Eumenes labiatus flavoniger, Eumenes punctatus, Eumenes asioboreus, Eumenes tripunctatus, Eumenes coarctatus, Eumenes rubrofemoratus* and *Eumenes rubronotatus;* species of the genus *Ancistrocerus* viz. *Ancistrocerus waldenii, Ancistrocerus catskill, Ancistrocerus albophaleratus* and *Ancistrocerus parietum; Delta esuriens,* a Hymenoptera species and *Leptochilus regulus.* The species of the genus *Eumenes* and *Ancistrocerus* arose from a common ancestor. The peptide tree also showed the relationship pattern of *P. flavopictum* to the species of the subfamily Polistinae and Vespinae. The most distantly related species to *P. flavopictum* from the phylogeny tree is found to be *Symmorphus crassicornis*, a wasp of the subfamily Eumeninae.

The present results indicated the significance of an identification system based on COI gene sequence. The result highlighted the significant sequence diversity of the COI gene of *P. flavopictum* from its allied species, thus enabling an easy discrimination of species.

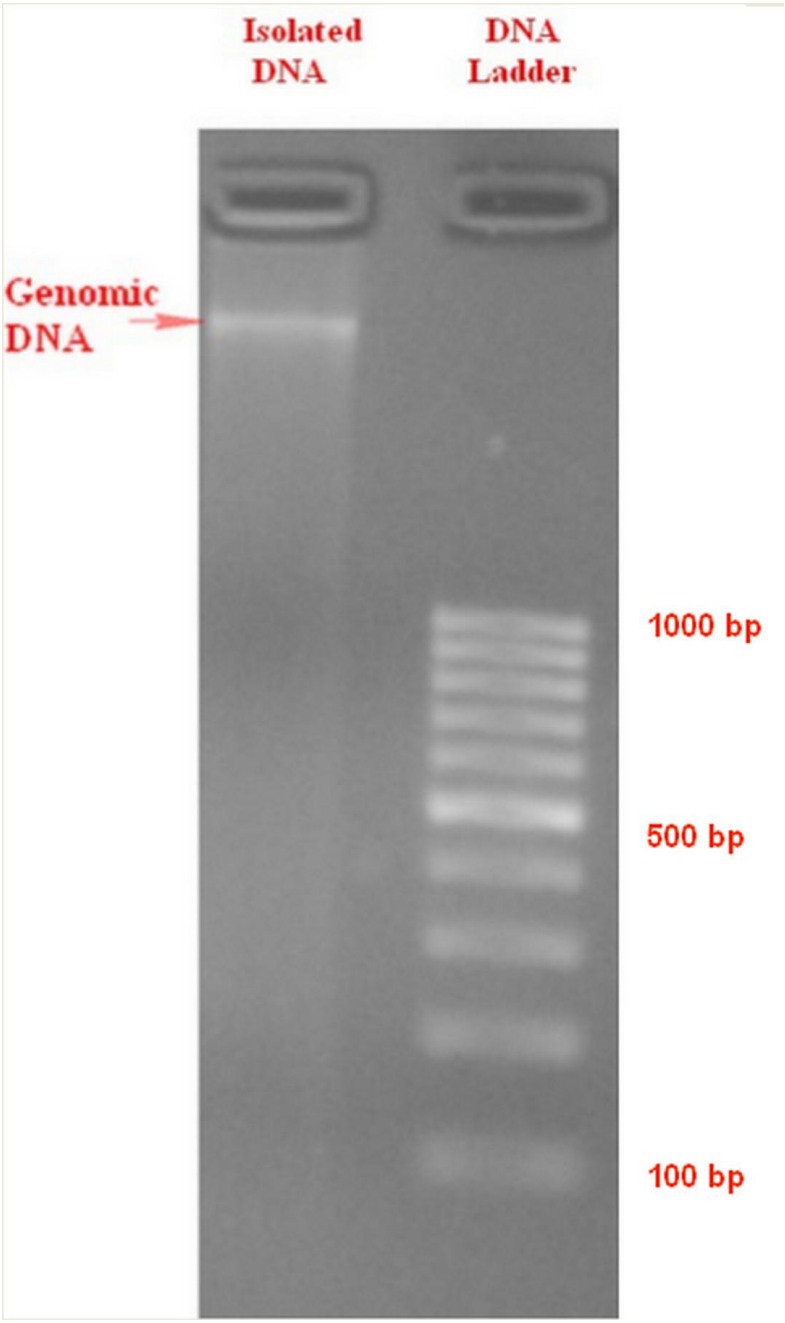

Fig.61. Gel picture of total DNA isolated from *Phimenes flavopictum* Voucher CUKAK5 .

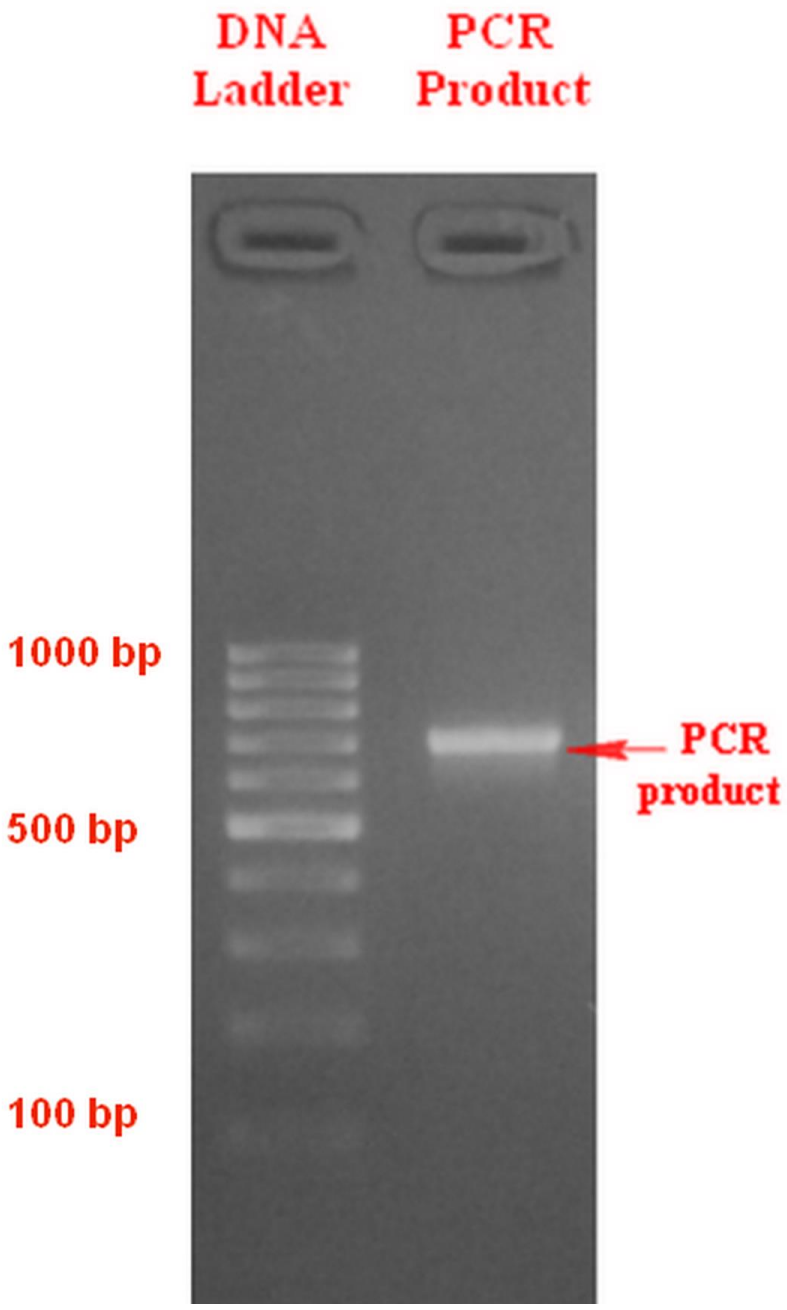

Fig.62. Gel picture showing the PCR product of partial COI gene of *Phimenes flavopictum* Voucher CUKAK5.

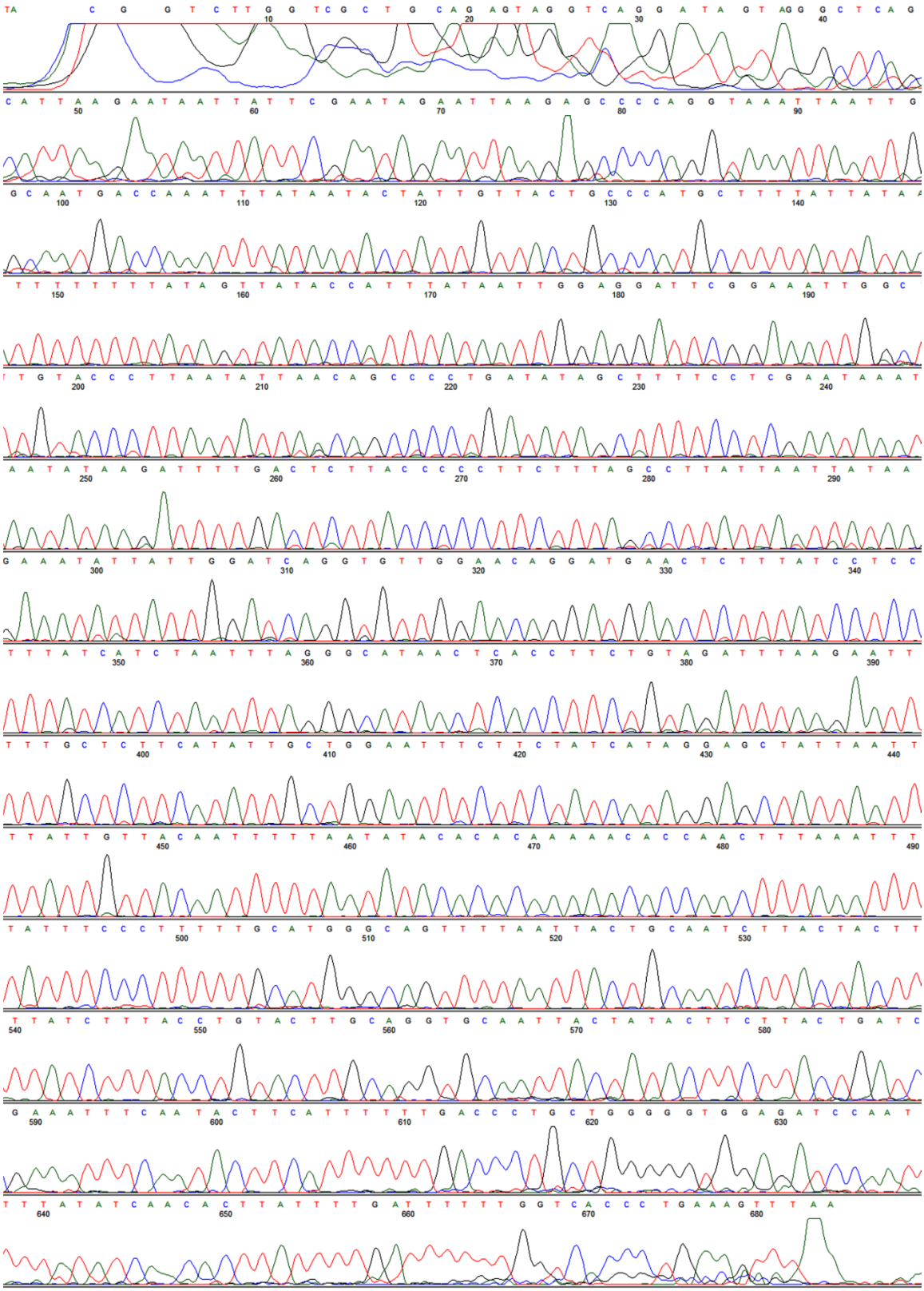

Fig.63. Sequencing chromatogram (forward sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *P. flavopictum* Voucher CUKAK5 (GenBank Accession Number: KM455120).

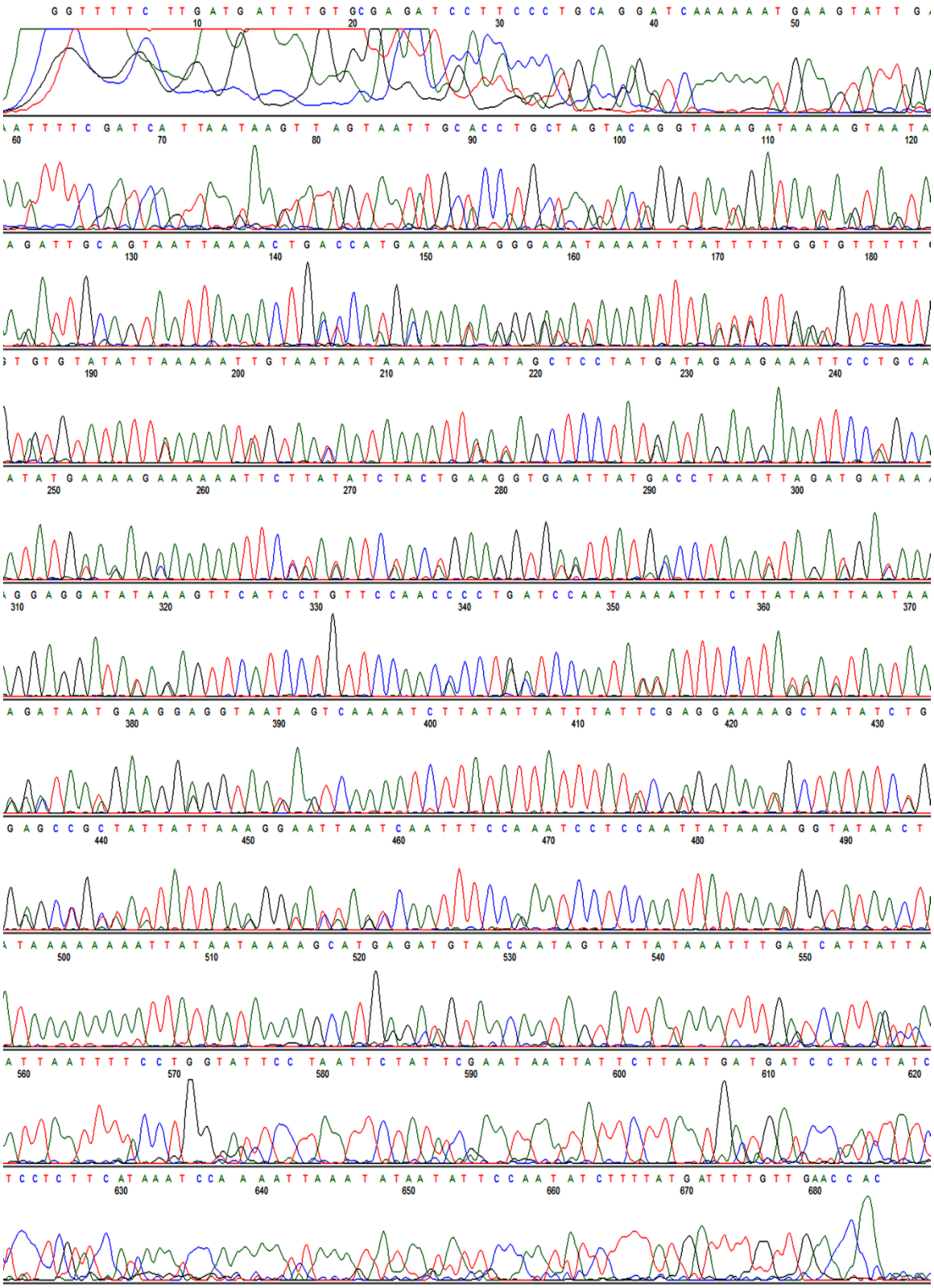

Fig.64. Sequencing chromatogram (reverse sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *P. flavopictum* Voucher CUKAK5 (GenBank Accession Number: KM455120).

>*P. flavopictum* Voucher CUKAK5 mitochondrial cytochrome oxidase subunit I (COI) partial nucleotide sequence, 658 bases

AATATTATATTTAATTTTTGGTTTATGAAGAGGGATAATTGGATCATCATT
AAGAATATTAATTCGTATAGAATTAGGAATACCAGGAAAATTAATTAATAA
TGATCAAATTTTTAATACTATTGTTACATCTCATGCTTTTATTATAATTTT
TTTTATAGTTATACCTTTTATAATTGGAGGATTTGGAAATTGATTAATTCC
TTTAATAATTGCGGCTCCAGATATAGCATTTCCACGTATAAATAATATAAG
ATTTTGATTATTACCTCCTTCATTATTTTTATTATTATTAAGAAATTATAT
TGGAGTAGGGGTTGGAACAGGATGAACTTTATATCCTCCTTTGTCTTCTAT
TTTAGGTCATAATACACCTTCAGTTGATATTGGAATTTTTTCTTTACATAT
TGCAGGAATTTCTTCTATTATAGGAGCTTTAAATTTTATTATTACTATTTT
AAATATACATACAAAAACTTTAAAAATAAATTTTTTACCTTTATTTTCTTG
ATCAGTTTTAATTACTGCATTTTTATTATTATTATCATTACCAGTTTTAGC
TGGAGCTATTACAATATTATTAACTGATCGAAATTTTAATACTTCTTTTTT
TGATCCTTCAGGGGGGGGTGATCCAATTTTATATCAACATTTATTT

Fig.65. Partial coding sequence of *P. flavopictum* Voucher CUKAK5 cytochrome oxidase subunit I (COI) gene (GenBank Accession Number: KM455120).

Score	Expect	Identities	Gaps	Strand
811 bits (439)	0.0	552/608 (91%)	2/608 (0%)	Plus/Plus

```
Query       AATATTATATTTAATTTTTGGTTTATGAAGAGGGATAATTGGATCATCATTAAGAATATT  60
            |||||||||||| |||||||||||||||||| |||||||||||||||||||||||||| |
Subject     AATATTATATTTTATTTTTGGTTTATGAAGAGGTATAATTGGATCATCATTAAGAATAAT  60

Query       AATTCGTATAGAATTAGGAATACCAGGAAAATTAATTAATAATGATCAAATTTTTAATAC  120
             |||||  |||||||||||||||||||||||||||||||||||||| |||||||| ||||||
Subject     TATTCGATTAGAATTAGGAATACCAGGAAAATTAATTAATAATGACCAAATTTATAATAC  120

Query       TATTGTTACATCTCATGCTTTTATTATAAttttttttATAGTTATACCTTTTATAATTGG  180
            |||||| || |||||||||||||||||||||| |||||||||||||| ||||||||||||
Subject     TATTGTAACTTCTCATGCTTTTATTATAATTTTCTTTATAGTTATACCATTTATAATTGG  180

Query       AGGATTTGGAAATTGATTAATTCCTTTAATAATTGCGGCTCCAGATATAGCATTTCCACG  240
            |||||||||||||||||| ||||| |||||||| | || || |||||||||||||| ||
Subject     AGGATTTGGAAATTGATTAGTTCCTCTAATAATTTCTGCCCCTGATATAGCATTTCCTCG  240

Query       TATAAATAATATAAGattttgattattacctccttcattatttttattattattaagaaa  300
            |||||||||||||||||||||||||||||| ||||| |||||  | ||||||||||||||
Subject     TATAAATAATATAAGATTTTGATTATTACCACCTTCTTTATTCCTTTTATTATTAAGAAA  300

Query       ttatattGG-AGTAGGGGTTGGAACAGGATGAACTTTATATCCTCCTTTGTCTTCTATTT  359
            || |||||| | | || || ||||||||||||||| | || || ||||| |||||||||
Subject     TTTTATTGGTAAT-GGTGTAGGAACAGGATGAACTCTTTACCCACCTTTATCTTCTATTA  359

Query       TAGGTCATAATACACCTTCAGTTGATATTGGAATTTTTTCTTTACATATTGCAGGAATTT  419
             |||||||||| | ||||| || ||||||||||||||||||||||||||||||||||||
Subject     CAGGTCATAATTCTCCTTCTGTAGATATTGGAATTTTTTCTTTACATATTGCAGGAATTT  419

Query       CTTCTATTATAGGAGCtttaaattttattattactattttaaatatacatacaaaaactt  479
            ||||||||||||||| |||||||| ||| |||| ||||||||||||||||||||   |
Subject     CTTCTATTATAGGAGCATTAAATTTCATTGTTACAATTTTAAATATACATACAAAATTAT  479

Query       taaaaataaatttttttaccttttattttcttgatcagttttaattactgcatttttattat  539
            ||||  |||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Subject     TAAATTTAAATTTTTTACCTTTATTTTCTTGATCAGTTTTAATTACTGCTTTTTTATTAT  539

Query       tattatcattaccagttttaGCTGGAGCTATTACAATATTATTAACTGATCGAAATTTTA  599
            |||||||||||||| |||||||| ||||| ||||| |||||||||||||||||||||||||
Subject     TATTATCATTACCTGTTTTAGCAGGAGCAATTACTATATTATTAACTGATCGAAATTTTA  599

Query       ATACTTCt  607
            |||| |||
Subject     ATACATCT  607
```

Fig.66. Nearest sequence match from the BLAST result of *P. flavopictum* Voucher CUKAK5 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455120). Query = *P. flavopictum;* Subject = *Eumenes rubronotatus* (GenBank Accession Number: KJ634030.1). Note that the nearest match is 91% similar to the sequence in the database depicting that the sequence obtained is novel.

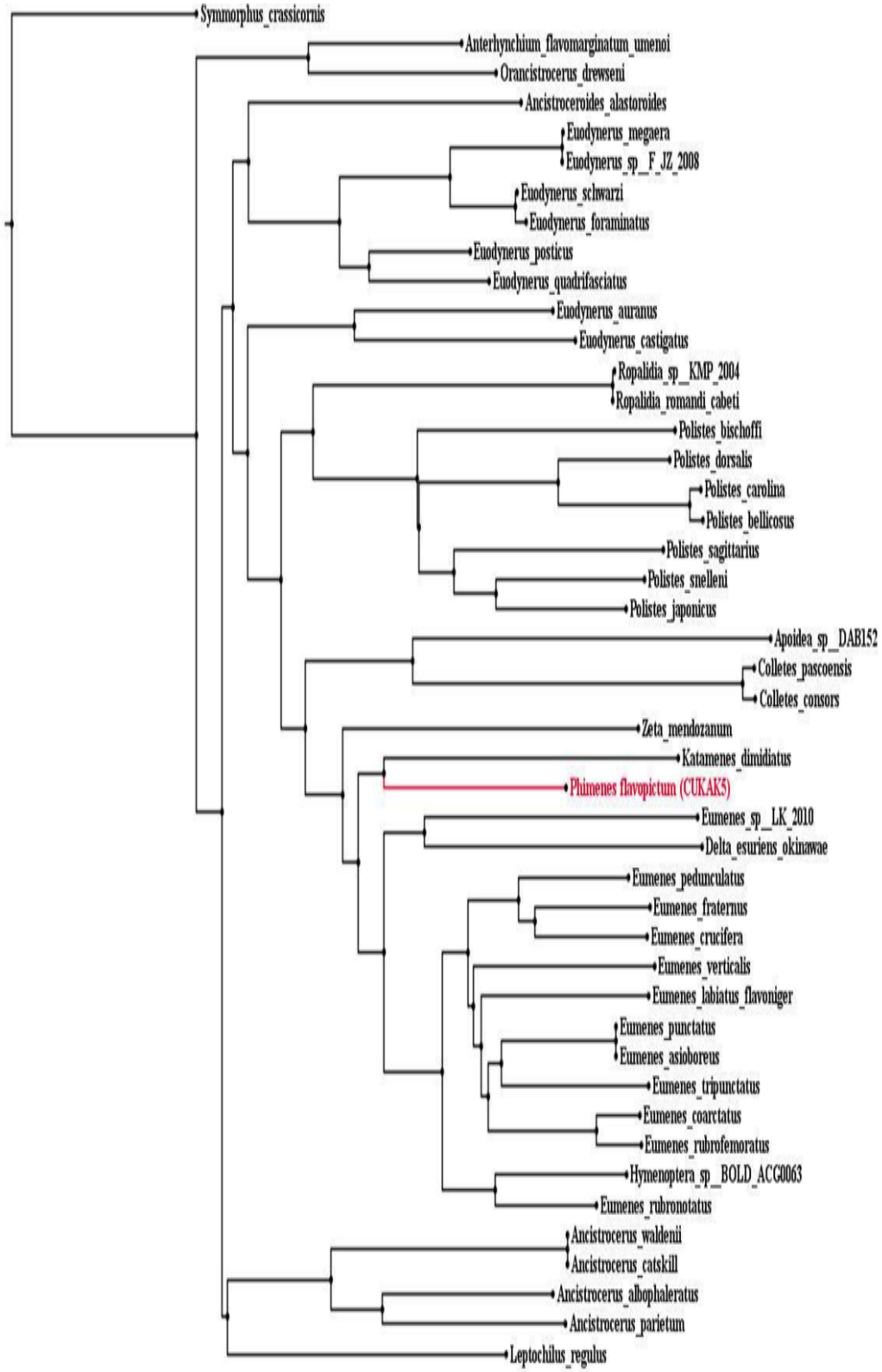

Fig.67. N-J tree plotted for *P. flavopictum* Voucher CUKAK5 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455120).

>*P. flavopictum* CUKAK5

MLYLIFGLWSGMIGSSLSMLIRMELGMPGKLINNDQIFNTIVTSHAFIMIFFMVMPF
MIGGFGNWLIPLMIAAPDMAFPRMNNMSFWLLPPSLFLLLLSNYIGVGVGTGWTLYP
PLSSILGHNTPSVDIGIFSLHIAGISSIMGALNFIITILNMHTKTLKMNFLPLFSWS
VLITAFLLLLSLPVLAGAITMLLTDRNFNTSFFDPSGGGDPILYQHLF

Fig.68. The conceptual translation product of the DNA sequence of mitochondrial cytochrome oxidase subunit I (COI) gene of *P. flavopictum* Voucher CUKAK5.

Score	Expect	Method	Identities	Positives	Gaps
370 bits (949)	4e-127	Compositional matrix adjust.	208/219(95%)	218/219 (99%)	0/219 (0%)

```
Query       MLYLIFGLWSGMIGSSLSMLIRMELGMPGKLINNDQIFNTIVTSHAFIMIFFMVMPFMIG  60
            MLYLIFG+WSGMIGSSLSM+IRMELG+PGKLINNDQI+NTIVTSHAFIMIFFMVMPFMIG
Subject     MLYLIFGMWSGMIGSSLSMIIRMELGIPGKLINNDQIYNTIVTSHAFIMIFFMVMPFMIG  60

Query       GFGNWLIPLMIAAPDMAFPRMNNMSFWLLPPSLFLLLLSNYIGVGVGTGWTLYPPLSSIL  120
            GFGN+L+PLMIAAPDMAFPRMNNMSFWLLPPSLFLLLLSNYIG GVGTGWTLYPPLSSIL
Subject     GFGNYLVPLMIAAPDMAFPRMNNMSFWLLPPSLFLLLLSNYIGSGVGTGWTLYPPLSSIL  120

Query       GHNTPSVDIGIFSLHIAGISSIMGALNFITILNMHTKTLKMNFLPLFSWSVLITAFLLL  180
            GHNTPSVDIGIFSLHIAGISSIMGALNFI+TILNMHTKTLK+N+LPLF+WSVLITAFLLL
Subject     GHNTPSVDIGIFSLHIAGISSIMGALNFIVTILNMHTKTLKLNYLPLFAWSVLITAFLLL  180

Query       LSLPVLAGAITMLLTDRNFNTSFFDPSGGGDPILYQHLF  219
            LSLPVLAGAITMLLTDRNFNTSFFDPSGGGDPILYQHLF
Subject     LSLPVLAGAITMLLTDRNFNTSFFDPSGGGDPILYQHLF  219
```

Fig.69. Nearest peptide sequence match from the BLAST result of *P. flavopictum* Voucher CUKAK5 cytochrome oxidase subunit I (COI) conceptual translation product. Query = *P. flavopictum* CUKAK5; Subject = *Delta esuriens okinawae* (GenBank Accession Number: BAP05526.1). Note that the nearest match is only 95% similar to the peptide sequence in the database depicting that the sequence obtained is novel.

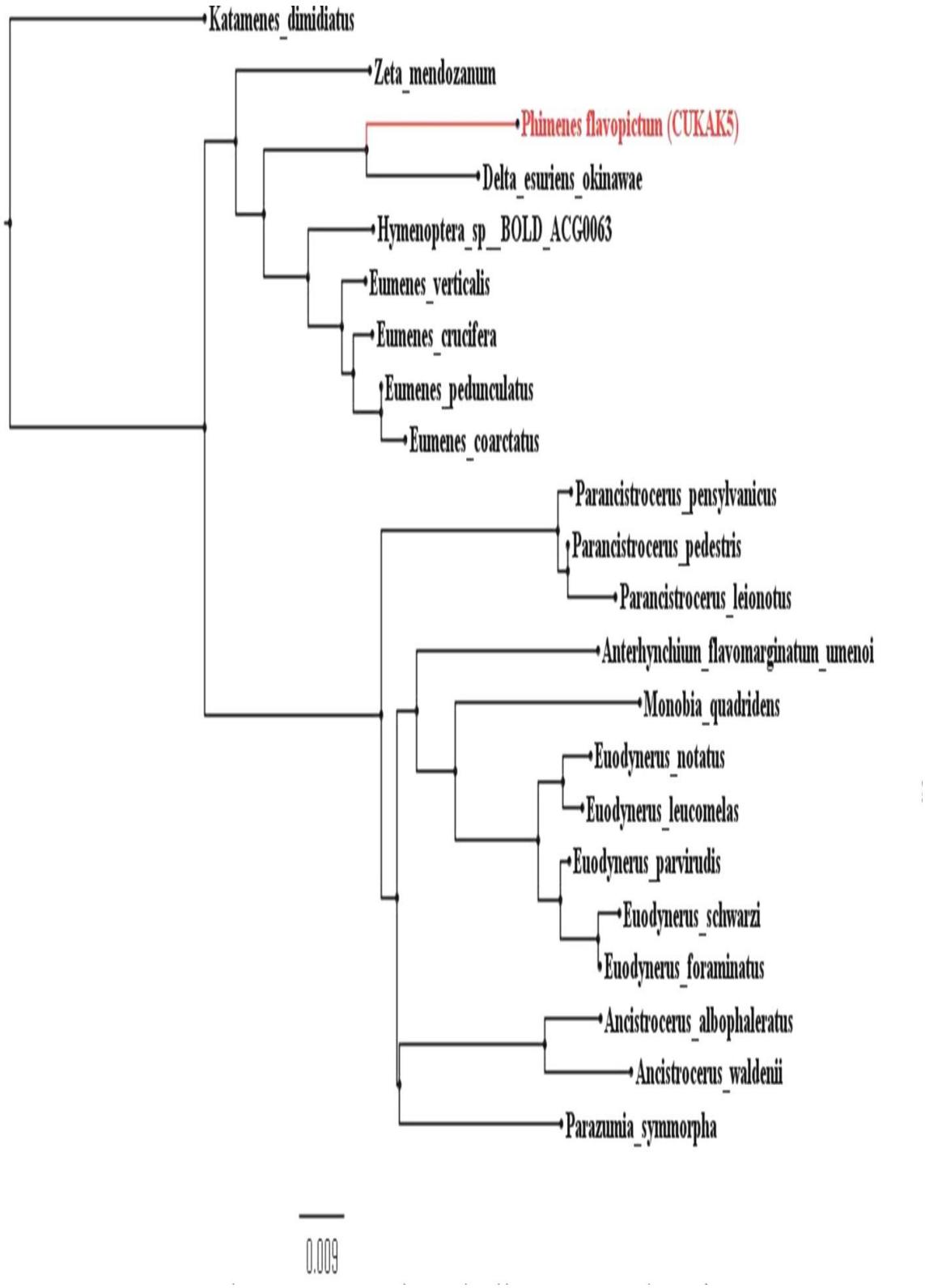

Fig.70. N-J tree plotted for conceptual translation product of *P. flavopictum* Voucher CUKAK5 cytochrome oxidase subunit I (COI) peptide sequence.

6. *Ropalidia spatulata*

Ropalidia spatulata (**Figures 71-74**), is a common paper wasp which builds nest using long fine plant fibers and wasp adult oral secretion, looking like a nest made of paper. It is also known for its pollination activity.

6.1. History of nomenclature

Icaria ferruginea (Fabricius). Saussure, 1853. *Etud. fam. Vespidae*, 2**:** 38; Bingham, 1877. *Fauna of British India, Hymenoptera*, 1: 386, 387; Rothnes, 1903. *Trans. Ent. Soc.* London, 1903: 107.

Ropalidia marginata indica. Van der Vecht, 1941. *Treubia*, 18: 121.

Ropalidia (Icariola) spathulata. Richards, 1978. *Aust. J. Zool. Suppl. Ser,* 61: 58.

Ropalidia spatulata. Van der Vecht, 1962. *Zool. Verh.,* 57: 9; Yamane and Yamane, 1979. *Insecta Matsumurana*, 15: 4, 6, 32.

(Lambert, 2002)

6.2. Diagnosis

The species collected was identical to those described by Lambert (2002). Body was reddish brown with yellow and black marking. Yellow marking as follows: anterior margin of clypeus broadly, a spot at base of mandible towards clypeus, a line below antennal scape, another line on basal margin of pronotum, two elongated marks on apical half of propodeum, a broad mark on fore coxa in front, middle metatarsus, hind metatarsus except a small basal part, second to fourth hind tarsal segments, a broad band

Fig.71. *Ropalidia spatulata* Voucher CUKAK6 lateral view (scale in cm).

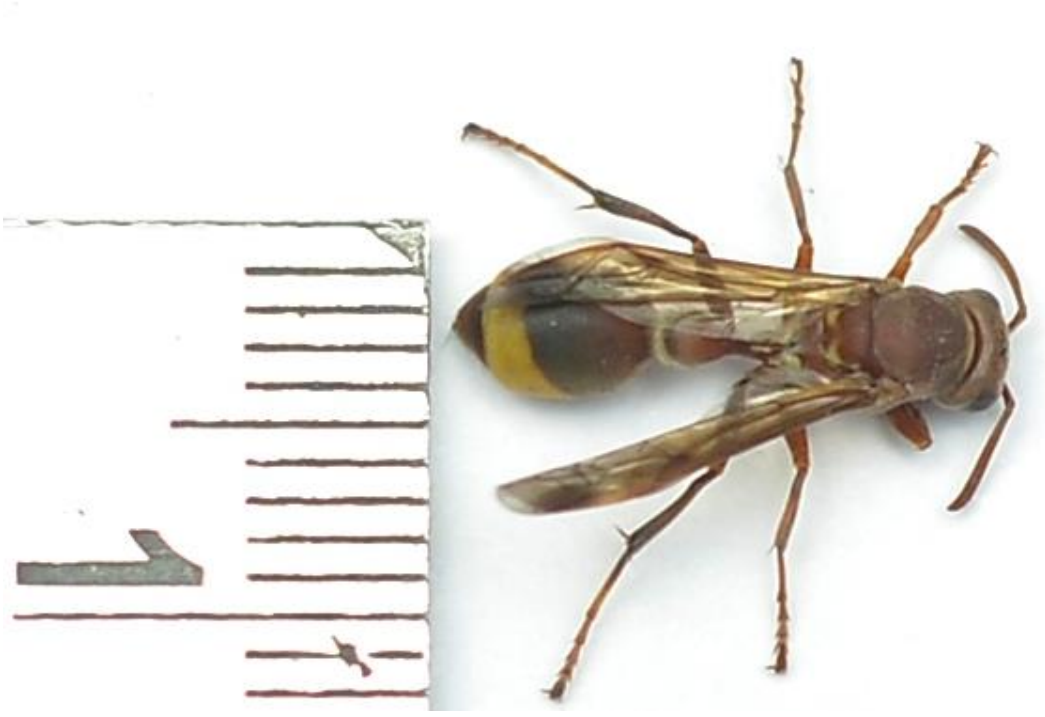

Fig.72. *Ropalidia spatulata* Voucher CUKAK6 upper view (scale in cm).

Fig. 73. *Ropalidia spatulata* Voucher CUKAK6 front view

Fig.74. *Ropalidia spatulata* Voucher CUKAK6 wing pattern.

on apical petiole, and another broad band on apical second metasomal tergite. Black mark as follows: a faint triangular mark at base of mandible just next to yellow mark, lateral margins of clypeus narrowly, a circular spot on antennal socket dorsally, ocelli along margin, a faint irregular band next to yellow basal margin of propodeum, a narrow margin on mesoscutum, a broad mark at base and apex of mesoscutum, postscutellum faintly at base, suture between mesopleuron and metapleuron, postscutellum and propodeum broadly, mesosternum, metapleuron, a faint mark surrounding two yellow marks on propodeum, and a band adjacent to apical yellow band on first and second metasomal tergite. Hind tibia black, dorsally. Wings with brownish tint veins, radial cell with apical cloud.

6.3. Systematic position

Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Hymenoptera; Apocrita; Aculeata; Vespoidea; Vespidae; Polistinae; *Ropalidia*; *spatulata.*

6.4. Distribution

It is reported from Nepal and in India reported from Kerala (Kojima *et al*., 2007).

6.5. Collection

Ropalidia spatulata, used in the present study was collected from Calicut District of Kerala at 11° 11' 0" North, 75° 51' 0" East.

6.6. PCR amplification of the cytochrome c oxidase subunit I gene of *Ropalidia spatulata* Voucher CUKAK6.

The gel pictures showing genomic DNA and the PCR product of COI gene of *Ropalidia spatulata* Voucher CUKAK6 are shown in **Figures 75 and 76**. The PCR of the COI gene fragment of *R. spatulata* from Calicut yielded a product of 484bp. The forward and reverse sequence chromatograms, COI gene sequence obtained, BLASTn result, conceptual translation product and BLASTp result are presented in **Figures 77-80, 82 and 83.** The sequence obtained is deposited in the GenBank (GenBank Accession Number: KM455121).

The COI sequence of *R. spatulata* in the present study can be used for its accurate taxonomic identification. The nucleotide BLAST against the nucleotide redundant database showed that the cytochrome c oxidase gene sequence obtained is novel. The hypervariable region of DNA of *R. spatulata* is 86% similar to that of *Ropalidia romandi cabeti* COI gene (GenBank Accession Number AF146677.1).

The conceptual translation of partial COI gene of *R. spatulata* yielded a peptide of 161 amino acids. The peptide BLAST of COI gene of *R. spatulata* showed 96% similarity to that of cytochrome oxidase subunit I of *Polybioides melainus* (ADD60380.1). The results of the BLASTp indicated that the peptide of the cytochrome

oxidase subunit I (COI) gene of *R. spatulata* collected from Calicut is novel. The mtDNA sequence of different wasps confirms the species differences of *R. spatulata* from others.

6.7. Molecular phylogeny of *Ropalidia spatulata* Voucher CUKAK6

The phylogenetic trees of DNA and peptide are plotted using Neighbour-Joining method and are exhibited in **Figures 81 and 84**.

The DNA tree showed that *R. spatulata* is closer to *Vespa bicolor*, having a single origin. They are closely related to the species of the genera *Dolichovespula* viz. *Dolichovespula arenaria* and *Dolichovespula pacifica; Vespula* viz. *Vespula vidua, Vespula consobrina* and *Vespula acadica*, having a common ancestor. The DNA tree also depicted the relationship pattern of *R. spatulata* to the species *Zeta mendozanum, Euodynerus quadrifasciatus,* species of the genus *Ropalidia, Mischocyttarus flavitarsis, Chartergillus communis*, species of the genus *Apoica, Protopolybia*, *Pseudopolybia* and *Polistes.* The most distantly related species to *R. spatulata* according to DNA tree is found to be *Belanogaster juncea juncea,* a species of the subfamily Polistinae.

The peptide tree showed that *R. spatulata* is more closely related to *Polybioides melainus* and the cluster containing the species of the genera *Chartergillus* viz. *Chartergillus amazonicus, Chartergillus communis; Polybia sericea* and *Metapolybia cingulata* having a single origin; *Pseudopolybia vespiceps, Agelaia pallipes, Apoica*

pallens, Apoica strigata, Apoica pallida, Apoica ambracarina, Apoica flavissima, Apoica gelida, Apoica thoracica, Apoica albimacula; species of the genus *Polistes* viz. *Polistes marginalis, Polistes chinensis antennalis, Polistes nimpha, Polistes gallicus, Polistes dominulus, Polistes japonicas, Polistes sagittarius, Polistes stigma, Polistes olivaceus, Polistes carolina;* species of the genus *Dolichovespula* viz. *Dolichovespula maculate, Dolichovespula media, Dolichovespula pacifica, Dolichovespula arenaria,* and *Dolichovespula albida;* species of the genus *Vespula* viz. *Vespula squamosa* and *Vespula germanica;* species of the genus *Vespa* viz. *Vespa basalis, Vespa affinis, Vespa ducalis, Vespa mandaiana, Vespa simillima, Vespa soror, Vespa bicolor, Vespa velutina, Vespa vivax* and *Vespa crabro*. All these species arose from a common ancestor. The species of the same genera like *Polistes, Vespa, Apoica* and *Dolichovespula* has a single origin. The peptide tree also showed the relationship pattern of *R. spatulata* with *Belanogaster juncea juncea*, of the subfamily Polistinae, which diverged from a common ancestor.

The present results indicated the significance of an identification system based on COI gene sequence. The result highlighted the significant sequence diversity of the COI gene of *R. spatulata* from its allied species, thus enabling an easy discrimination of species.

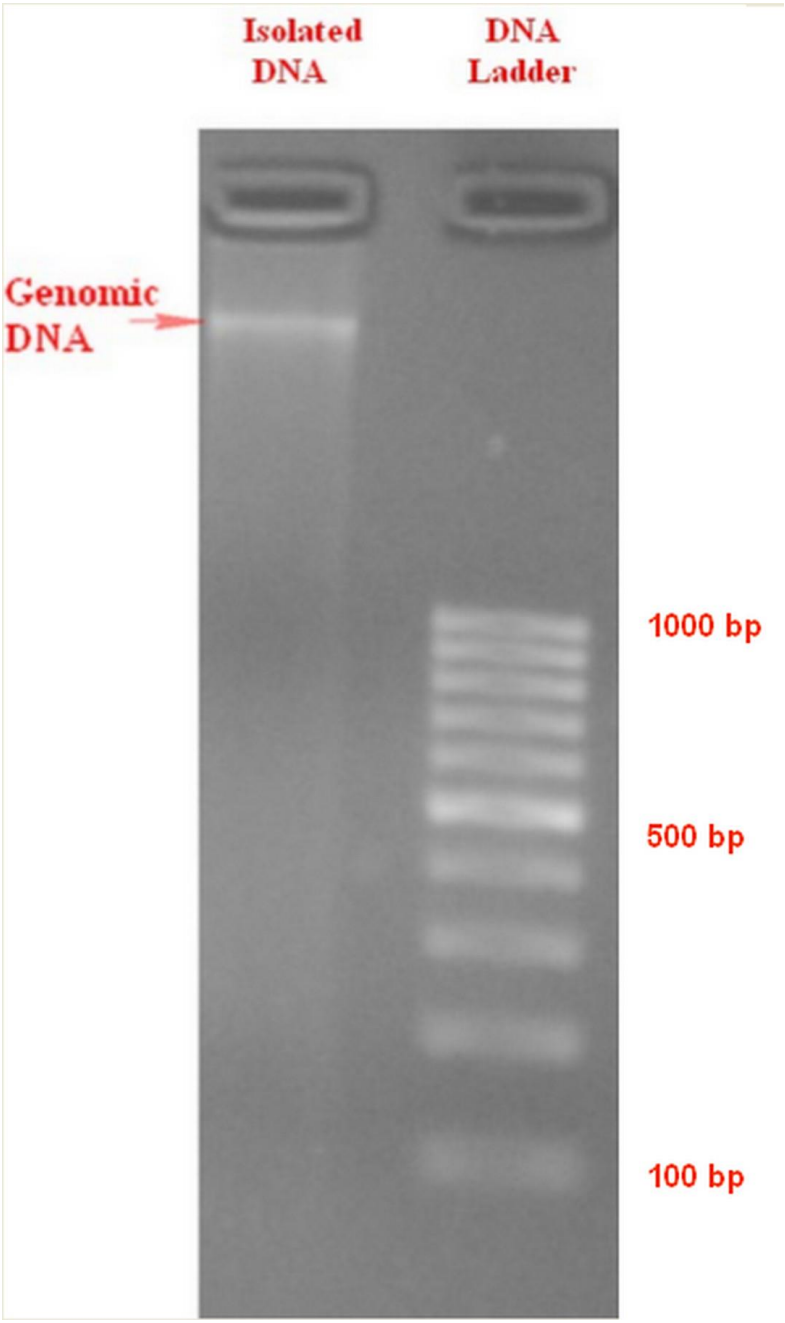

Fig.75. Gel picture of total DNA isolated from *Ropalidia spatulata* Voucher CUKAK6.

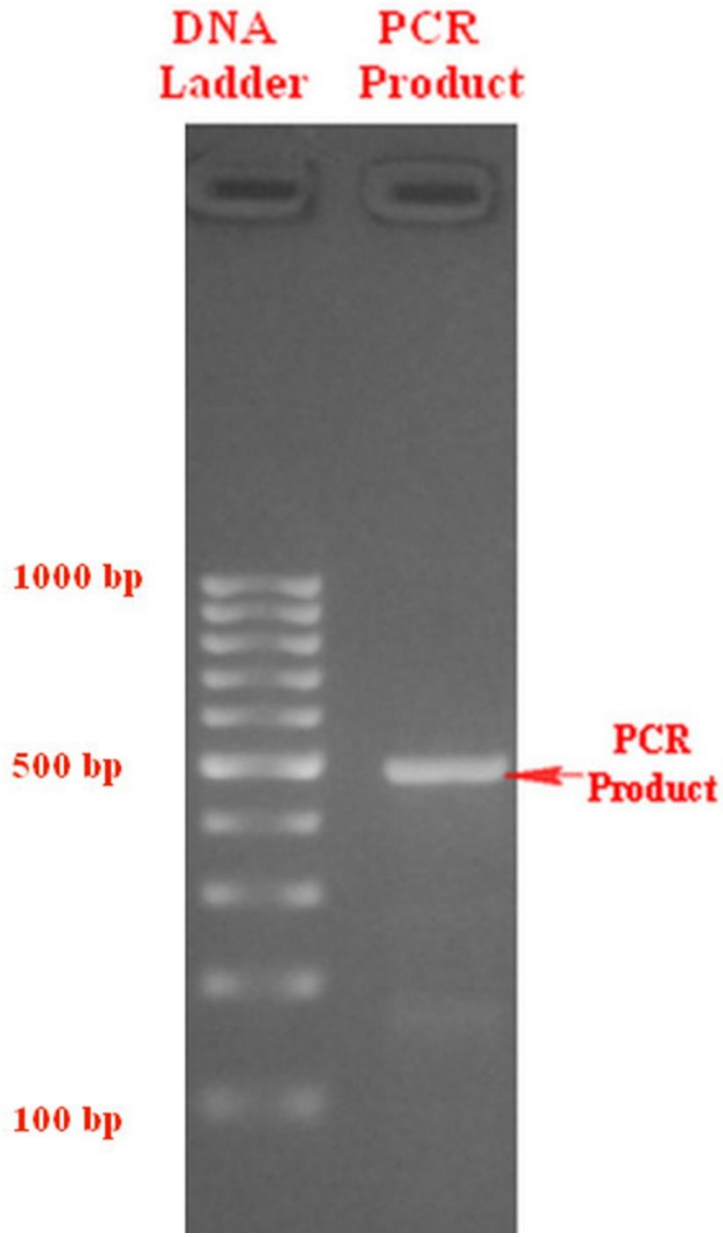

Fig.76. Gel picture showing the PCR product of partial COI gene of *Ropalidia spatulata* Voucher CUKAK6.

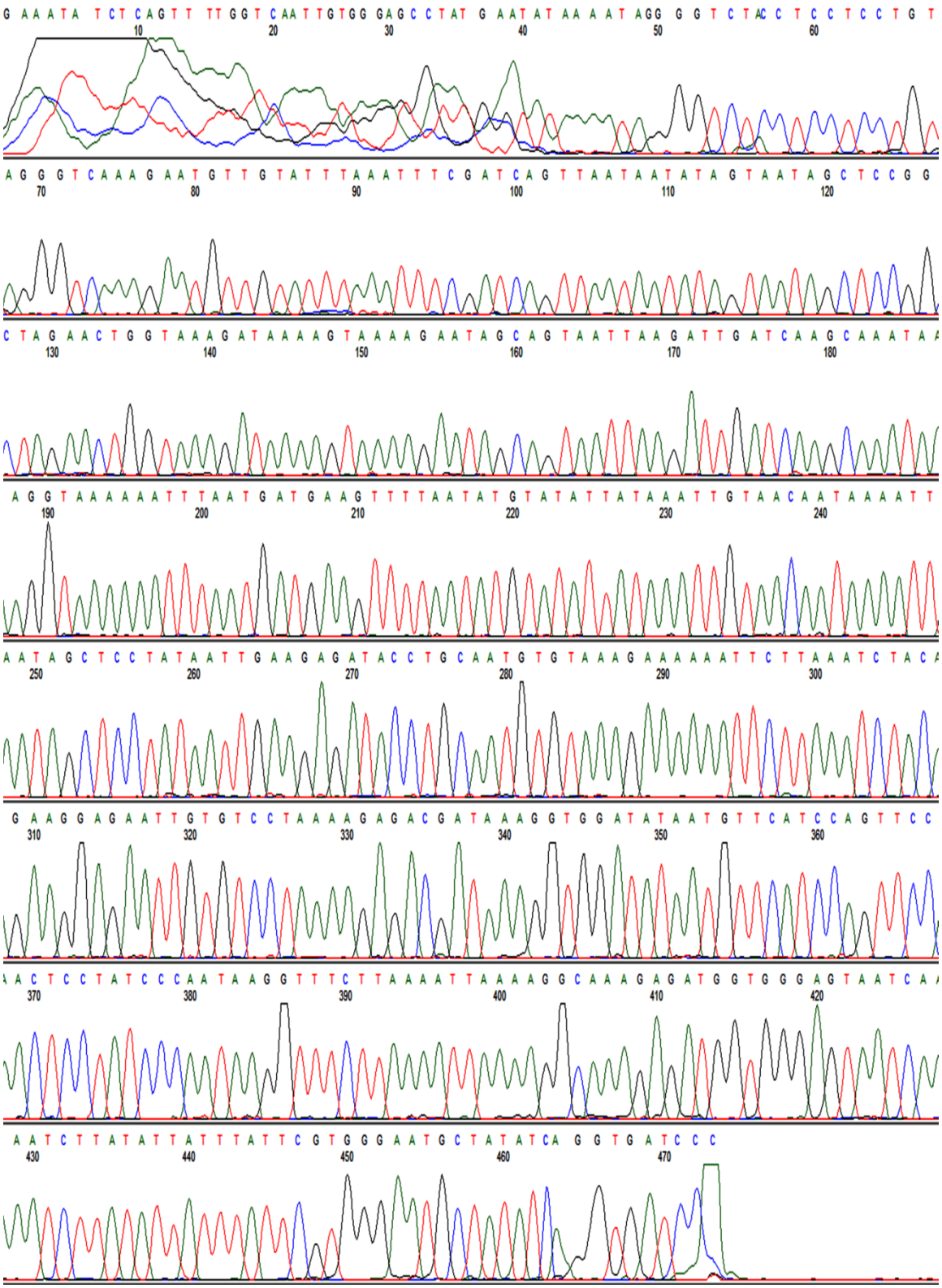

Fig.77. Sequencing chromatogram (forward sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *Ropalidia spatulata* Voucher CUKAK6 (GenBank Accession Number: KM455121).

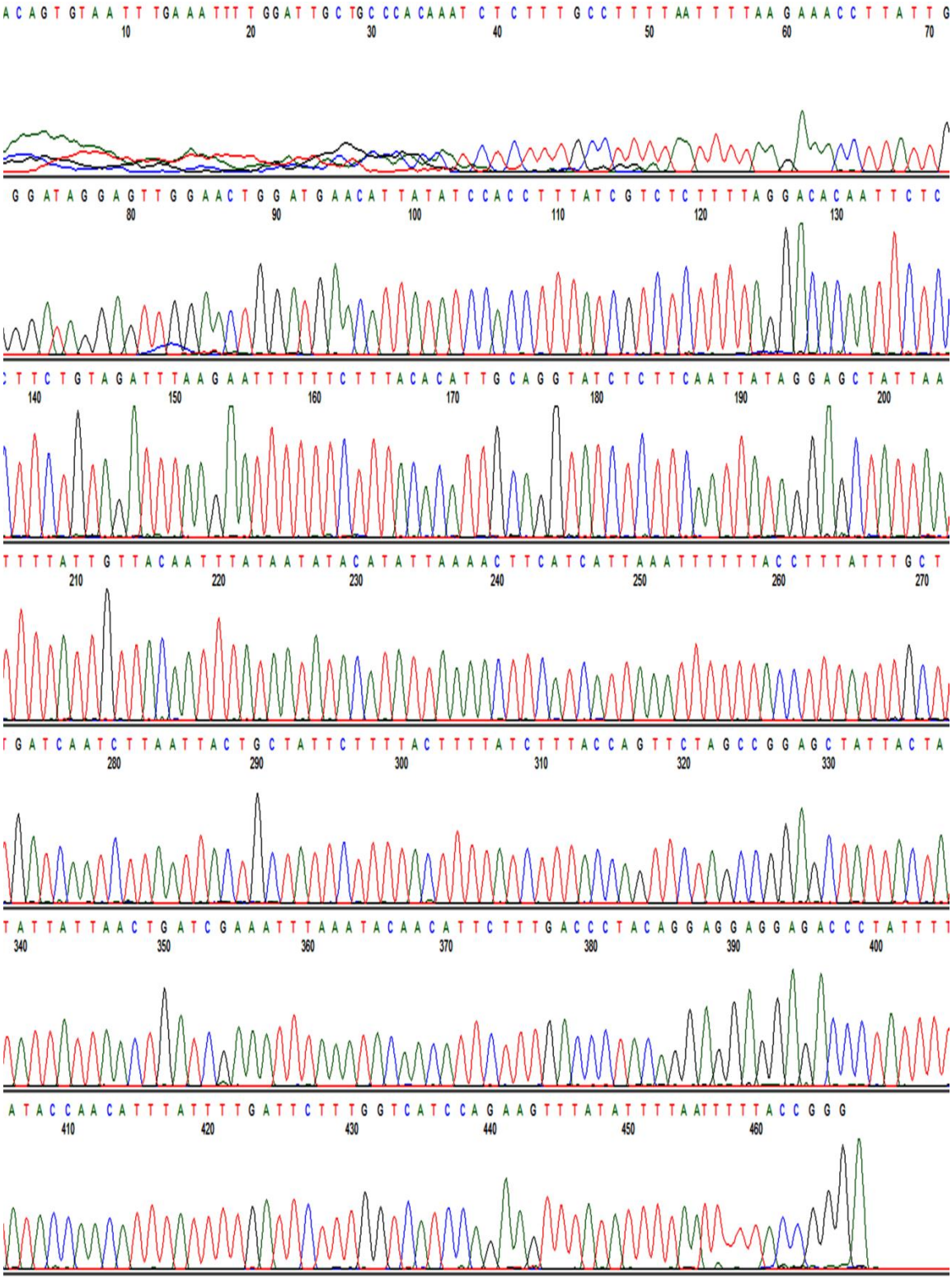

Fig.78. Sequencing chromatogram (reverse sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *Ropalidia spatulata* Voucher CUKAK6 (GenBank Accession Number: KM455121).

>*R. spatulata* Voucher CUKAK6 mitochondrial cytochrome oxidase subunit I (COI) partial nucleotide sequence, 484 bases

TCACCTGATATAGCATTCCCACGAATAAATAATATAAGATTTTGATTACTC
CCACCATCTCTTTGCCTTTTAATTTTAAGAAACCTTATTGGGATAGGAGTT
GGAACTGGATGAACATTATATCCACCTTTATCGTCTCTTTTAGGACACAAT
TCTCCTTCTGTAGATTTAAGAATTTTTTCTTTACACATTGCAGGTATCTCT
TCAATTATAGGAGCTATTAATTTTATTGTTACAATTTATAATATACATATT
AAAACTTCATCATTAAATTTTTTACCTTTATTTGCTTGATCAATCTTAATT
ACTGCTATTCTTTTACTTTTATCTTTACCAGTTCTAGCCGGAGCTATTACT
ATATTATTAACTGATCGAAATTTAAATACAACATTCTTTGACCCTACAGGA
GGAGGAGACCCTATTTTATACCAACATTTATTTTGATTCTTTGGTCATCCA
GAAGTTTATATTTTAATTTTTACCG

Fig.79. Partial coding sequence of *R. spatulata* Voucher CUKAK6 cytochrome oxidase subunit I (COI) gene (GenBank Accession Number: KM455121).

Score	Expect	Identities	Gaps	Strand
431 bits(233)	5e-117	354/414(86%)	2/414(0%)	Plus/Plus

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Query       CCTGATATAGCATTCCCACGAATAAATAATATAAGATTTTGATTACTCCCACCATCTCTT  63
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Subject     CCTGATATAGCATTTCCACGAATAAATAATATAAGATTTTGATTATTACCTCCATCATTA  297

Query       TGCCTTTTAATT-TTAAGAAACCTTATTGGGATAGGAGTTGGAACTGGATGAACATTATA  122
            ||  || | ||| |  |||||  ||||||| | ||| || ||||| ||||||||  |||||
Subject     TG-TTTATTATTATCTAGAAATTTTATTGGAACAGGTGTAGGAACAGGATGAACTTTATA  356

Query       TCCACCTTTATCGTCTCTTTTAGGACACAATTCTCCTTCTGTAGATTTAAGAATTTTTTC  182
            |||||| ||||| ||  || ||||||| ||||| ||||| || |||||||||| || ||
Subject     TCCACCATTATCATCAATTATAGGACATAATTCACCTTCAGTTGATTTAAGAATCTTCTC  416

Query       TTTACACATTGCAGGTATCTCTTCAATTATAGGAGCTATTAATTTTATTGTTACAATTTA  242
              | || |||||||| || ||||||||||||||||||||||||||||||| ||||||
Subject     ACTTCATATTGCAGGAATTTCTTCAATTATAGGAGCTATTAATTTTATTGTAACAATTAC  476

Query       TAATATACATATTAAAACTTCATCATTAAATTTTTTACCTTTATTTGCTTGATCAATCTT  302
             |||||||||| |||||||||  |||||||||||| |||||||| ||||||||  |
Subject     AAATATACATATCAAAACTTCATCTATAAATTTTTTACCCTTATTTGCCTGATCAATTCT  536

Query       AATTACTGCTATTCTTTTACTTTTATCTTTACCAGTTCTAGCCGGAGCTATTACTATATT  362
            |||||| || ||| | |||||||||||||||||||| |||| ||||| ||||||||||
Subject     AATTACAGCAATTTTATTACTTTTATCTTTACCAGTTTTAGCTGGAGCCATTACTATATT  596

Query       ATTAACTGATCGAAATTTAAATACAACATTCTTTGACCCTACAGGAGGAGGAGA  416
            |||||||||||||||||||||||||||||| ||||| || ||||||||||||||
Subject     ATTAACTGATCGAAATTTAAATACAACATTTTTTGATCCAACAGGAGGAGGAGA  650
```

Fig.80. Nearest sequence match from the BLAST result of *R. spatulata* Voucher CUKAK6 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455121). Query = *R. spatulata* Voucher CUKAK6; Subject = *Ropalidia romandi cabet* (GenBank Accession Number: AF146677.1). Note that the nearest match is only 86% similar to the nucleotide sequence in the database depicting that the sequence obtained is novel.

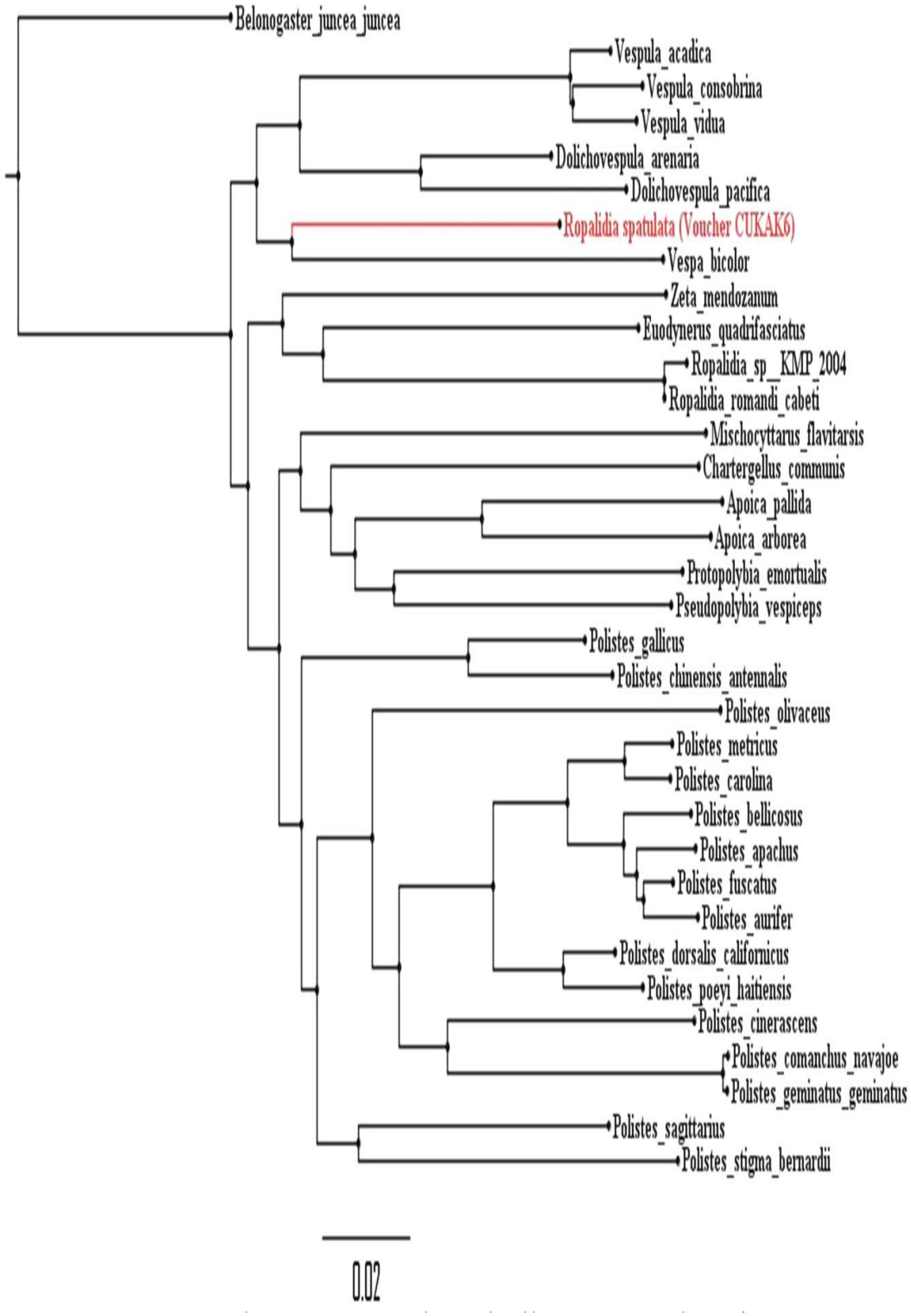

Fig.81. N-J tree plotted for *R. spatulata* Voucher CUKAK6 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455121).

>*R. spatulata* Voucher CUKAK6

SPDMAFPRMNNMSFWLLPPSLCLLILSNLIGMGVGTGWTLYPPLSSLLGHNSPSVDL
SIFSLHIAGISSIMGAINFIVTIYNMHIKTSSLNFLPLFAWSILITAILLLLSLPVL
AGAITMLLTDRNLNTTFFDPTGGGDPILYQHLFWFFGHPEVYILIFT

Fig.82. The conceptual translation product of the DNA sequence of mitochondrial cytochrome oxidase subunit I (COI) gene of *R. spatulata* Voucher CUKAK6.

Score	Expect	Method	Identities	Positives	Gaps
268 bits(685)	4e-86	Compositional matrix adjust.	152/158(96%)	154/158(97%)	0/158(0%)

```
Query       PDMAFPRMNNMSFWLLPPSLCLLILSNLIGMGVGTGWTLYPPLSSLLGHNSPSVDLSIFS  61
            PDMAFPRMNNMSFWLLPPSL LLILSNLIGMGVGTGWTLYPPLSS+LGHNSPSVDLSIFS
Subject     PDMAFPRMNNMSFWLLPPSLFLLILSNLIGMGVGTGWTLYPPLSSILGHNSPSVDLSIFS  132

Query       LHIAGISSIMGAINFIVTIYNMHIKT SLNFLPLFAWSILITAILLLLSLPVLAGAITML  121
            LHIAGISSIMGAINFIVTI NMHIKT S+NFLPLFAWSILITAILLLLSLPVLAGAITML
Subject     LHIAGISSIMGAINFIVTIINMHIKT SMNFLPLFAWSILITAILLLLSLPVLAGAITML  192

Query       LTDRNLNTTFFDPTGGGDPILYQHLFWFFGHPEVYILI  159
            LTDRNLNTTFFDP GGGDPILYQHLFWFFGHPEVYILI
Subject     LTDRNLNTTFFDPIGGGDPILYQHLFWFFGHPEVYILI  230
```

Fig.83. Nearest peptide sequence match from the BLAST result of *R. spatulata* Voucher CUKAK6 cytochrome oxidase subunit I (COI) conceptual translation product. Query = *R. spatulata* Voucher CUKAK6; Subject=*Polybioides melainus* (GenBank Accession Number: ADD60380.1). Note that the nearest match is only 96% similar to the peptide sequence in the database depicting that the sequence obtained is novel.

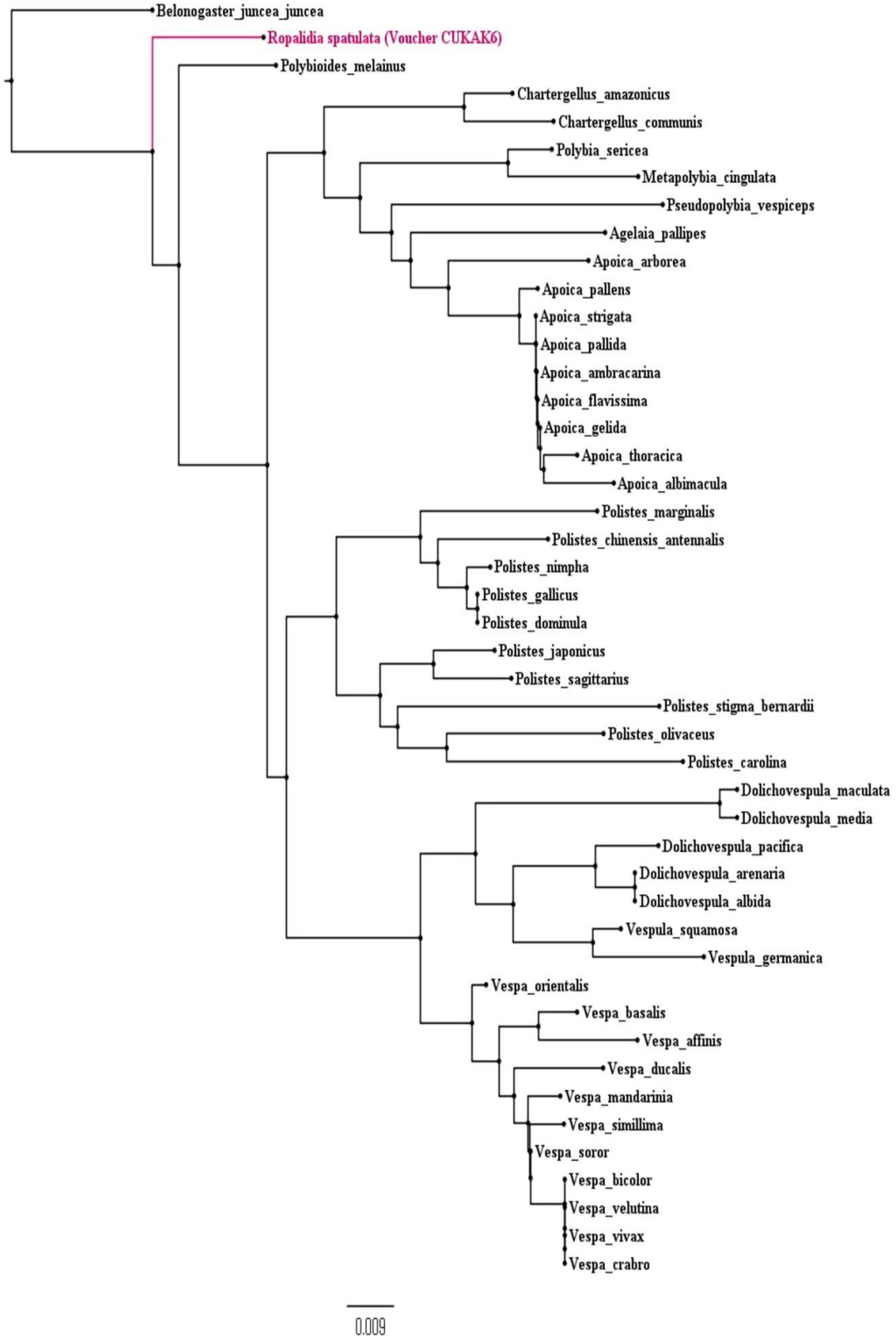

Fig.84. N-J tree plotted for conceptual translation product of *R. spatulata* Voucher CUKAK6 cytochrome oxidase subunit I (COI) peptide sequence.

7. *Polistes strigosus*

Polistes strigosus (**Figures 85-88**)**,** is a paper wasp which builds its nest using long fine plant fibers and wasp adult oral secretion, looking like a nest made of paper. It has a pollination activity and it is also used in pest management in biocontrol programmes against small insect pests of plantations.

7.1. History of nomenclature

Polistes strigosus. Bequaert, 1940. *Trans. Amer. Ent. Soc*, 66: 269-272.

(Lambert *et al*., 2012)

7.2. Diagnosis

The species collected was identical to those described by Lambert *et al*, 2012. Body was reddish brown with black and yellow markings. Yellow marking as follows: Apex of first tergite faintly, second tergite almost completely and apex of third tergite. The black markings are: supraclypeal area, a black band on vertex; antennal flagellum dorsally, upper margin of clypeus, mesoscutum except one median and two latral small marks, two large median marks on propodeum, legs except femora partly and foretibia almost entirely red. Wings brownish-yellow; veins yellow, apical cloud absent.

Fig.85. *Polistes strigosus* Voucher CUKAK7 lateral view (scale in cm)

Fig.86. *Polistes strigosus* Voucher CUKAK7 upper view (scale in cm)

Fig.87. *Polistes strigosus* Voucher CUKAK7 front view

Fig.88. *Polistes strigosus* Voucher CUKAK7 wing pattern.

7.3. Systematic position

Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Hymenoptera; Apocrita; Aculeata; Vespoidea; Vespidae; Polistinae; *Polistes*; *strigosus.*

7.4. Distribution

In India it is reported from Assam, Bihar, Delhi, Kerala, Manipur, Sikkim, Tripura, Uttarakhand and West Bengal (Lambert *et al*., 2012).

7.5. Collection

Polistes strigosus, used in the present study was collected from Ernakulum District of Kerala at 10° 7' 0" North, 76° 21' 0" East.

7.6. PCR amplification of the cytochrome c oxidase I gene of *Polistes strigosus* Voucher CUKAK7

The gel pictures showing the genomic DNA and PCR product of COI gene of *Polistes strigosus* Voucher CUKAK3 are given in **Figures 89 and 90.** The PCR of the COI gene fragment of *Polistes strigosus* yielded a product of 484bp. The forward and reverse sequence chromatograms, COI gene sequence obtained, BLASTn result, conceptual translation product and BLASTp result are presented in **Figures 91-94, 96**

and 97. The sequence obtained is deposited in the GenBank (GenBank Accession Number: KM455122).

The COI sequence of *P. strigosus* in the present study can be used for its accurate taxonomic identification. The nucleotide BLAST against the nucleotide redundant database showed that the cytochrome oxidase gene sequence obtained is novel.

The hypervariable region of DNA of *P. strigosus* is 99% similar to that of *Polistes sagittarius* COI gene (GenBank Accession Number: GU596922.1).

The conceptual translation of partial COI gene of *P. strigosus* yielded a peptide of 160 amino acids. The peptide blast of COI gene of *P. strigosus* showed 99% similarity to that of cytochrome oxidase subunit I of *P. sagittarius* (GenBank Accession Number: ADD60362.1). The results of the BLASTp indicated that the peptide of the cytochrome oxidase subunit I (COI) gene of *P. strigosus* collected from Ernakulum is novel. The mtDNA sequence of different wasps confirms the species differences of *P. strigosus* from others.

7.7. Molecular Phylogeny of *Polistes strigosus* Ernakulam Voucher CUKAK7

The phylogenetic trees of DNA and peptide are plotted using Neighbor-Joining method and are exhibited in **Figures 95 and 98.** The DNA tree showed that *P. strigosus*

is more closely related to *Polistes sagittarius* (GenBank Accession Number: GU596922.1). Pickett and Carpenter (2010) reported that *P. sagittarius* was more closely related to *Polistes stigma* than other *Polistes* species without considering the *Polistes strigosus* CO1 sequence in the phylogeny analysis. *Polistes strigosus* and *Polistes sagittarius* has a common ancestor.

The DNA tree showed their close relationship to the cluster containing the species of the genus *Polistes* viz. *Polistes humilis, Polistes nipponensis, Polistes formosanus*, and *Polistes japonicus*. They in turn share a common ancestor with the cluster contaiing the *Polistes* species viz. *Polistes biguttatus, Polistes californicus, Polistes bellicosus, Polistes carolina, Polistes metricus, Polistes perplexus, Polistes apaches, Polistes fuscatus* and *Polistes aurifer*.

The DNA tree also depicted the relationship pattern of *Polistes* species with the wasps *Mischocyttarus mexicanus cubicula* and *Mischocyttarus mastigophorus,* having a common ancestor; species *Polistes bischoffi, Polistes hellenicus, Polistes chinenesis antennalis, Allobatus brunneus* and *Polistes gallicus*; *Polistes dominula* having a common ancestor and also to the species *Polistes olivaceus* and *Dolichovespula arenaria* from a single origin.

The peptide tree also showed the close relationship of *Polistes strigosus* to *Polistes sagittarius,* having a single origin. They are in turn related to *Polistes japonicus* with a common ancestor. *P. strigosus* share a common ancestor with the species of the genus *Polistes* viz. *Polistes olivaceus, Polistes bicolor, Polistes geminates geminates, Polistes comanchus navajoe, Polistes Carolina, Polistes dorsalis californicus, Polistes major major, Polistes stigma bernardii, Polistes snelleni, Polistes formosanus, Polistes marginalis, Polistes chinensis antennalis, Polistes nimpha, Polistes gallicus* and *Polistes dominula*.

This cluster having a common ancestor in turn share a single origin with wasps of the subfamily Vespinae viz. *Vespula squamosa, Dolichovespula arenaria, Dolichovespula albida, Vespa orientalis, Vespa ducalis, Vespa basalis, Vespa affinis, Vespa simillima, Vespa mandariana, Vespa soror, Vespa crabro, Vespa vivax* and *Vespa velutina*. The peptide tree also showed the relationship of *P. strigosus* with the wasps *Mischocyttarus mastigophorus* and *Mischocyttarus latior* of the subfamily Polistinae.

The present results indicated the significance of an identification system based on COI gene sequence. The result highlighted the significant sequence diversity of the COI gene of *P. strigosus* from its allied species, thus enabling an easy discrimination of species.

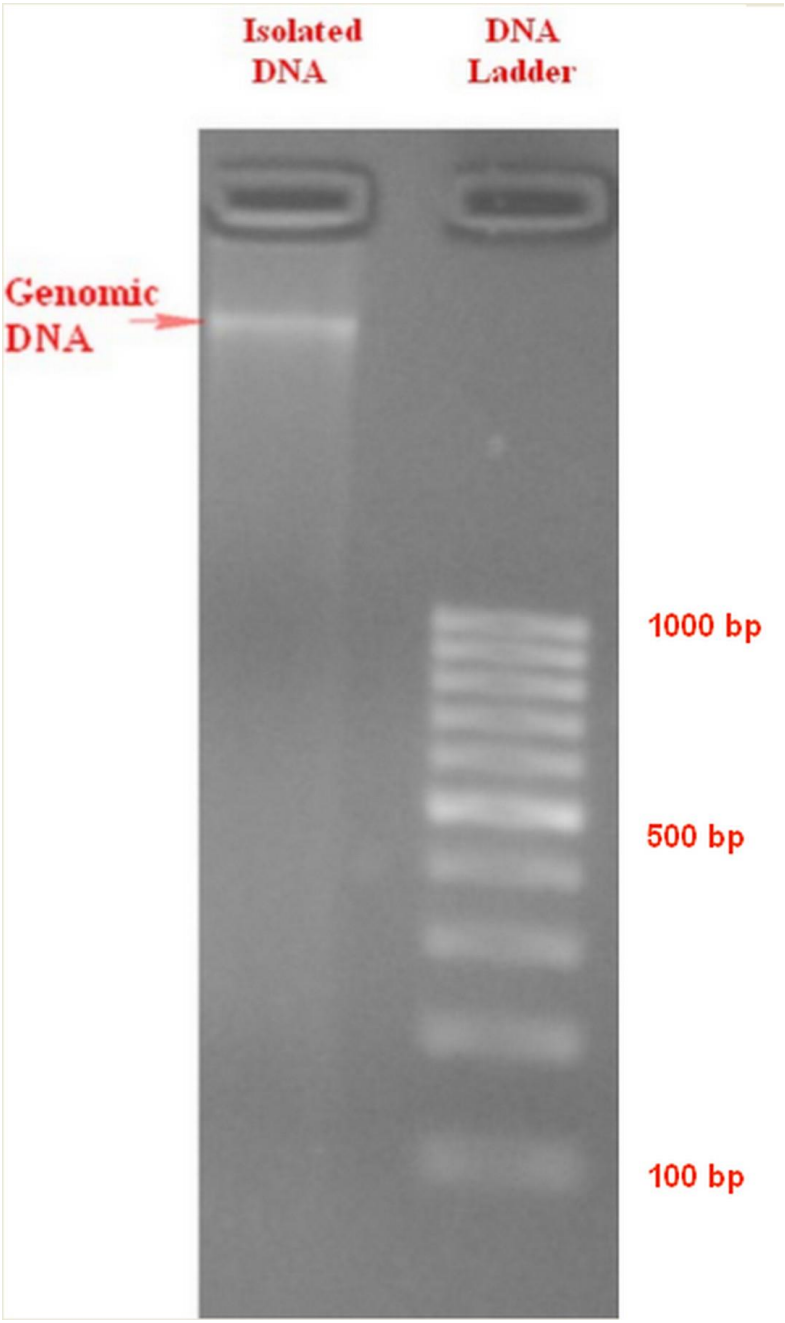

Fig.89. Gel picture of total DNA isolated from *Polistes strigosus* Voucher CUKAK7.

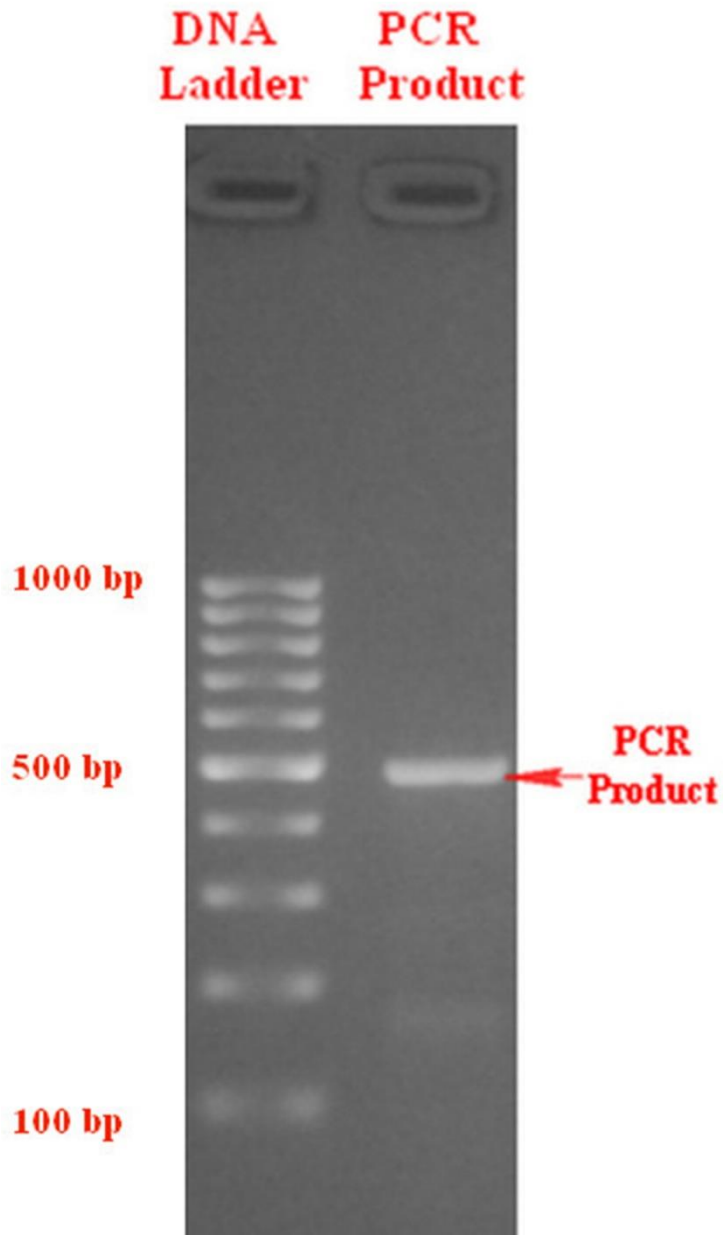

Fig.90. Gel picture showing the PCR product of partial COI gene of *Polistes strigosus* Voucher CUKAK7.

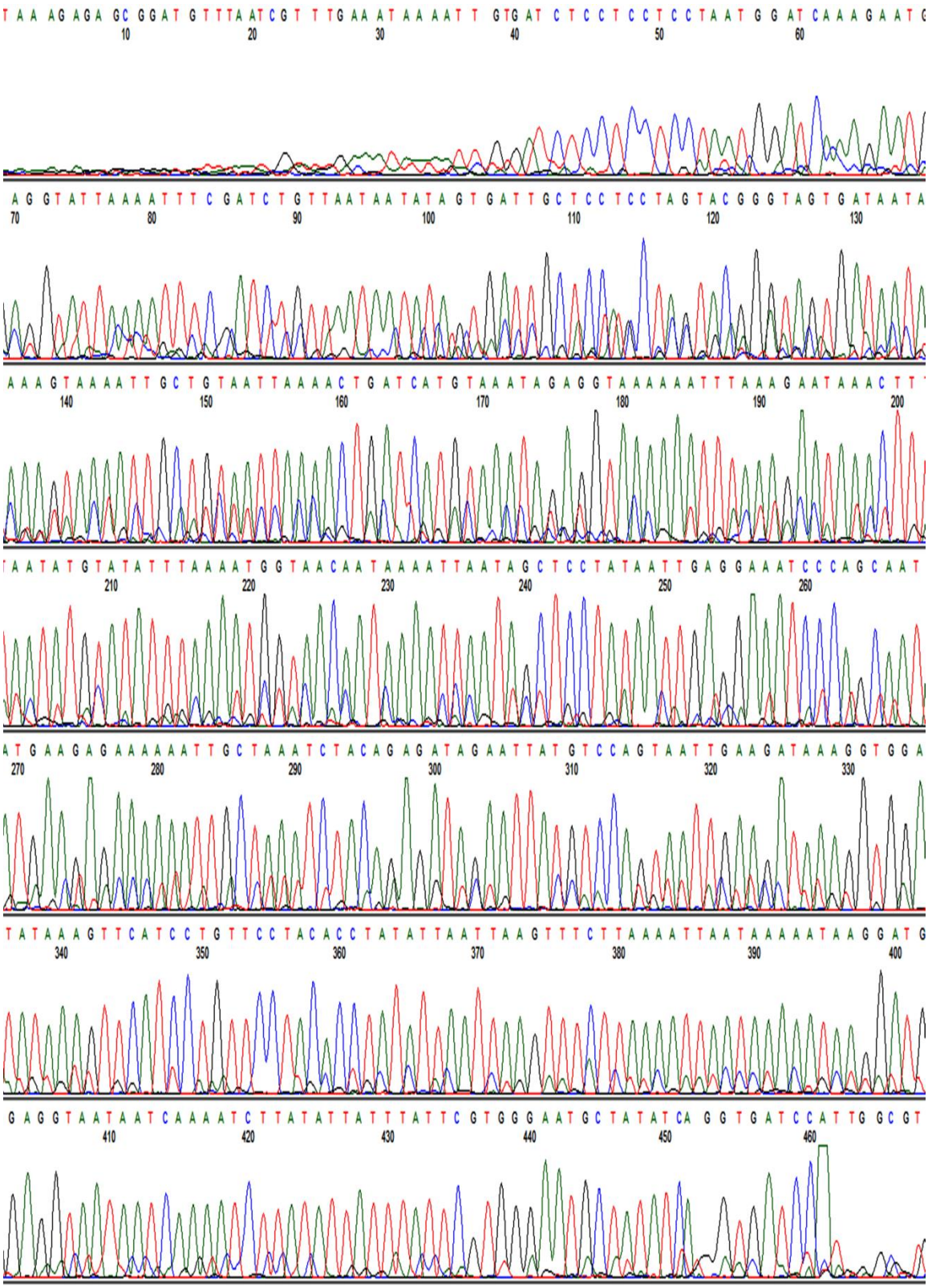

Fig.91. Sequencing chromatogram (forward sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *P. strigosus* Voucher CUKAK7 (GenBank Accession Number: KM455122).

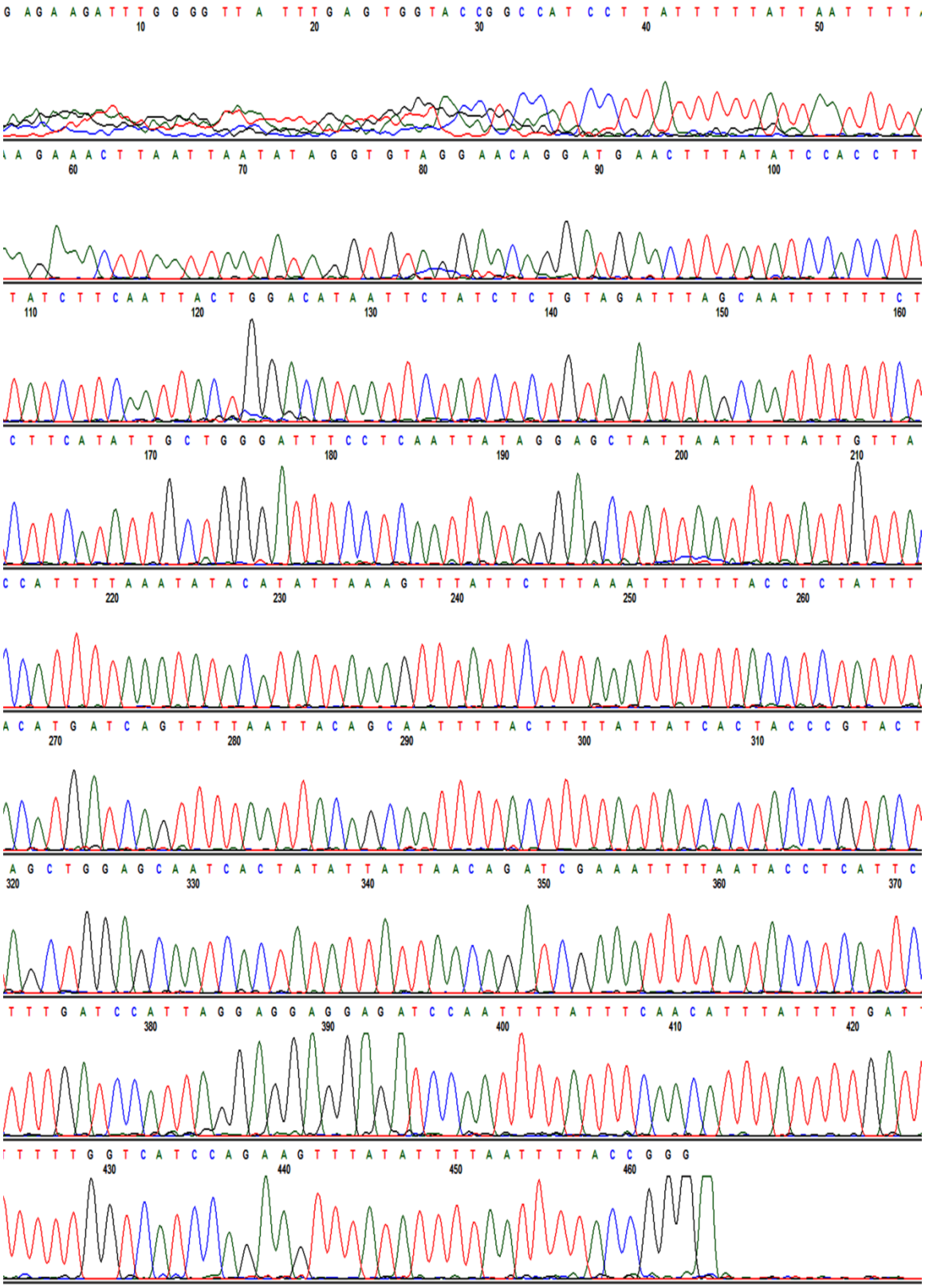

Fig.92. Sequencing chromatogram (reverse sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *P. strigosus* Voucher CUKAK7 (GenBank Accession Number: KM455122).

>*P. strigosus* Voucher CUKAK7 mitochondrial cytochrome oxidase subunit I (COI) partial nucleotide sequence, 484 bases

ATGGATCACCTGATATAGCATTCCCACGAATAAATAATATAAGATTTTGAT
TATTACCTCCATCCTTATTTTTATTAATTTTAAGAAACTTAATTAATATAG
GTGTAGGAACAGGATGAACTTTATATCCACCTTTATCTTCAATTACTGGAC
ATAATTCTATCTCTGTAGATTTAGCAATTTTTTCTCTTCATATTGCTGGGA
TTTCCTCAATTATAGGAGCTATTAATTTTATTGTTACCATTTTAAATATAC
ATATTAAAGTTTATTCTTTAAATTTTTTACCTCTATTTACATGATCAGTTT
TAATTACAGCAATTTTACTTTTATTATCACTACCCGTACTAGCTGGAGCAA
TCACTATATTATTAACAGATCGAAATTTTAATACCTCATTCTTTGATCCAT
TAGGAGGAGGAGATCCAATTTTATTTCAACATTTATTTTGATTTTTTGGTC
ATCCAGAAGTTTATATTTTAATTTT

Fig.93. Partial coding sequence of *P. strigosus* Voucher CUKAK7 (GenBank Accession Number: KM455122) cytochrome oxidase subunit I (COI) gene.

Score	Expect	Identities	Gaps	Strand
841 bits(455)	0.0	469/476(99%)	0/476(0%)	Plus/Plus

```
Query       CCTGATATAGCATTCCCACGAATAAATAATATAAGATTTTGATTATTACCTCCATCCTTA  68
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Subject     CCTGATATAGCATTTCCACGAATAAATAATATAAGATTTTGATTATTACCTCCATCCTTA  279

Query       TTTTTATTAATTTTAAGAAACTTAATTAATATAGGTGTAGGAACAGGATGAACTTTATAT  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     TTTTTATTAATTTTAAGAAACTTAATTAATATAGGTGTAGGAACAGGATGAACTTTATAT  339

Query       CCACCTTTATCTTCAATTACTGGACATAATTCTATCTCTGTAGATTTAGCAATTTTTTCT  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     CCACCTTTATCTTCAATTACTGGACATAATTCTATCTCTGTAGATTTAGCAATTTTTTCT  399

Query       CTTCATATTGCTGGGATTTCCTCAATTATAGGAGCtattaattttattgttaccatttta  248
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Subject     CTTCATATTGCTGGAATTTCCTCAATTATAGGAGCTATTAATTTTATTGTTACCATTTTA  459

Query       aatatacatattaaagtttattctttaaatttttacctctatttacatgatcagtttta  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     AATATACATATTAAAGTTTATTCTTTAAATTTTTTACCTCTATTTACATGATCAGTTTTA  519

Query       attacagcaattttacttttattaTCACTACCCGTACTAGCTGGAGCAATCACTATATTA  368
            |||||||||||||||||||||||| |||||||||||||||||||| |||||||||
Subject     ATTACAGCAATTTTACTTTTATTATCATTACCCGTACTAGCTGGAGCAATTACTATATTA  579

Query       TTAACAGATCGAAATTTTAATACCTCATTCTTTGATCCATTAGGAGGAGGAGATCCAatt  428
            |||||||| |||||||||||||||||||||||||||||||||| ||||||||||||
Subject     TTAACAGACCGAAATTTTAATACCTCATTCTTTGATCCATTAGGAGGGGGAGATCCAATT  639

Query       ttatttcaacatttattttgatttttggtcatccagaagtttatatttaatttt  484
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Subject     TTATTCCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTTT  695
```

Fig.94. Nearest sequence match from the BLAST result of *P. strigosus* Voucher CUKAK7 cytochrome oxidase subunit I (COI) gene sequence (GenBank Accession Number: KM455122). Query = *P. strigosus* Voucher CUKAK7; Subject = *Polistes sagittarius* (GenBank Accession Number: GU596922.1). Note that the nearest match is 99% similar to the nucleotide sequence in the database depicting that the sequence obtained is novel.

Fig.95. N-J tree plotted for *P. strigosus* Voucher CUKAK7 cytochrome oxidase subunit I (COI) gene sequence (Genbank Accession Number: KM455122).

> *P. strigosus* Voucher CUKAK7

GSPDMAFPRMNNMSFWLLPPSLFLLILSNLINMGVGTGWTLYPPLSSITGHNSISVD
LAIFSLHIAGISSIMGAINFIVTILNMHIKVYSLNFLPLFTWSVLITAILLLLSLPV
LAGAITMLLTDRNFNTSFFDPLGGGDPILFQHLFWFFGHPEVYILI

Fig.96. The conceptual translation product of the DNA sequence of mitochondrial cytochrome oxidase subunit I (COI) gene of *P. strigosus* Voucher CUKAK7.

Score	Expect	Method	Identities	Positives	Gaps
310 bits (795)	1e-102	Compositional matrix adjust.	159/160(99%)	160/160(100%)	0/160(0%)

```
Query       GSPDMAFPRMNNMSFWLLPPSLFLLILSNLINMGVGTGWTLYPPLSSITGHNSISVDLAI  60
            G+PDMAFPRMNNMSFWLLPPSLFLLILSNLINMGVGTGWTLYPPLSSITGHNSISVDLAI
Subject     GAPDMAFPRMNNMSFWLLPPSLFLLILSNLINMGVGTGWTLYPPLSSITGHNSISVDLAI  131

Query       FSLHIAGISSIMGAINFIVTILNMHIKVYSLNFLPLFTWSVLITAILLLLSLPVLAGAIT  120
            FSLHIAGISSIMGAINFIVTILNMHIKVYSLNFLPLFTWSVLITAILLLLSLPVLAGAIT
Subject     FSLHIAGISSIMGAINFIVTILNMHIKVYSLNFLPLFTWSVLITAILLLLSLPVLAGAIT  191

Query       MLLTDRNFNTSFFDPLGGGDPILFQHLFWFFGHPEVYILI  160
            MLLTDRNFNTSFFDPLGGGDPILFQHLFWFFGHPEVYILI
Subject     MLLTDRNFNTSFFDPLGGGDPILFQHLFWFFGHPEVYILI  231
```

Fig.97. Nearest peptide sequence match from the BLAST result of *P. strigosus* Voucher CUKAK7 cytochrome oxidase subunit I (COI) conceptual translation product. Query = *P. strigosus* Voucher CUKAK7; Subject = *Polistes sagittarius* (GenBank Accession Number: ADD60362.1). Note that the nearest match is 99% similar to the peptide sequence in the database depicting that the sequence obtained is novel.

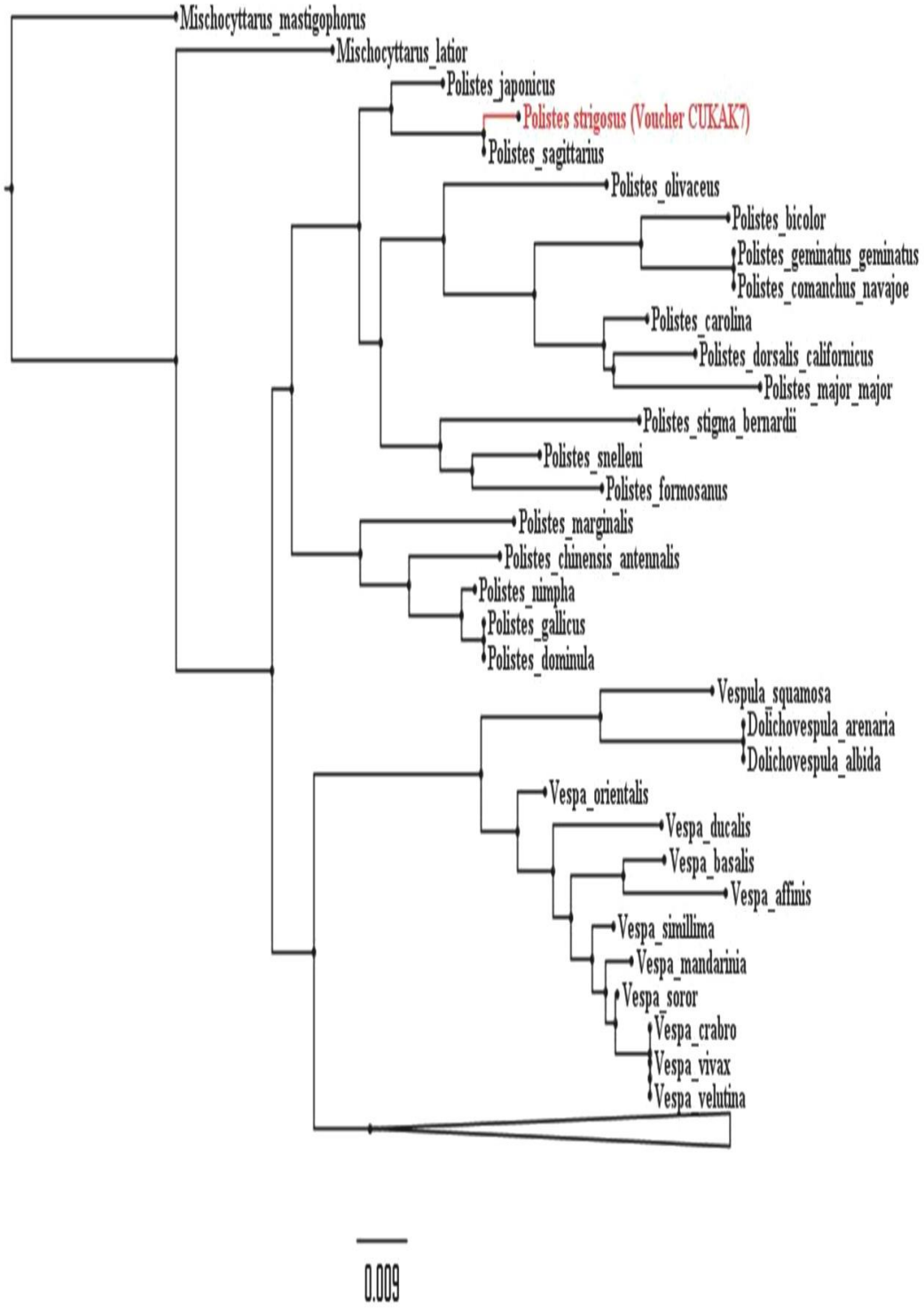

Fig.98. N-J tree plotted for conceptual translation product of *P. strigosus* Voucher CUKAK7 cytochrome oxidase subunit I (COI) peptide sequence.

8. *Ropalidia jacobsoni*

Ropalidia jacobsoni (**Figures 99-102**), is a common paper wasp which builds its nest in both indoor and outdoor using long fine plant fibers and wasp adult oral secretions.

8.1. History of nomenclature

Icaria jacobsoni. du Buysson, 1908. *Notes Leiden Mus,* 30: 123

Ropalidia variegata jacobsoni. Van der Vecht, 1941. *Treubia,* 18: 156, 157.

Ropalidia (Icariola) Jacobsoni. Richards, 1978. *Aust. J. Zool. Suppl. Ser,* 61: 58.

Ropalidia (Anthreneida) variegata jacobsoni. Van der Vecht, 1962. *Zool. Verh,* 57: 29; Yamane and Yamane, 1979. *Insecta Matsumurana,* 15: 32.

Ropalidia(Anthreneida) jacobsoni jacobsoni. Das and Gupta, 1983. *Oriental Ins,* 17: 418
(Lambert, 2002)

8.2 Diagnosis

The species collected was identical to those described by Lambert (2002). Body was reddish brown with yellow and black markings. Yellow marking as follows: clypeus except a characteristic black mark, mandible except for black reddish-brown teeth, inner orbit up to ocular sinus narrowly, a line below the antennal scape, lower sides of all antennal segment, narrow line on pronotal carina, basal and lateral margin of scutellum, two marks on postscutellum, a small mark on upper part of mesopleuron, two broad marks on propodeum seperated by black mark on median groove, fore and middle coxae

Fig.99. *Ropalidia jacobsoni* lateral view (scale in cm)

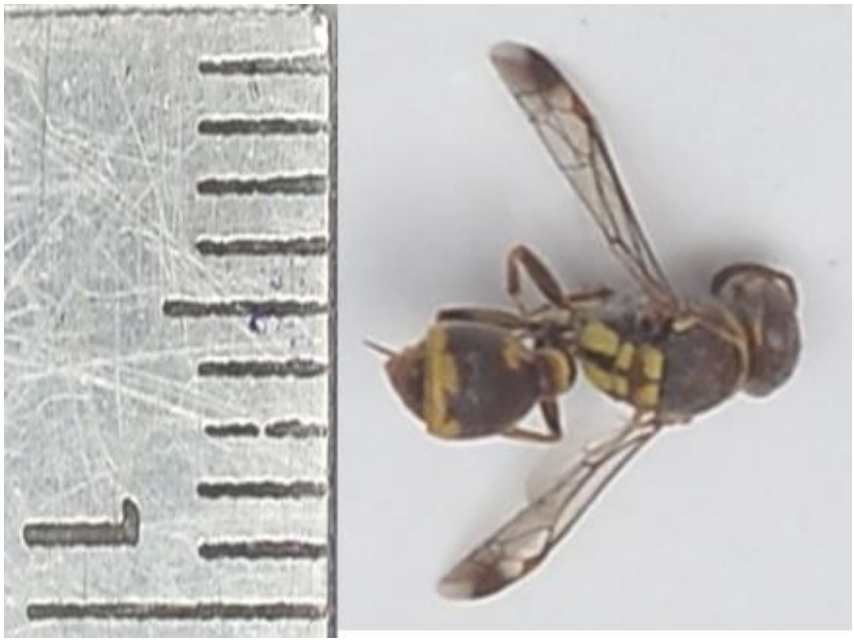

Fig.100. *Ropalidia jacobsoni* upper view (scale in cm)

Fig.101. *Ropalidia jacobsoni* front view

Fig. 102. *Ropalidia jacobsoni* wing pattern.

in front, a line on hindcoxa laterally another line below forefemur, a small mark on middle femur laterally, narrow line on tibia above, foretarsi, second metasomal tergite with a large irregular mark on each side at base and an apical band. The black marking as follows: a mark at base to centre on clypeus, supraclypeal area, a mark enclosing ocellus, a large spot above antennal socket, margins of mesoscutum narrowly, apical margin towards scutellum broadly, dorsal metapleuron, basal and apical margins of propodeum and broadly along median groove, Wings transparent hyaline with apical half of radial cell brown; stigma yellow, veins brown.

8.3. Systematic position

Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota; Neoptera; Endopterygota; Hymenoptera; Apocrita; Aculeata; Vespoidea; Vespidae; Polistinae; *Ropalidia*; *jacobsoni.*

8.4. Distribution

In India it is reported from Delhi, Utter Pradesh, Rajasthan, Maharashtra, Karnataka, Kerala, Tamil Nadu, and Assam. It is also distributed in Myanmar, Sumatra, Bangka, Java, Lombok and Sulawesi (Lambert, 2002).

8.5. Collection

*Ropalidia jacobsoni**,*** used in the present study was collected from Trivandrum District of Kerala at 8° 29' 0" North, 76° 55' 0" East.

8.6. PCR amplification of the cytochrome c oxidase subunit I gene of *Ropalidia jacobsoni* Voucher CUKAK8

The gel pictures showing genomic DNA and the PCR product of COI gene of *Ropalidia jacobsoni* are shown in **Figures 103 and 104**. The PCR of the COI gene fragment of *R. jacobsoni* from Trivandrum yielded a product of 353bp. The forward sequence chromatogram, COI gene sequence obtained, BLASTn result, conceptual translation product and BLASTp result are presented in **Figures 105-108, 110 and 111.**

The COI sequence of *R. jacobsoni* in the present study can be used for its accurate taxonomic identification. The nucleotide BLAST against the nucleotide redundant database showed that the cytochrome oxidase gene sequence obtained is novel.

The hypervariable region of DNA of *R. jacobsoni* is 98% similar to that of *R. quinquecinctum* (GenBank Accession Number: AB969818.1).

The conceptual translation of COI gene of *R. jacobsoni* yielded a peptide of 117 amino acids. The peptide blast of COI gene of *R. jacobsoni* showed 99% of similarity to that of cytochrome oxidase subunit I of *R. quinquecinctum* (GenBank Accession Number: BAP05525.1). The results of the BLASTp indicated that the peptide of the cytochrome oxidase subunit I (COI) gene of *R. jacobsoni* collected from Trivandrum is novel. The

mtDNA sequence of different wasps confirms the species differences of *R. jacobsoni* from others.

8.7. Molecular phylogeny of *Ropalidia jacobsoni* Voucher CUKAK8

The phylogenetic trees of DNA and peptide are plotted using Neighbour-Joining method and are exhibited in **Figures 118 and 121**. The DNA phylogenetic tree showed that *R. jacobsoni* is more closely related to *R. quinquecinctum,* having a single origin. They are closely related to the species *Metapolybia singulata, Apoica flavissima, Apoica albimacula, Apoica pallida, Apoica ambracarina* by a common ancestry.

All these species have a single parent which diverged into another clade containing the species of the genus *Symmorphus* viz. *Symmorphus allobrogus* and *Symmorphus cristatus;* genus *Euodynerus* viz. *Euodynerus megaera* and *Euodynerus forminatus*; *Vespula germanica*; *Orancistrocerus drewsoni* and *Anterhynchium flavomarginatum umenoi*; *Tachyancistrocerus rhodensis* and a Vespidae species. The DNA tree showed the relationship pattern of *R. jacobsoni* to the species of the genus *Ancistrocerus* viz. *Ancestrocerus campestris, Ancestrocerus ichneumonideus, Ancestrocerus parietinus, Ancestrocerus albophaleratus*; a Hymenoptera species and *Discoelius zonalis.* The most distantly related dspecies to *R. jacobsoni* according to DNA phylogeny tree is found to be *Vespa ducalis* of the subfamily Vespinae.

The peptide tree also depicted the close relationship between *R. jacobsoni* and *Rhynchium quinquecinctum*, having a single origin. They are closely related to the species *Anterhynchium flavomarginatum unmenoi* and *Orancistrocerus drewsoni* sharing a common ancestor. *R. jacobsoni* also showed close relationship with species *Partancistrocerus perennis, Parancistrocerus pedestris* and *Parancistrocerus pensylvanicus* having a single origin and species *Ancistrocerus waldenii, Ancestrocerus campestris* and *Ancestrocerus albophaleratus* which arose from a common ancestor. The peptide tree showed the relationship pattern of *R. jacobsoni* to the species of the genus *Apoica* viz. A*poica pallida, Apoica strigata, Apoica pallens* and a Vespidae species; species of genus *Dolichovespula* viz. *Dolichovespula arenaria* and *albida;* species of *Vespa* genus viz. *Vespa velutina, Vespa simillima, Vespa mandariana japonica* and *Vespa mandartiana,* species of genus *Polistes* viz. *Polistes semenowi, Polistes chinensis, Polistes chinensis antennalis, Polistes riparius, Polistes helveticus, Polistes gallicus, Polistes biglumis, Polistes hellenicus, Polistes bischoffi, Polistes metricuss, Polistes dominula, Polistes sulcifer* and *Polistes nimpha. R. jacobsoni* is distantly related to the species *Odynerus spinipes* of the subfamily Eumeninae. The present results indicated the significance of an identification system based on COI gene sequence. The result highlighted the significant sequence diversity of the COI gene of *R. jacobsoni* from its allied species, thus enabling an easy discrimination of species.

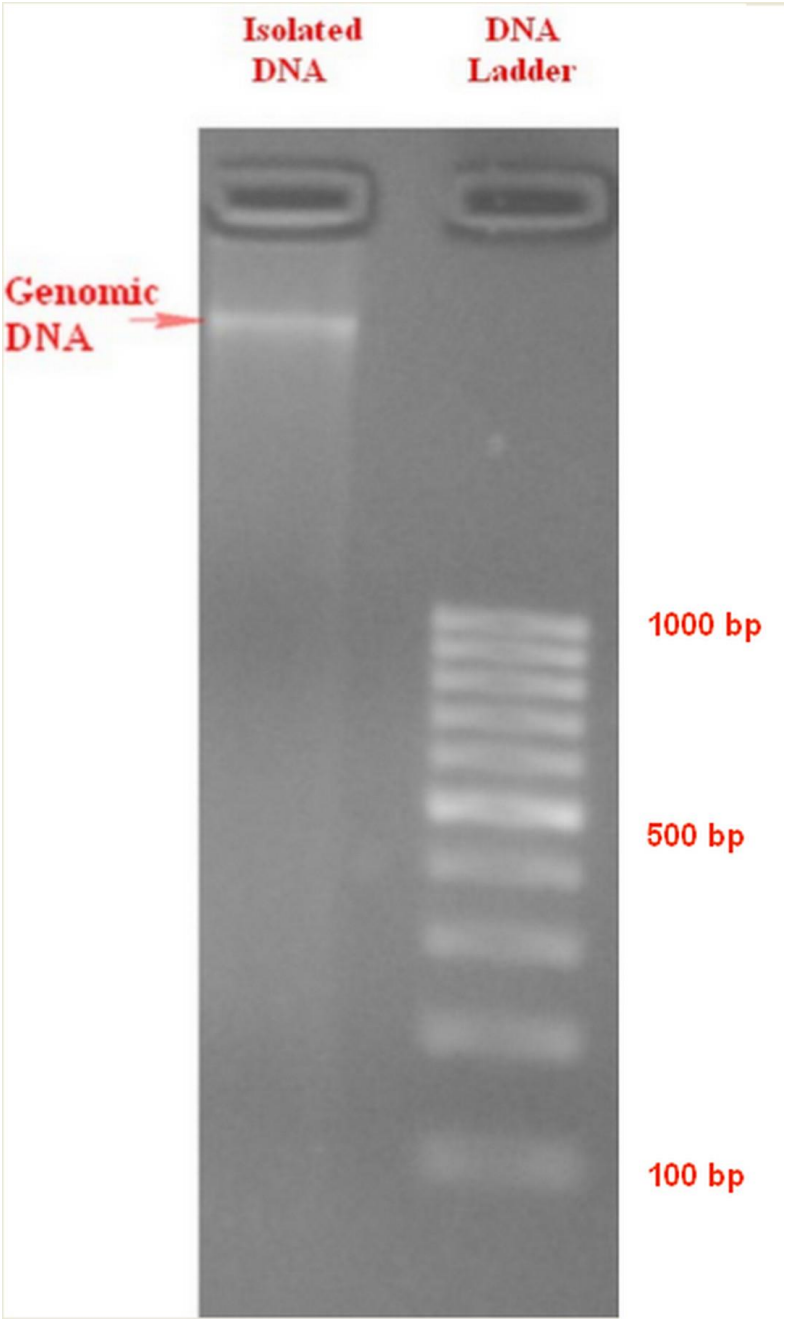

Fig.103. Gel picture of total DNA isolated from *Ropalidia jacobsoni* Voucher CUKAK8.

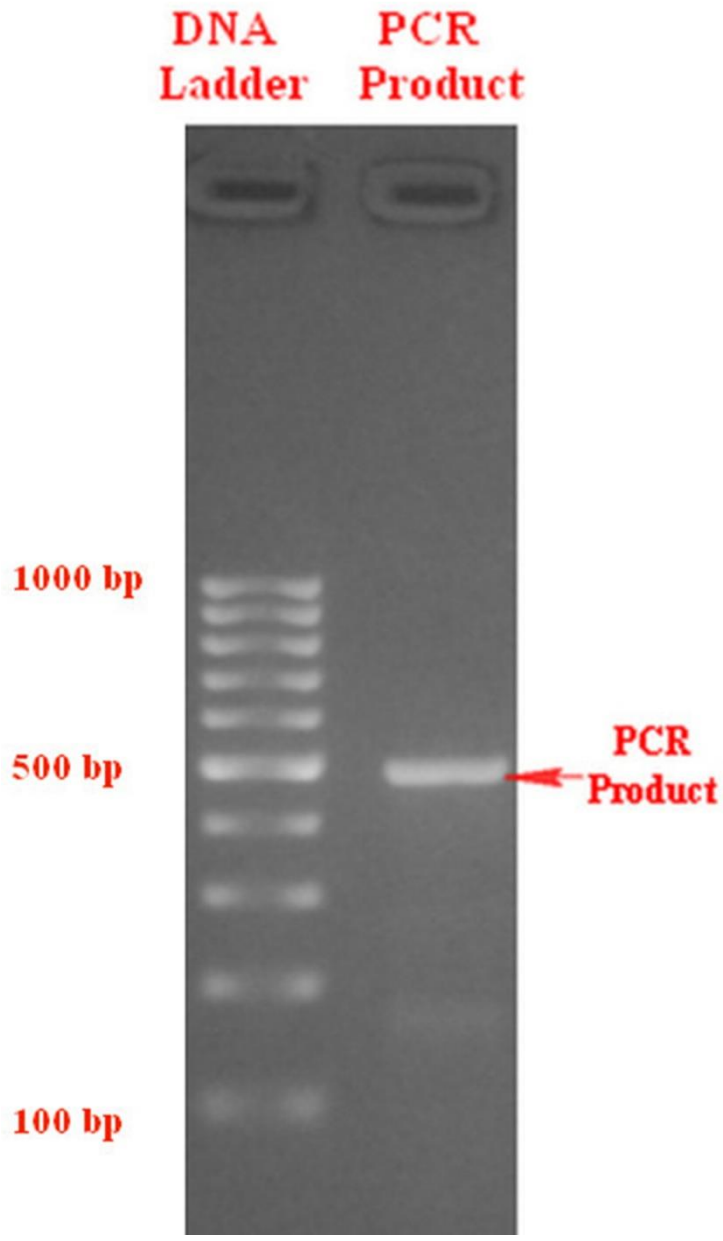

Fig.104. Gel picture showing the PCR product of partial COI gene of *Ropalidia jacobsoni* Voucher CUKAK8.

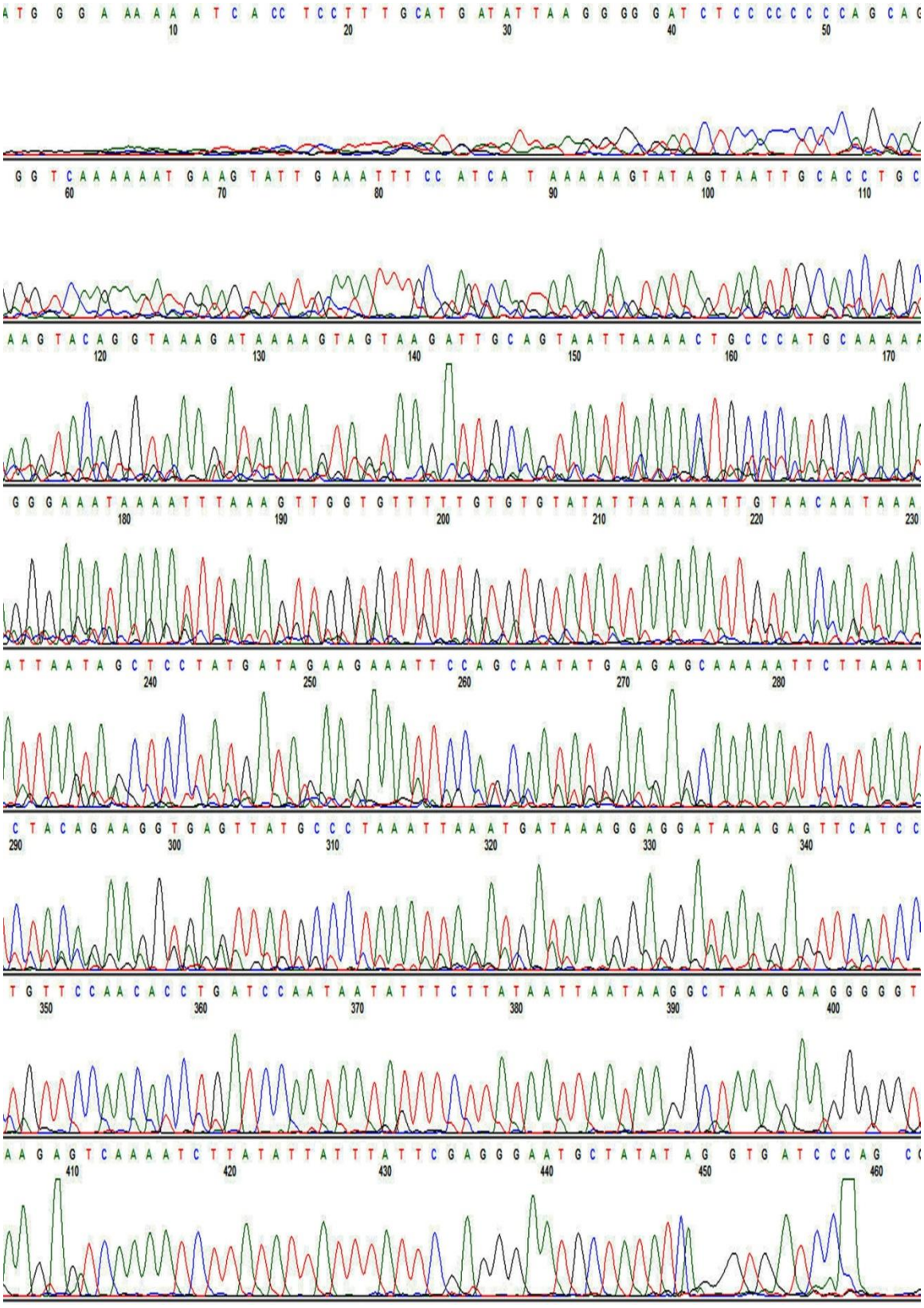

Fig.105. Sequencing chromatogram (forward sequence) showing partial coding sequence of cytochrome oxidase subunit I (COI) of *R. jacobsoni* Voucher CUKAK8.

>*R. jacobsoni* Voucher CUKAK8 mitochondrial cytochrome oxidase subunit I (COI) partial nucleotide sequence, 353 bases

TAGCATTCCCTCGAATAAATAATATAAGATTTTGACTCTTACCCCCTTCTT
TAGCCTTATTAATTATAAGAAATATTATTGGATCAGGTGTTGGAACAGGAT
GAACTCTTTATCCTCCTTTATCATTTAATTTAGGGCATAACTCACCTTCTG
TAGATTTAAGAATTTTTGCTCTTCATATTGCTGGAATTTCTTCTATCATAG
GAGCTATTAATTTTATTGTTACAATTTTTAATATACACACAAAAACACCAA
CTTTAAATTTTATTTCCCTTTTTGCATGGGCAGTTTTAATTACTGCAATCT
TACTACTTTTATCTTTACCTGTACTTGCAGGTGCAATTACTATACTT

Fig.106. Partial coding sequence of *R. jacobsoni* Voucher CUKAK8 cytochrome oxidase subunit I (COI) gene.

Score	Expect	Identities	Gaps	Strand
614 bits (332)	3e-172	346/353 (98%)	0/353 (0%)	Plus/Plus

```
Query       TAGCATTCCCTCGAATAAATAATATAAGATTTTGACTCTTACCCCCTTCTTTAGCCTTAT  60
            |||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     TAGCTTTTCCTCGAATAAATAATATAAGATTTTGACTCTTACCCCCTTCTTTAGCCTTAT  287

Query       TAATTATAAGAAATATTATTGGATCAGGTGTTGGAACAGGATGAACTCTTTATCCTCCTT  120
            |||||||||||||||||||| |||||||||| |||||||||||||||||||||||||||
Subject     TAATTATAAGAAATATTATTGGGTCAGGTGTTGGGACAGGATGAACTCTTTATCCTCCTT  347

Query       TATCATTTAATTTAGGGCATAACTCACCTTCTGTAGATTTAAGAATTTTTGCTCTTCATA  180
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     TATCATCTAATTTAGGGCATAACTCACCTTCTGTAGATTTAAGAATTTTTGCTCTTCATA  407

Query       TTGCTGGAATTTCTTCTATCATAGGAGCTATTAATTTTATTGTTACAATTTTTAATATAC  240
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Subject     TTGCTGGAATTTCTTCTATCATAGGAGCTATTAATTTTATTGTCACAATTTTTAATATAC  467

Query       ACACAAAAACACCAACTTTAAATTTTATTTCCCTTTTTGCATGGGCAGTTTTAATTACTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject     ACACAAAAACACCAACTTTAAATTTTATTTCCCTTTTTGCATGGGCAGTTTTAATTACTG  527

Query       CAATCTTACTACTTTTATCTTTACCTGTACTTGCAGGTGCAATTACTATACTT  353
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||
Subject     CAATCTTATTACTTTTATCTTTACCTGTACTTGCAGGTGCAATTACTATACTT  580
```

Fig.107. Nearest sequence match from the BLAST result of *R. jacobsoni* Voucher CUKAK8 cytochrome oxidase subunit I (COI) gene sequence. Query = *R. jacobsoni* Voucher CUKAK8; Subject = *Rhynchium quinquecinctum* (GenBank Accession Number: AB969818.1). Note that the nearest match is only 98% to the sequence in the NCBI database depicting the sequence obtained here is novel.

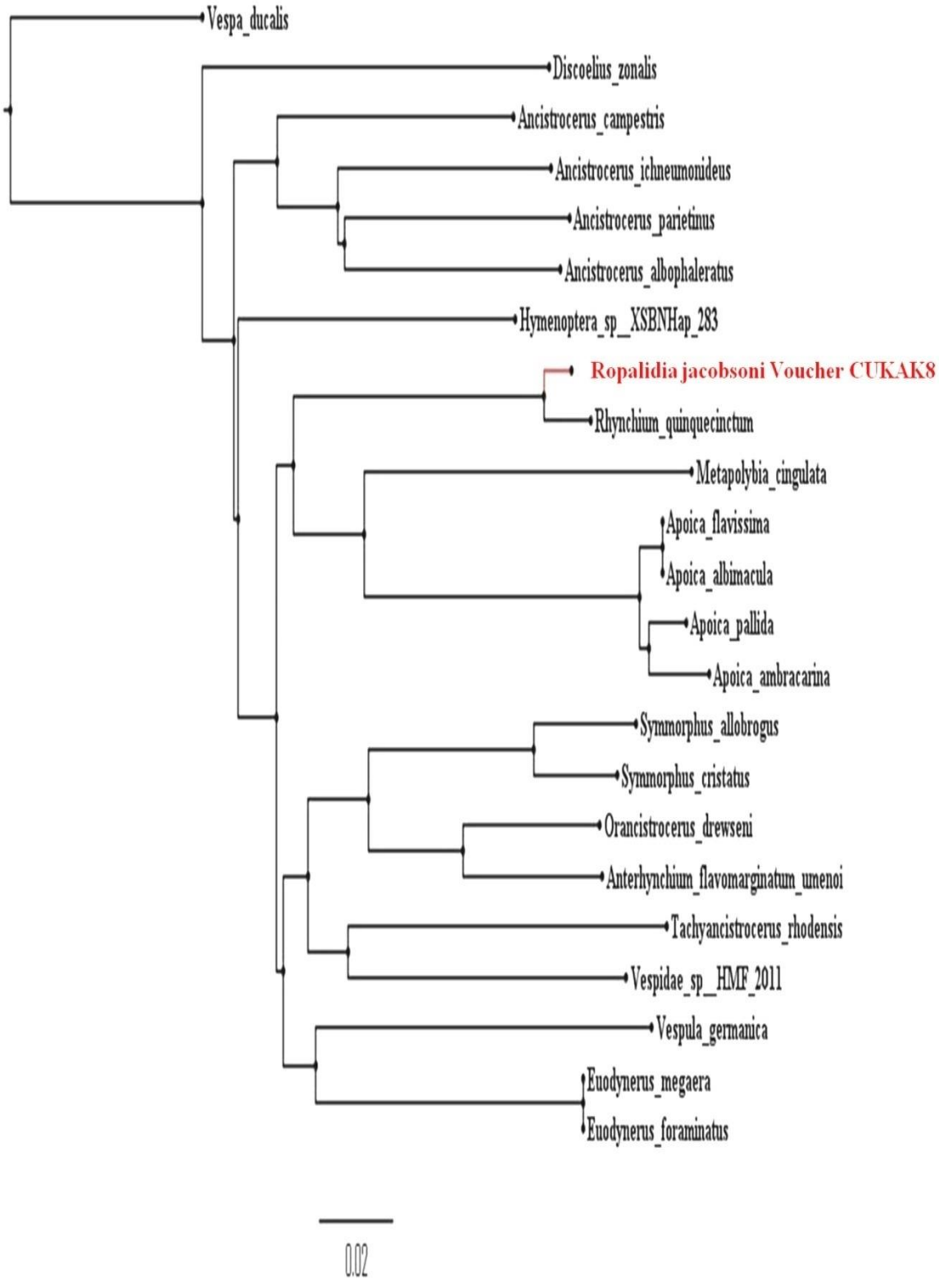

Fig.108. N-J tree plotted for *R. jacobsoni* Voucher CUKAK8 cytochrome oxidase subunit I (COI) gene sequence.

>*R. jacobsoni* Voucher CUKAK8

AFPRMNNMSFWLLPPSLALLIMSNIIGSGVGTGWTLYPPLSFNLGHNSPSVDLSIFA
LHIAGISSIMGAINFIVTIFNMHTKTPTLNFISLFAWAVLITAILLLLSLPVLAGAI
TML

Fig.109. The conceptual translation product of the DNA sequence of mitochondrial cytochrome oxidase subunit I (COI) gene of *R. jacobsoni* Voucher CUKAK8.

Score	Expect	Method	Identities	Positives	Gaps
218 bits (556)	3e-69	Compositional matrix adjust.	116/117 (99%)	116/117 (99%)	0/117 (0%)

```
Query       AFPRMNNMSFWLLPPSLALLIMSNIIGSGVGTGWTLYPPLSFNLGHNSPSVDLSIFALHI  60
            AFPRMNNMSFWLLPPSLALLIMSNIIGSGVGTGWTLYPPLS NLGHNSPSVDLSIFALHI
Subject     AFPRMNNMSFWLLPPSLALLIMSNIIGSGVGTGWTLYPPLSSNLGHNSPSVDLSIFALHI  136

Query       AGISSIMGAINFIVTIFNMHTKTPTLNFISLFAWAVLITAILLLLSLPVLAGAITML  117
            AGISSIMGAINFIVTIFNMHTKTPTLNFISLFAWAVLITAILLLLSLPVLAGAITML
Subject     AGISSIMGAINFIVTIFNMHTKTPTLNFISLFAWAVLITAILLLLSLPVLAGAITML  193
```

Fig.110. Nearest peptide sequence match from the BLAST result of *R. jacobsoni* Voucher CUKAK8 cytochrome oxidase subunit I (COI) conceptual translation product. Query = *R. jacobsoni* Voucher CUKAK8; Subject = *Rhynchium quinquecinctum* (GenBank Accession Number: BAP05525.1). Note that the nearest match is only 99% to the sequence in the NCBI database depicting the sequence obtained here is novel.

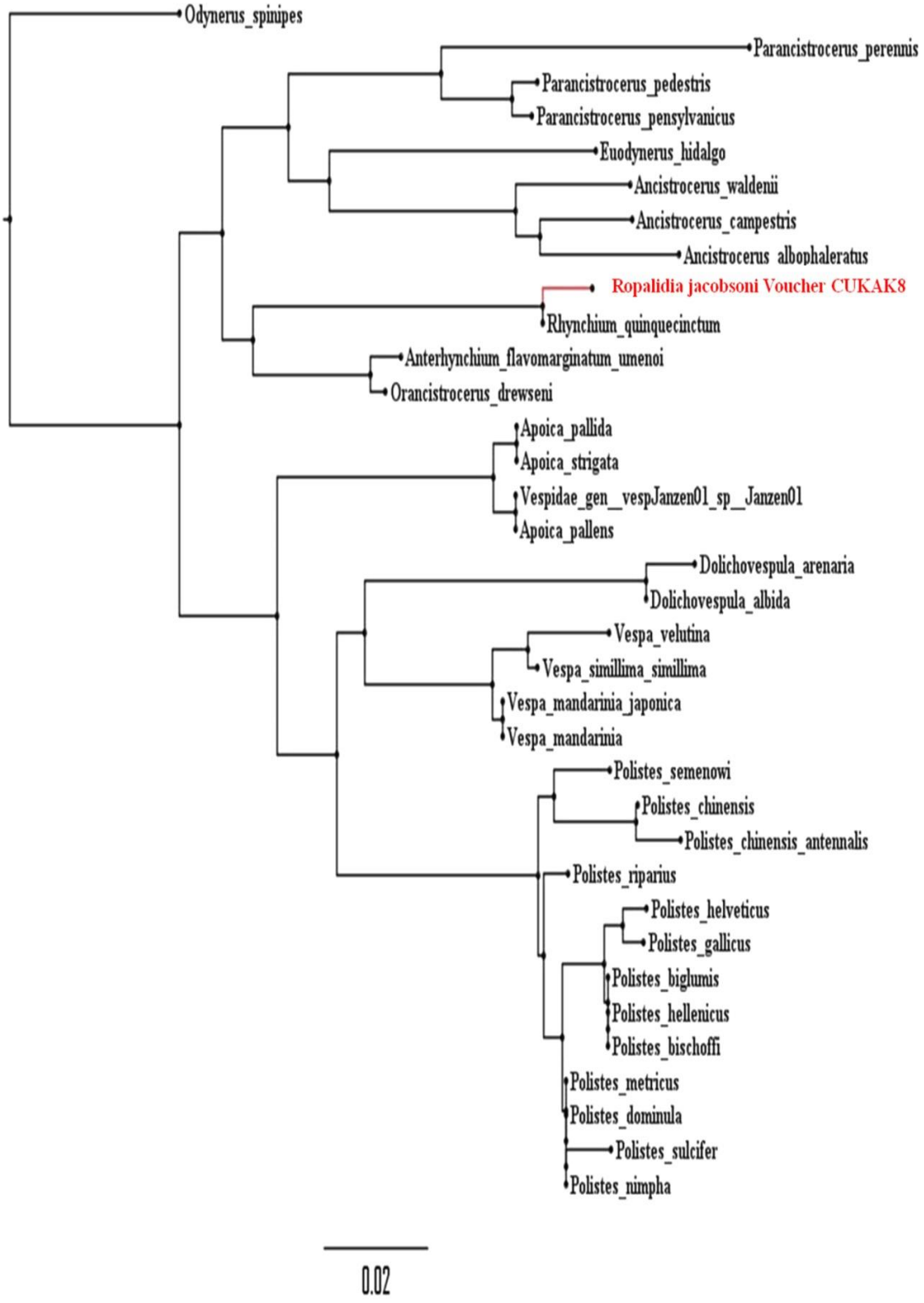

Fig.111. N-J tree plotted for conceptual translation product of *R. jacobsoni* Voucher CUKAK8 cytochrome oxidase subunit I (COI) peptide sequence.

9. Genetic relationships between the eight wasps studied

The relationship among all the 8 species of wasps studied in the present work was analyzed using ClustalW programme (http://www.genome.jp/tools/clustalw/). The close relation among all the species confirms the taxonomic positions of them under a single family 'Vespidae'. According to nucleotide sequence analysis, *Rhynchium brunneum* and *R. spatulata* is found to be the most closely related wasps having 99% identity. They have a single origin and are closely related to *Vespa tropica* by a common ancestor, which diverged into two clades; one of *Vespa tropica* and the other containing the *R. brunneum + Ropalidia jacobsoni* cluster. *Vespa tropica, Rhynchium brunneum* and *Ropalidia jacobsoni* have a sister relationship with *Ropalidia spatulata + Polistes strigosus* cluster, which inturn arose from a single ancestor and are placed under the same subfamily Polistinae. These six wasps are equally related to *Delta pyriforme* by a common ancestor. The wasps *Phimenes flavopictum* and *Delta conoideum* has a common ancestor which diverged into three clades viz. the cluster containing *Delta pyriforme, Vespa tropica* and *Rhynchium brunneum + Ropalidia spatulata; Polistes strigosus + Ropalidia spatulata; Phimenes flvopictum* and *Delta conoideum.*

The most distantly related species are *Rhynchium brunneum* and *Polistes strigosus*, which are of different genus and represent the subfamilies Eumeninae and

Polistinae respectively (**Figure 113**). The phylogeny analyzes supported the ClustalW results. The species *Rhynchium brunneum* and *R. jacobsoni* are the most closely related species along with *Polistes strigosus* and *Ropalidia spatulata*, having a common ancestor, according to COI partial nucleotide sequence phylogenetic tree. *Delta conoideum* and *Phimenes flavopictum* are also closely related species having a single origin (**Figure 114**). The phylogeny analysis of the eight selected wasps showed the interrelationships among the species and their pattern of evolutionary divergence.

```
Sequence 1: V.tropica_Voucher_CUKAK1       658bp
Sequence 2: R.brunneum_Voucher_CUKAK2      606bp
Sequence 3: D.pyriforme_Voucher_CUKAK3     658bp
Sequence 4: D.conoideum_Voucher_CUKAK4     621bp
Sequence 5: P.flavopictum_Voucher_CUKAK5   658bp
Sequence 6: R.spatulata_Voucher_CUKAK6     484bp
Sequence 7: P.strigosus_Voucher_CUKAK7     484bp
Sequence 8: R.jacobsoni_Voucher_CUKAK8     353bp

P.flavopictum_Voucher_CUKAK5      AATATTATATTTAATTTTTGGTTTATGAAGAGGGATAATTGGATCATCAT
D.conoideum_Voucher_CUKAK4        AATACTTTATTTAATTTTTGGATTATGAAGTGGAATAATTGGATCTTCTT
D.pyriforme_Voucher_CUKAK3        AATACTTTATTTTATATTTGGTTTATGAAGAGGAATAATTGGAGCATCAT
V.tropica_Voucher_CUKAK1          AATACTATATTTTATTTTTGCATTATGATCCGGTACTTTAGGAGCATCCA
R.brunneum_Voucher_CUKAK2         --------------------------------GGAATAGTAGGCTCAGCAT
R.jacobsoni_Voucher_CUKAK8        --------------------------------------------------
P.strigosus_Voucher_CUKAK7        --------------------------------------------------
R.spatulata_Voucher_CUKAK6        --------------------------------------------------

P.flavopictum_Voucher_CUKAK5      TAAGAATATTAATTCGTATAGAATTAGGAATACCAGGAAAATTAATTAAT
D.conoideum_Voucher_CUKAK4        TAAGAATAATTATTCGTATAGAATTAGGAATACCTGGAAAATTAATTAAT
D.pyriforme_Voucher_CUKAK3        TAAGAATATTAATTCGAATAGAATTAGGTATACCTGGAAAATTAATTAAT
V.tropica_Voucher_CUKAK1          TAAGACTTATTATCCGAGCAGAATTAAGATCTCCCGGTAACTTAATTAAT
R.brunneum_Voucher_CUKAK2         TAAGAATAATTATTCGAATAGAATTAAGAGCCCCAGGTAAATTAATTGGC
R.jacobsoni_Voucher_CUKAK8        --------------------------------------------------
P.strigosus_Voucher_CUKAK7        --------------------------------------------------
R.spatulata_Voucher_CUKAK6        --------------------------------------------------

P.flavopictum_Voucher_CUKAK5      AATGATCAAATTTTTAATACTATTGTTACATCTCATGCTTTTATTATAAT
D.conoideum_Voucher_CUKAK4        AATGATCAAATTTATAATACAGTTGTTACATCTCATGCTTTTATTATAAT
D.pyriforme_Voucher_CUKAK3        AATGATCAAATCTATAATACTATTGTTACATCACATGCTTTTATTATAAT
V.tropica_Voucher_CUKAK1          AATGATCAAATTTATAACACCATTATTACTGCTCATGCCTTCATTATAAT
R.brunneum_Voucher_CUKAK2         AATGACCAAATTTATAATACTATTGTTACTGCCCATGCTTTTATTATAAT
R.jacobsoni_Voucher_CUKAK8        --------------------------------------------------
P.strigosus_Voucher_CUKAK7        --------------------------------------------------
R.spatulata_Voucher_CUKAK6        --------------------------------------------------

P.flavopictum_Voucher_CUKAK5      TTTTTTTATAGTTATACCTTTTATAATTGGAGGATTTGGAAATTGATTAA
D.conoideum_Voucher_CUKAK4        TTTTTTTATAGTTATACCTTTTATAATTGGAGGATTTGGTAATTGATTAA
D.pyriforme_Voucher_CUKAK3        TTTTTTTATAGTAATACCATTCATAATTGGAGGATTTGGAAATTGATTAA
V.tropica_Voucher_CUKAK1          TTTTTTTATAGTTATACCTTTCATAATTGGAGGATTTGGAAATTGATTAA
R.brunneum_Voucher_CUKAK2         TTTTTTTATAGTTATACCATTTATAATTGGAGGATTCGGAAATTGGCTTG
R.jacobsoni_Voucher_CUKAK8        --------------------------------------------------
P.strigosus_Voucher_CUKAK7        --------------------------------------------------
R.spatulata_Voucher_CUKAK6        --------------------------------------------------

P.flavopictum_Voucher_CUKAK5      TTCCTTTAATAATTGCGGCTCCAGATATAGCATTTCCACGTATAAATAAT
D.conoideum_Voucher_CUKAK4        TTCCTTTAATAATTTCAGCTCCTGATATAGCTTTTCCTCGAATAAATAAT
D.pyriforme_Voucher_CUKAK3        TTCCACTTATAATTTCCGCTCCTGATATAGCTTTCCCACGAATAAATAAT
V.tropica_Voucher_CUKAK1          TTCCTATAATACTAGGAACTCCAGATATAGCTTTCCCTCGAATAAATAAT
R.brunneum_Voucher_CUKAK2         TACCCTTAATATTAACAGCCCCTGATATAGCTTTTCCTCGAATAAATAAT
R.jacobsoni_Voucher_CUKAK8        ----------------------------TAGCATTCCCTCGAATAAATAAT
P.strigosus_Voucher_CUKAK7        ------------ATGGATCACCTGATATAGCATTCCCACGAATAAATAAT
R.spatulata_Voucher_CUKAK6        -----------------TCACCTGATATAGCATTCCCACGAATAAATAAT
                                                   **** ** ** ** *********

P.flavopictum_Voucher_CUKAK5      ATAAGATTTTGATTATTACCTCCTTCATTATTTTTATTATTATTAAGAAA
D.conoideum_Voucher_CUKAK4        ATAAGATTTTGATTACTTCCACCATCTTTATTTTTTTTATTATTAAGAAA
D.pyriforme_Voucher_CUKAK3        ATAAGATTTTGATTACTTCCTCCATCATTATTTTTTCTACTATTAAGAAA
V.tropica_Voucher_CUKAK1          ATAAGATTTTGACTTTTACCTCCTTCATTATTCCTACTAATTATAAGAAA
R.brunneum_Voucher_CUKAK2         ATAAGATTTTGACTCTTACCCCCTTCTTTAGCCTTATTAATTATAAGAAA
R.jacobsoni_Voucher_CUKAK8        ATAAGATTTTGACTCTTACCCCCTTCTTTAGCCTTATTAATTATAAGAAA
P.strigosus_Voucher_CUKAK7        ATAAGATTTTGATTATTACCTCCATCCTTATTTTTATTAATTTTAAGAAA
R.spatulata_Voucher_CUKAK6        ATAAGATTTTGATTACTCCCACCATCTCTTTGCCTTTTAATTTTAAGAAA
                                  ************ *  * ** ** **  *     *  ** *  *******
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```
P.flavopictum_Voucher_CUKAK5      TTATATTGGAGTAGGGGTTGGAACAGGATGAACTTTATATCCTCCTTTGT
D.conoideum_Voucher_CUKAK4        TTTTATTGGTATAGGAATAGGAACAGGATGAACATTATATCCTCCATTAT
D.pyriforme_Voucher_CUKAK3        TTATATTGGAACAGGAGTTGGAACAGGATGAACCTTATATCCTCCCCTCT
V.tropica_Voucher_CUKAK1          TTTTATTGGTGGAGGCGTAGGAACTGGTTGAACTCTTTACCCCCCTTTAT
R.brunneum_Voucher_CUKAK2         TATTATTGGATCAGGTGTTGGAACAGGATGAACTCTTTATCCTCCTTTAT
R.jacobsoni_Voucher_CUKAK8        TATTATTGGATCAGGTGTTGGAACAGGATGAACTCTTTATCCTCCTTTAT
P.strigosus_Voucher_CUKAK7        CTTAATTAATATAGGTGTAGGAACAGGATGAACTTTATATCCACCTTTAT
R.spatulata_Voucher_CUKAK6        CCTTATTGGGATAGGAGTTGGAACTGGATGAACATTATATCCACCTTTAT
                                      ***     ***  * ***** ** *****  * ** ** **  * *

P.flavopictum_Voucher_CUKAK5      CTTCTATTTTAGGTCATAATACACCTTCAGTTGATATTGGAATTTTTTCT
D.conoideum_Voucher_CUKAK4        CTTCTATTCTTGGACATAATTCACCTTCTATTGATATTGGAATTTTTTCT
D.pyriforme_Voucher_CUKAK3        CATCAATTATAGGACATAATTCCCCATCTGTTGATATTGGTATTTTTTCT
V.tropica_Voucher_CUKAK1          CATCAATTACCGGCCATAATTCACCATCAGTAGATTTAGGTATTTTTTCT
R.brunneum_Voucher_CUKAK2         CATCTAATTTAGGGCATAACTCACCTTCTGTAGATTTAAGAATTTTTGCT
R.jacobsoni_Voucher_CUKAK8        CATTTAATTTAGGGCATAACTCACCTTCTGTAGATTTAAGAATTTTTGCT
P.strigosus_Voucher_CUKAK7        CTTCAATTACTGGACATAATTCTATCTCTGTAGATTTAGCAATTTTTTCT
R.spatulata_Voucher_CUKAK6        CGTCTCTTTTAGGACACAATTCTCCTTCTGTAGATTTAAGAATTTTTTCT
                                  * *    *   ** ** **  *    **  * *** *    ****** **

P.flavopictum_Voucher_CUKAK5      TTACATATTGCAGGAATTTCTTCTATTATAGGAGCTTTAAATTTTATTAT
D.conoideum_Voucher_CUKAK4        CTTCATATTGCAGGAATTTCTTCAATTATAGGAGCATTAAATTTTATTGT
D.pyriforme_Voucher_CUKAK3        TTACATATTGCAGGAATTTCATCTATTATAGGAGCATTAAATTTTATTGT
V.tropica_Voucher_CUKAK1          CTTCATATTGCTGGCATCTCTTCTATTATAGGAGCAATTAATTTCATTGT
R.brunneum_Voucher_CUKAK2         CTTCATATTGCTGGAATTTCTTCTATCATAGGAGCTATTAATTTTATTGT
R.jacobsoni_Voucher_CUKAK8        CTTCATATTGCTGGAATTTCTTCTATCATAGGAGCTATTAATTTTATTGT
P.strigosus_Voucher_CUKAK7        CTTCATATTGCTGGGATTTCCTCAATTATAGGAGCTATTAATTTTATTGT
R.spatulata_Voucher_CUKAK6        TTACACATTGCAGGTATCTCTTCAATTATAGGAGCTATTAATTTTATTGT
                                   * ** ***** ** ** ** ** ** ********  * ***** *** *

P.flavopictum_Voucher_CUKAK5      TACTATTTTAAATATACATACAAAAACTTTAAAAATAAATTTTTTACCTT
D.conoideum_Voucher_CUKAK4        TACAATTTTAAATATACATACAAAAACCTTAAATTTAAATTTTTTACCTT
D.pyriforme_Voucher_CUKAK3        TACTATCTTAAATATACATACAAAAATATTAAAATTAAATTACTTACCAC
V.tropica_Voucher_CUKAK1          TACAATCTTAAATATACACATTAAAACTCATTCATTAAATTTTTTACCTT
R.brunneum_Voucher_CUKAK2         TACAATTTTTAATATACACACAAAAACACCAACTTTAAATTTTATTTCCC
R.jacobsoni_Voucher_CUKAK8        TACAATTTTTAATATACACACAAAAACACCAACTTTAAATTTTATTTCCC
P.strigosus_Voucher_CUKAK7        TACCATTTTAAATATACATATTAAAGTTTATTCTTTAAATTTTTTACCTC
R.spatulata_Voucher_CUKAK6        TACAATTTATAATATACATATTAAAACTTCATCATTAAATTTTTTACCTT
                                  *** ** *  ******** *  ***          ******   *  *

P.flavopictum_Voucher_CUKAK5      TATTTTCTTGATCAGTTTTAATTACTGCATTTTTATTATTATTATCATTA
D.conoideum_Voucher_CUKAK4        TATTTGTTTGATCTGTATTAATTACAGCTATTTTATTATTATTATCTCTT
D.pyriforme_Voucher_CUKAK3        TATTTGCATGATCAGTTTTAATTACAGCAATCTTATTATTATTATCATTA
V.tropica_Voucher_CUKAK1          TATTTGCATGATCAGTATTAATTACAGCTTTTCTTTTACTTCTCTCTCTT
R.brunneum_Voucher_CUKAK2         TTTTTGCATGGGCAGTTTTAATTACTGCAATCTTACTACTTTTATCTTTA
R.jacobsoni_Voucher_CUKAK8        TTTTTGCATGGGCAGTTTTAATTACTGCAATCTTACTACTTTTATCTTTA
P.strigosus_Voucher_CUKAK7        TATTTACATGATCAGTTTTAATTACAGCAATTTTACTTTTATTATCACTA
R.spatulata_Voucher_CUKAK6        TATTTGCTTGATCAATCTTAATTACTGCTATTCTTTTACTTTTATCTTTA
                                  * ***   **  *  * ******** **  *  *  *  *  * **  *

P.flavopictum_Voucher_CUKAK5      CCAGTTTTAGCTGGAGCTATTACAATATTATTAACTGATCGAAATTTTAA
D.conoideum_Voucher_CUKAK4        CCAGTTTTAGCAGGAGCAATTACTATATTACTTACAGATCGAAATTTTAA
D.pyriforme_Voucher_CUKAK3        CCAGTTCTAGCAGGAGCAATCACTATATTATTAACAGATCGAAATTTTAA
V.tropica_Voucher_CUKAK1          CCCGTTTTAGCTGGAGCAATTACTATACTTTTAACAGATCGAAATTTCAA
R.brunneum_Voucher_CUKAK2         CCTGTACTTGCAGGTGCAATTACTATACTTCTTACTGATCGAAATTTCAA
R.jacobsoni_Voucher_CUKAK8        CCTGTACTTGCAGGTGCAATTACTATACTT--------------------
P.strigosus_Voucher_CUKAK7        CCCGTACTAGCTGGAGCAATCACTATATTATTAACAGATCGAAATTTTAA
R.spatulata_Voucher_CUKAK6        CCAGTTCTAGCCGGAGCTATTACTATATTATTAACTGATCGAAATTTAAA
                                  ** **  * ** ** ** ** ** *** *


P.flavopictum_Voucher_CUKAK5      TACTTCTTTTTTTGATCCTTCAGGGGGGGGTGATCCAATTTTATATCAAC
D.conoideum_Voucher_CUKAK4        TACATCTTTTTTTGATCCTTC----------------------------
D.pyriforme_Voucher_CUKAK3        TACATCATTCTTTGATCCATCAGGAGGAGGAGATCCAATTCTTTATCAAC
V.tropica_Voucher_CUKAK1          TACATCATTTTTTGATCCAACAGGAGGTGGCGATCCTATCCTATACCAAC
R.brunneum_Voucher_CUKAK2         TACTTCATTTTTTGACCCTGCTGGGGGTGGAGATCCA-------------
R.jacobsoni_Voucher_CUKAK8        ---------------------------------------------------
P.strigosus_Voucher_CUKAK7        TACCTCATTCTTTGATCCATTAGGAGGAGGAGATCCAATTTTATTTCAAC
R.spatulata_Voucher_CUKAK6        TACAACATTCTTTGACCCTACAGGAGGAGGAGACCCTATTTTATACCAAC
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P.flavopictum_Voucher_CUKAK5      ATTTATTT-----------------------------------------
D.conoideum_Voucher_CUKAK4        ------------------------------------------------
D.pyriforme_Voucher_CUKAK3        ATTTATTC-----------------------------------------
V.tropica_Voucher_CUKAK1          ACTTATTC-----------------------------------------
R.brunneum_Voucher_CUKAK2         ------------------------------------------------
R.jacobsoni_Voucher_CUKAK8        ------------------------------------------------
P.strigosus_Voucher_CUKAK7        ATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTTT----
R.spatulata_Voucher_CUKAK6        ATTTATTTTGATTCTTTGGTCATCCAGAAGTTTATATTTTAATTTTTACC


P.flavopictum_Voucher_CUKAK5      -
D.conoideum_Voucher_CUKAK4        -
D.pyriforme_Voucher_CUKAK3        -
V.tropica_Voucher_CUKAK1          -
R.brunneum_Voucher_CUKAK2         -
R.jacobsoni_Voucher_CUKAK8        -
P.strigosus_Voucher_CUKAK7        -
R.spatulata_Voucher_CUKAK6        G
```

Fig.113. The similarity among the nucleotide sequence of the partial COI gene from the eight different wasps species selected in this study using ClustalW.

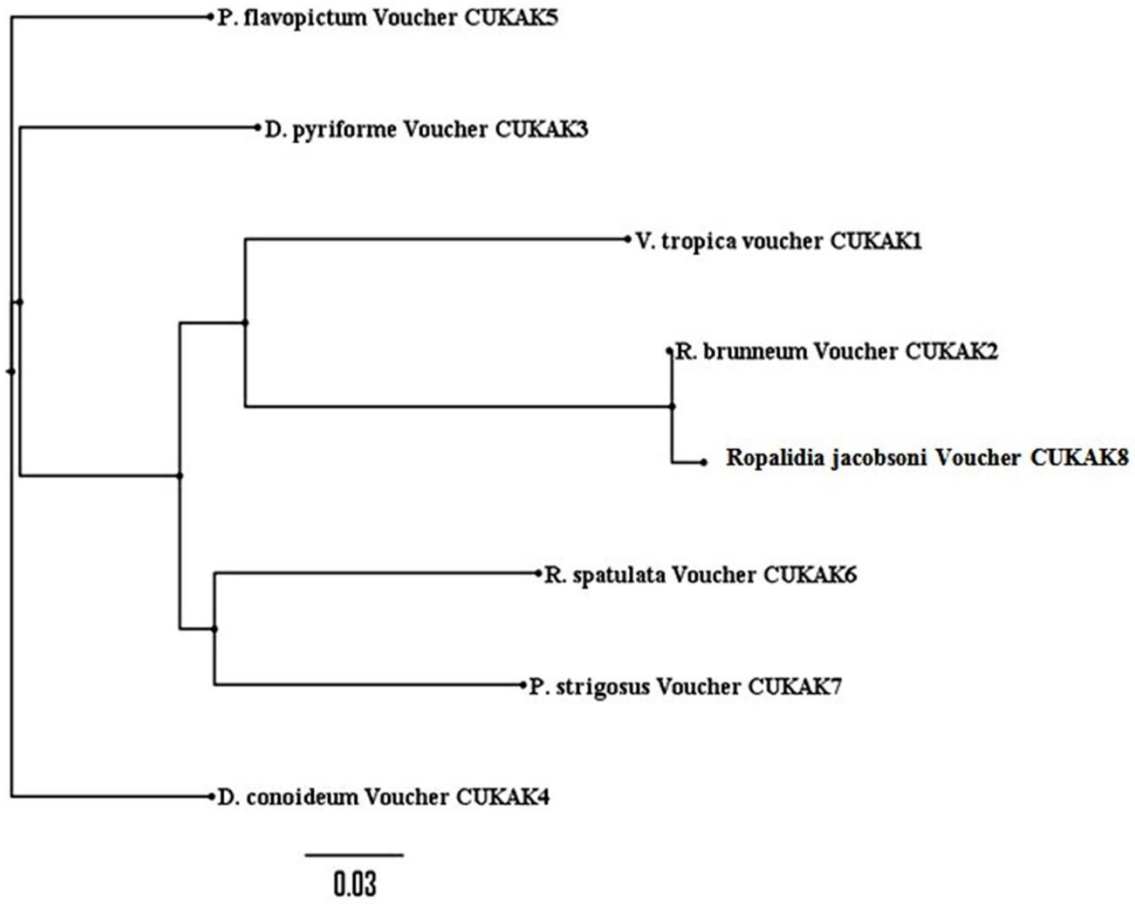

Fig.114. The phylogenetic relationship between the nucleotide sequences of the partial COI gene from the eight wasp species selected in this study using N-J method.

SUMMARY

The family Vespidae is a diverse family of wasps comprising nearly all the known eusocial wasps and many solitary wasps which has a worldwide distribution. They are increasingly used in agricultural pest control as they prey mostly on pest insects and have little impact on crops. Some of them are known to be good pollinators of plants. Their diverse biological characteristics make them excellent for basic studies in genetics, ecology, behavior, development and evolution.

About 75,000 species of wasps are known to date in which nearly 5000 species are coming under the family Vespidae. 105 species of Vespidae wasps are reported from India among which 51 species are from Kerala. But only 2 molecular barcode data of COI gene from family Vespidae is reported from India. The molecular genetic study of wasps has attracted many biologists, geneticists and entomologists. Molecular analysis of the mitochondrial gene of wasps provided an unambiguous data for the origin and phylogeny analysis of these species.

The complete genome sequencing of wasps have been carried out in three species which revealed almost 7000 genes that have recognizable counter parts in humans. The wasp genome sequencing significantly augments the opportunities to generate molecular barcodes for the species, study their genetics, phylogeny and evolution along with its

various applications in the field of Integrated Pest Management, medicine, vaccine, drug development etc.

In the present study, a brief survey was conducted on the Vespidae wasps of Kerala and eight species of wasps coming under different subfamilies of Vespidae were selected for the study. They were collected from various places of Kerala, photographed and stored for the molecular analysis. The partial sequence of mitochondrial cytochrome c oxidase subunit I gene of 8 wasps coming under the family Vespidae from Kerala were amplified and sequenced. The DNA sequences obtained were novel and can be used as a molecular barcode for accurate identification and phylogeny analysis of the species.

The detailed studies on the genetic structure of mitochondrial COI gene were conducted. The similarity of the nucleotide and conceptually translated peptide sequence of the COI gene of the selected wasp species were analyzed using BLASTn and BLASTp tools. The BLAST results showed similarity to the evolutionarily related species of wasps, suggesting the importance of mitochondrial COI gene in the phylogenetic studies of wasps. The sequence can be used to analyze the evolutionary divergence ratio of species and also the nucleotide substitution rate in the mitochondrial genome.

The COI sequences obtained in the present study can also be used in molecular systematic studies of the family Vespidae. It can assign an unidentified species to its

correct taxonomic status by comparing with the COI sequences in the database. N-J tree for molecular phylogeny analysis of all the 8 species of wasps were constructed. It clearly depicted the relationship of each species of wasps to other species of same genus, family or others.

The PCR of COI gene of the great banded hornet *Vespa tropica*, collected from Trichur District of Kerala yielded a product of 658bp which was novel. Its conceptual translation product yielded a product of 219 amino acids. It showed close realtion to the wasp *Vespa ducalis* of the same genus with 91% identity in BLASTn and 99% identity in BLASTp analysis. The phylogeny tree (N-J tree) also depicted the relationship of *Vespa tropica*, to its closely related species *Vespa ducalis* having a acommon ancestor, followed by other species of the same genus and subfamily. The phylogeny tree also depicted the relationship pattern of *V. tropica* to species of other genus and subfamily.

The PCR of COI gene of the potter wasp *Rhynchium brunneum*, collected from Kasaragod District of Kerala yielded a product of 606bp which was novel. The conceptual translation product yielded a product of 202 amino acids. It is found more closely related to the wasp *Rhynchium quinquecinctum* of the same genus, with 99% identity in BLASTn and 100% identity in BLASTp. The result showing 100% identity can be explained by the degeneracy of genetic code where more than one codon codes for

a single amino acid. The phylogeny tree (N-J tree) supported the close relation of *Rhynchium brunneum* to *Rhynchium quinquecinctum,* which arose from a single origin. The phylogeny trees also showed the relationship pattern of *R. brunneum* with other species of the subfamily Eumeninae and Vespinae.

The PCR of COI gene of the potter wasp *Delta pyriforme* collected from Malappuram District of Kerala yielded a product of 658bp which was novel. The conceptual translation yielded a product of 219 amino acids. It showed more closer relation to the wasp *Eumenes sp*. of the same subfamily with 99% identity in BLASTn and 100% identity in BLASTp analysis. The phylogeny tree (N-J tree) showed the relationship of *Delta pyriforme* to its related species *Eumenes sp*. having a common ancestor and coming under the same sub family Eumeninae. The phylogeny tree also explained the pattern of relationship of *D. pyriforme* with other species of the subfamily Eumeninae and their mode of evolutionary divergence.

The PCR of COI gene of the potter wasp *Delta conoideum* collected from Alappuza District of Kerala yielded a product of 621bp which was novel. The conceptual translation product yielded a product 207 amino acids. It is found more closely related to *Eumenes asioboreus* with 90 % identity and *Eumenes verticalis* with 95% identity in the BLASTn and BLASTp analyses respectevely. The phylogeny tree depicted the close

relationship of *Delta conoideum* to the species of the genus *Eumenes*, and the wasp *Delta esuriens okinawae* coming under the same subfamily Eumeninae. They have a common ancestor, which diverged into another clade conatining the species of the genus *Eumenes.* The phylogeny tree also showed the relationship pattern of *D. conoideum* with species of the subfamily Eumeninae, Polistinae and Vespinae.

The PCR of the COI gene of tiger striped potter wasp, *Phimenes flavopictum* collected from Kottayam District yielded a product of 658bp which was novel. The conceptual translation product yielded a product of 219 amino acids. It showed more similarity to *Eumenes rubronotatus* and *Delta esuriens okinawae* with 91% similarity in BLASTn and 95% similarity in BLASTp respectively. The phylogeny tree (N-J tree) showed the close relation of *Phimenes flavopictum* to *Delta esuriens okinawae* under the same subfamily Eumeninae and having a single origin. They showed a sister relationship with the cluster containing the species of the genus *Eumenes* through a common ancestor. The phylogeny tree also showed the pattern of relationship of *P. flavopictum* with the species of the subfamily Eumeninae and their mode of divergence which took place in the evolutionary process.

The PCR of COI gene of the common paper wasp *Ropalidia spatulata* collected from Calicut District of Kerala yielded a product of 484bp which was novel. The

conceptual translation product yielded a product of 161 amino acids. It showed more similarity to *Ropalidia romandi cabeti* with 86% similarity in BLASTn and 96% similarity in BLASTp analysis. The phylogeny tree (N-J tree) depicted the relationship of *Ropalidia spatulata* with *Ropalidia romandi*, *Vespa bicolor* and *Polybioides melainus* and other species of the subfamily Polistinae and Vespinae. The phylogeny tree depicted the close relationship of species of the subfamily Polistinae to *R. spatulata* and their pattern of evolutionary divergence.

The PCR of COI gene of the paper wasp *Polistes strigosus* collected from Ernakulam District of Kerala yielded a product of 484bp which was novel. The conceptual translation product yielded a product of 160 amino acids. It is found to be more closer to *Polistes sagittarius* with 99% similarity in BLASTn and 99% similarity in BLASTp analysis. The phylogeny tree also supported the similarity of *Polistes strigosus* to *Polistes sagittarius*, having a common ancestor and coming under the same genus and subfamily. The phylogeny tree showed the pattern of relationship of *P. strigosus* with other species of the subfamily Polistinae and their evolutionary divergence from a common ancestor.

The PCR of COI gene of the paper wasp *Ropalidia jacobsoni* collected from Trivandrum District of Kerala yielded a product of 353bp which was novel. The

conceptual translation product yielded a product of 117 amino acids. It showed 98% identity to *Rhynchium quinquecinctum* in BLASTn analysis and 99% identity to *Rhynchium quinquecinctum* in BLASTp analysis. The phylogeny tree supported the similarity of *Ropalidia spatulata* to *Rhynchium quinquecinctum.* The phylogeny tree also showed the close relation of *R. jacobsoni* to other species of the subfamily Polistinae and their mode of evolutionary divergence from a common ancestor.

The ClustalW and phylogeny analysis between the eight wasps selected for the present study indicated the close relation of *Polistes strigosus* to *Roplalidia spatulata* and *Ropalidia jacobsoni* to *Rhynchium brunneum*. *Delta conoideum, Delta pyriforme* and *Phimenes flavopictum,* which come under the same subfamily Eumeninae are also found more closely related to each other than to other wasps. The analysis allowed the study of interspecies relationship pattern between the selected species of wasps.

The importance of the present study lies in the application of these results in field of systematics of Vespidae. This can be used for accurate identification of a species even from a damaged and degraded sample, or from any stage in the lifecycle of a species like larval or pupal. The identification of predator wasps of many agricultural pest insects using molecular data is a potential tool that can be used in the field of integrated pest management.

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