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SEQUENCING AND PHYLOGENETIC ANALYSIS OF THE MITOCHONDRIAL CYTOCHROME C OXIDASE SUBUNIT I OF *OXYCARENUS LAETUS* (HEMIPTERA: LYGAEIDAE)

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ABSTRACT: Dusky cotton bug *Oxycarenus laetus* (Hemiptera:Lygaeidae) is a pest of cotton resulting huge losses. Identification of this bug species is a difficult task due to its (*O.laetus*) similar morphology to other members of the genus Oxycarenus. Hence, this study focuses on the aspect of species identification with the help of mitochondrial Cytochrome C Oxidase subunit 1 (COI) gene which can serve as a potential barcode for *O.laetus*. The sequenced COI segment was found to be 688bp long and it was deposited in the Genbank database and the family analysis and phylogenetic analysis was performed and the results indicated that the sequenced segement belongs to the Hemipteran Lygaeidae family.
Keywords: *Oxycarenus laetus*, COI (Cytochrome C Oxidase subunit 1), Phylogeny, NCBI, Hemiptera & Lygaeidae.

INTRODUCTION

Bug monitoring and control methods rely on timely and accurate identification of species present. Proper identification of bug species is problematic, time-consuming and requires an expert taxonomist, authenticated specimens, old literature, etc. Also nymphs and damaged specimen cannot be easily assigned to proper species (Tembe 2009).

DNA barcoding aims at identifying organisms by assessing their degree of DNA sequence similarity to a set of reference taxa. The standard sequence used for this purpose is the mitochondrial COI gene fragment amplified by the "universal primers" of Folmer et al (1994). DNA barcoding is generally considered as a reliable, cost-effective and easy molecular identification tool with a wide applicability across metazoan taxa (Hebert 2004, 2004a & 2005; Smith 2008; Hajibabaei 2006). As such it could be very useful to routinely identify difficult taxa of economic and medical importance. This particularly holds for many insect taxa that comprise large numbers of notorious pest species or disease vectors, whose identification often requires highly specialised taxonomic skills. Several studies showed that it is a reliable tool for the molecular identification of Lepidoptera (Hebert 2004; Hajibabaei 2006; Burns 2008), Hymenoptera (Smith 2008; Fisher 2008), Coleoptera (Greenstone 2005) and Diptera species (Smith 2006 & 2007). However, studies on the Hemiptera are lagging behind inspite of its usefulness in phylogenetic analysis. Mitochondrial DNA (mtDNA) has been one of the most widely used molecular markers for phylogenetic studies in animals, because of its simple genomic structure (Avise 2004). Though mtDNA sequence data have proved valuable in determining phylogenetic relationships, the choice of gene is also of great significance (Simon *et al*. 1994; Lunt *et al*. 1996). The 13 protein-coding genes in the animal mitochondrial genome are better targets because indels are rare since most lead to a shift in the reading frame. There is no compelling a priori reason to focus analysis on a specific gene, but the cytochrome c oxidase I gene (COI) does have two important advantages.

First, the universal primers for this gene are very robust, enabling recovery of its 5' end from representatives of most, if not all, animal phyla (Folmer et al. 1994; Zhang & Hewitt 1997). Second, COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene. In common with other protein coding genes, its third-position nucleotides show a high incidence of base substitutions, leading to a rate of molecular evolution that is about three times greater than that of 12S or 16S rDNA (Knowlton & Weigt 1998). In fact, the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogeographic groups within a single species (Cox & Hebert 2001; Wares & Cunningham 2001). Although COI may be matched by other mitochondrial genes in resolving such cases of recent divergence, this gene is more likely to provide deeper phylogenetic insights than alternatives such as cytochrome b (Simmons 2001) because changes in its amino acid sequence occur more slowly than those in this, or any other, mitochondrial gene (Lynch 1993).

One hundred and sixty two species of phytophagous insects have been recorded on the cotton crop in India, of which 24 species have attained pest status and nine are key pests in one or more cotton growing zones of the country (Sundramurthy,1992; Dhawan, 2000). In the family of Oxycarenidae there are 24 small genera and one large genus, Oxycarenus, which contains about 55 species, of which atleast 6 species are listed as pests, especially on malvaceous plants such as cotton and hibiscus.

Dusky cotton bug Oxycarenus laetus Kirby (Hemiptera : Lygaeidae) is one such minor pest of cotton and its chief importance lies in the fact that the adults and nymphs get crushed at the time of ginning, thus staining the lint and lowering the market value of cotton and other end products of this plant (Sweet 2000).

Surprisingly there are a very few true bug (Hemiptera) COI sequences deposited so far even though bugs are known to cause severe damage to plants and transmit viruses. As we write this research report there were a total of 243 insect mitochondrial genomes to be sequenced and deposited in the NCBI database, and quite a good number of COI gene sequences, but O.laetus. Hence in this research activity we have given importance to sequence the COI gene sequence of this economically important pest species which can serve as a molecular barcode for this species in future.

MATERIALS AND METHODS

Collection of Sample

Dusky cotton bug O. laetus was collected from the cotton fields of Dindivanam, Tamilnadu, India. The field collected insects were transferred to a plastic container and maintained as a mass culture. Once the insects laid eggs they were transferred to separate containers in order to keep track of the growth stage of the insects. The experiments were carried out on the F1 generation and the freshly moulted adults were used for the study.

DNA Extraction

The method of Andrew et al (1996) was followed to extract the DNA. An adult cotton bug was placed in a centrifuge tube containing 300µl of Homogenization buffer and 75µl of lysis buffer and ground using a thin glass rod. The whole content was incubated at 65°C for 30min (under a controlled environment). To this 17µl of 8M Potassium acetate was added. The tubes were incubated in ice for 30min overnight. The incubated samples were centrifuged at 15000 rpm for 15min at 4°C. The aqueous phase was transformed to a new tube and 400µl of absolute ethanol was added (to precipitate the Nucleic Acid content from the aqueous phase). Precipitated Nucleic Acid is pelleted out by centrifuging at 12000rpm for 15min at 4°C. Pellet was retained. Air dried Pellet was dissolved in 20µl of T.E buffer and Stored at 20°C for further use. The DNA concentration and purity were assesed by spectrophotometry using a spectrophotometer. DNA purity was determined by calculating the absorbance ratio A260/280.

Amplification and Sequencing of 5'COI gene region:

The reaction was performed in a final volume of 20 µl, containing 9 µl of MilliQ water, 1µl of 100ng/µl genomic DNA, 2.5µl of dNTPs, 2 µl of Taq buffer, 2.5 µl of forward (5' – GGT CAA CAA ATC ATA AAG ATA TTG G – 3') and reverse (5' – TAA ACT TCA GGG TGA CCA AAA AAT CA – 3') primers each, the Taq DNA polymerase 0.5µl. The samples were initially denatured at 94°C for 3 mins followed by the annealing at 47°C for a minute, the final extension was set at 72°C for 7mins and the whole process was continued for 40 cycles. The reaction was performed using a PCR instrument. The amplified product was electrophored through a 1% agarose gel, at 50V for 30 mins and the DNA fragments in the gel were visualized using a UV trans-illuminator. The purified PCR product was sequenced with the help of a sequence scanner v1 from ABI.

Phylogenetic Analysis

The COI sequence obtained after sequencing was translated and its family was assessed with the help of InterProScan database (Zdobnov 2001). Similar sequences to ours are downloaded from the NCBI database after performing a search for similar sequences using the Blast tool (Altschul 1997). These sequences were subjected to multiple sequence alignment and a phylogenetic analysis was performed with the help of MEGA4 tool (Tamura 2007).

RESULTS AND DISCUSSIONS

The COI sequence of *O.laetus* was submitted to the Genbank database holding an accession number HQ908084 (table.1). The amino acid sequence of the corresponding COI gene was also updated under the accession number ADZ05746, which turned out to contain 222 amino acids. Base statistics of the *O.laetus* COI are presented in table.2. It can be seen from the table that the fragment is rich in AT content as expected with thymine occurring most frequently followed by the others in the order A, C & G. The AT% stood at 67.2 in comparison to GC% at 32.8.

The protein entry was subjected to family confirmation by searching the InterProScan database and the results indicate a very high and significant match confirming our sequence to be a part of Cytochrome C Oxidase subunit1 family with good query coverage (figure.1). The Blast search (figure.2) indicated that the sequenced segment to be closely related to insect species from the Order Hemiptera.

The multiple sequence alignment of the similar sequences was done using ClustalW and the evolutionary history was inferred using the Neighbor-Joining method (Saitou 1987). The bootstrap (Felsenstein 1985) consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. The analysis involved 42 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 203 positions in the final dataset. There were a total of 511 positions including gaps after the alignment out of which 148 were variable sites, 363 were conserved sites and 95 being parsimoniously informative sites.

The phylolenetic tree (figure.3) shows that all the species belonging to the lygaeids being clubbed together with high bootstrap score of 98, 95& 91 for the species belonging to the genus Geocoris, Oxycarenus & Cymus respectively. The tree built with the hemipteran entries was divided in to two major
clusters and a few smaller clusters.

The first major cluster was further subdivided in to one big sub-cluster with a low but acceptable bootstrap value of 73, where the species belonging to the genus Laryngodus were grouped together with a good bootstrap score of 94 and similarly the species belonging to Ligyrocoris genus formed a single clade with a score of 74, the fact that these genus's being a member of lygaeidae family. Interestingly, this sub- cluster also accommodated an entry from the Coleopteran species along with the Ligyrocoris, that too with a fine 94 bootstrap score. However the second sub-cluster comprises the members of Berytidae family with a healthy bootstrap score of 89.

The second major cluster at a score of 58 accommodated the Coreidae member Anasa spp., with the species from the genus Dysdercus (Pyrrhocoridae), at a very good 99 bootstrap score. Our entry *O.laetus*

was found to be clustered with the members of the genus Oxycarenus expectedly. Several other taxas were also presented in this tree out of which most accounting to be lygaeids, which gives us an idea about the tree being a polyphyletic.

Table.1: Sequence entry in Genbank.

```
LOCUS       HQ908084        688 bp    DNA     linear   INV 09-MAR-2011
DEFINITION  Oxycarenus laetus cytochrome oxidase subunit 1 (COI) gene,
partial     cds; mitochondrial.
ORIGIN
        1 acctttttct tttttatttt ggtatatgat ccggtatagt tggatcatca ttaagatgaa
       61 ttatccgtat tgaattaggt caatcagggt cttttattgg tgatgatcaa atttataatg
      121 ttgtagtcac agcacatgca tttattataa ttttctttat agttatacct attataattg
      181 gaggatttgg aaattgatta gtcccattaa taattggagc cccagatata gccttccccc
      241 gaataaataa tataagattc tgactattac ccccatcatt aacactcctg ttatcaagta
      301 gcttagtaga aataggagca ggaacaggat gaacagttta tccaccttta tctaatagat
      361 tattccatag aggagcatct gtagatttag caatttttc cctacattta gcaggtgtat
      421 catcaatttt aggagcaatc aactttatct caactattat taatatacga ccagctggta
      481 taacccttga acgaatcccc ctctttgttt gatcagtagg aattactgca ttattattat
      541 tattatcatt accagtatta gctggagcca ttactatatt attaacagac cgaaatttca
      601 atacatcatt ctttgaccct acaggaggag gtgaccctat ttttatacca acatttattt
      661 tgatttttg gtcacccctg aagttgaa
```

Table.2: Base Statistics of *O.laetus* COI.

Genbank ID: HQ908084	**G+C Content = 32.8%**	**A+T Content = 67.2**
Nucleotide	**Count**	**Percentage**
A	209	30.38
T	253	36.77
G	105	15.26
C	121	17.59

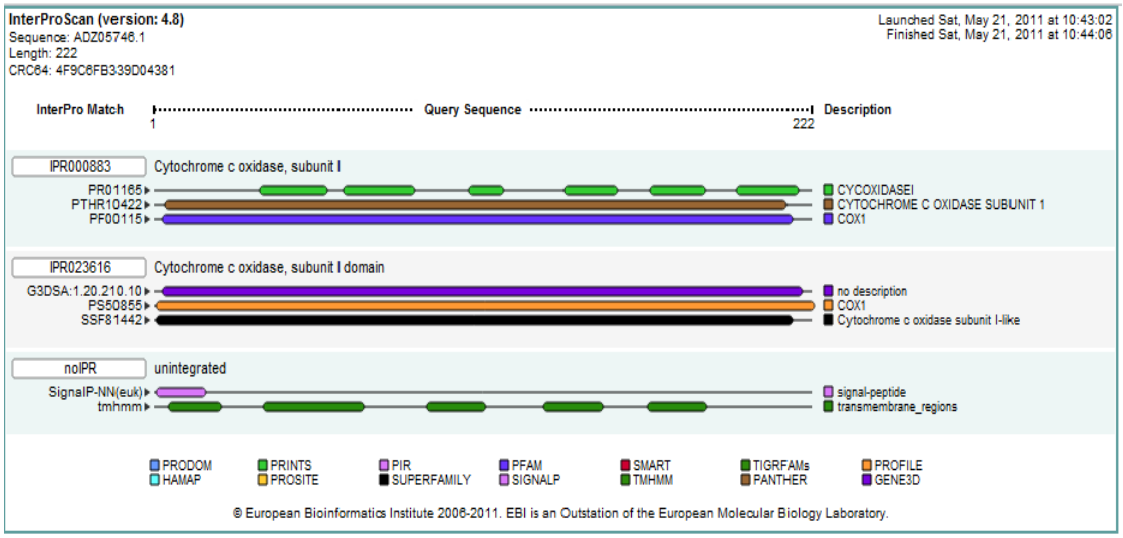

Figure.1: InterProScan Analysis.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ908084.1	Oxycarenus laetus cytochrome oxidase subunit 1 (COI) gene, partial cds; mitc	1271	1271	100%	0.0	100%
GU681993.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00044 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681988.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00026 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681987.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00025 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681985.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00027 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681984.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00030 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681983.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00029 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681982.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00032 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681981.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00031 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681980.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00034 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681979.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00033 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681978.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00036 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681977.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00035 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681976.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00038 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681975.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00037 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681973.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00039 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681971.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00041 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681969.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00043 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681968.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00047 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681967.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00046 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681965.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00048 cytochrome oxidase s	1182	1182	94%	0.0	99%
GU681986.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00028 cytochrome oxidase s	1177	1177	94%	0.0	99%
GU681974.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00040 cytochrome oxidase s	1177	1177	94%	0.0	99%
GU681970.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00045 cytochrome oxidase s	1177	1177	94%	0.0	99%
GU681989.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00024 cytochrome oxidase s	1171	1171	94%	0.0	99%
GU681972.1	Hemiptera sp. BOLD:AAB4491 voucher NIBGE IMB-00042 cytochrome oxidase s	1168	1168	94%	0.0	99%
AY252994.1	Udeocoris nigroaeneus cytochrome oxidase subunit I gene, partial cds; mitoch	754	754	97%	0.0	87%
AY253130.1	Neoneides muticus cytochrome oxidase subunit I gene, partial cds; mitochond	749	749	97%	0.0	86%
AY253138.1	Pachygrontha sp. WCW-2003 cytochrome oxidase subunit I gene, partial cds;	739	739	97%	0.0	86%
HM416203.1	Hemiptera sp. BOLD:AAC2393 voucher 09BBEHE-120 cytochrome oxidase subu	728	728	94%	0.0	87%
GU692437.1	Hemiptera sp. BOLD:AAC2393 voucher 09BBEHE-071 cytochrome oxidase subu	728	728	94%	0.0	87%
GU692436.1	Hemiptera sp. BOLD:AAC2393 voucher 09BBEHE-050 cytochrome oxidase subu	728	728	94%	0.0	87%
HQ105991.1	Neoneides muticus voucher CNC-HEM-0914 cytochrome oxidase subunit 1 (CO	726	726	93%	0.0	87%
HQ105716.1	Harmostes reflexulus voucher CNC-HEM-0869 cytochrome oxidase subunit 1 ((	725	725	93%	0.0	87%
HQ105714.1	Harmostes reflexulus voucher CNC-HEM-0871 cytochrome oxidase subunit 1 ((	725	725	94%	0.0	86%

Figure. 2: Blast Results.

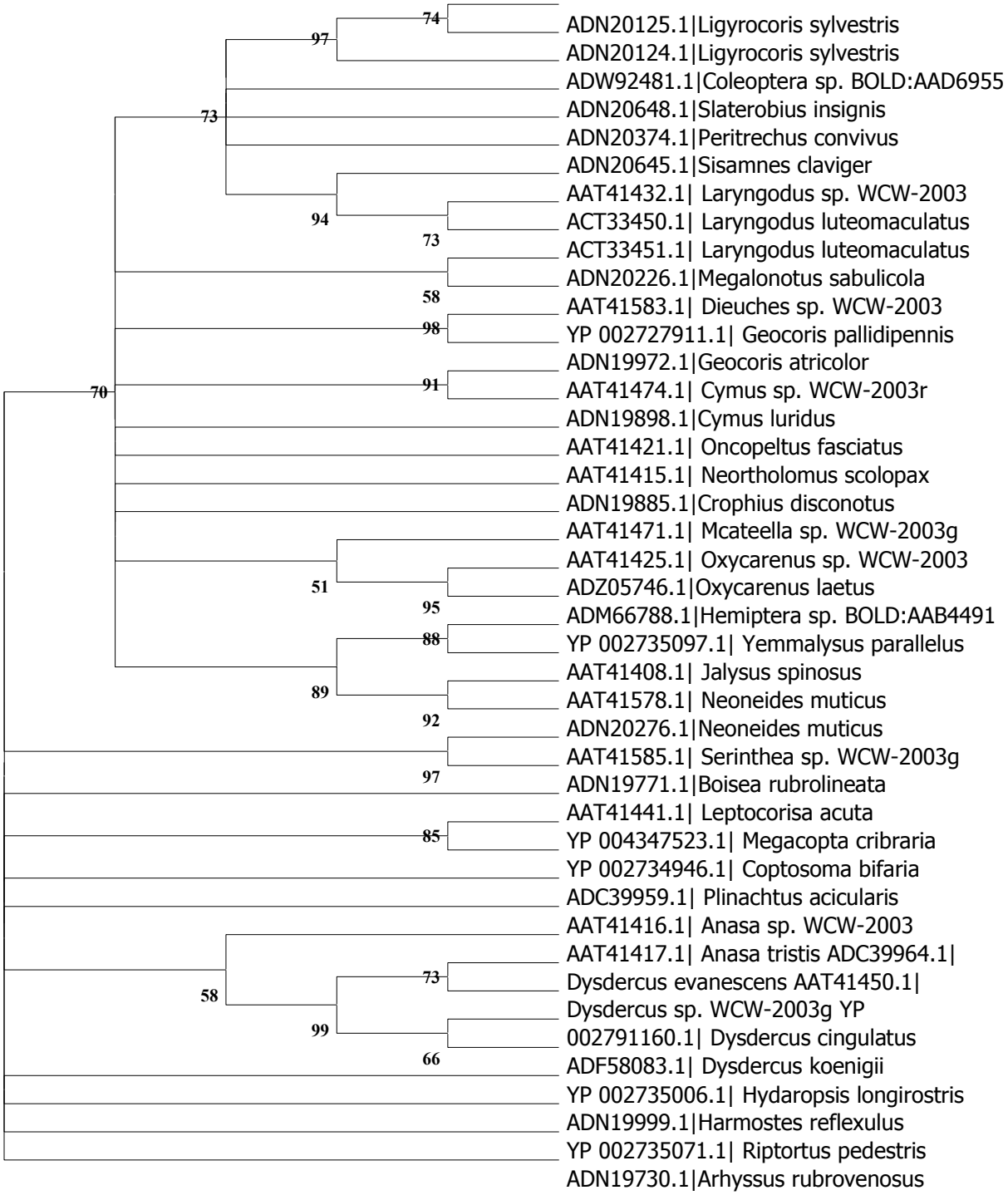

Figure.3: Phylogenetic tree.

CONCLUSION

Oxycarenus laetus is one of the pests of cotton and it is highly confused with other members of this genus due to its almost similar morphology. DNA barcoding is one of the ways of identifying the species. There was rarely an entry in the Genbank database for the species *O.laetus*. This study was undertaken with an aim to sequence the mitochondrial Cytochrome C Oxidase subunit 1 for the species *Oxycarenus laetus* Kirby. The sequenced segment was found to be 688bp long and rich in AT content. It was confirmed from the Interproscan database search that the sequenced segment belongs to the Cytochrome C Oxidase subunit 1 family and also showing close proximity towards the Hemipteran species to which it belongs. From the phylogenetic studies we also infer that the sequence was clustered with an entry corresponding to Oxycarenus spp., with a high bootstrap score. Considering the difficulty in identifying the most diverse world of insect species', this exercise will be of great help and the sequence will serve as an exclusive DNA barcode for this species, which can be utilized for species identification and other taxonomic studies in future.

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