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DNA BARCODING AND PHYLOGENETIC INFERENCE OF THE ENDEMIC SPECIES ONYCHOGOMPHUS MALABARENSIS (ANISOPTERA: GOMPHIDAE) USING MITOCHONDRIAL CYTOCHROME OXIDASE SUBUNIT I MARKER GENE

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ABSTRACT

The Gomphidae represents one of the fast flying dragonfly families of the insect order Odonata, widely distributed in Palaertic, Ethiopian and Oriental regions. Members can be easily diagnosed by having a club like swelling at the base of abdomen (Clubtails) and also black stripes could be seen on their yellow or green coloured thorax. They are habitat specialist and ecologically important as indicators of clean aquatic ecosystem and predators certain dragonflies also. *Onychogomphus malabarensis* is an endemic Gomphidae member reported only from the forest ecosystem of Palakkad District of Kerala. Here we amplified the cytochrome oxidase I gene and its sequence was deposited in the Gen Bank (KX503058) for future references. The nucleotide BLAST analysis showed that this species is having 100% sequence similarity to *Ophiogomphus anomalus* reported from America (KX890962). The entire study states that the molecular barcode is a new report to NCBI and their phylogeny inferred their specific taxonomic position among other Gomphidae members.

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INTRODUCTION

Taxonomy is the field of basic science helping for providing identity to every species by integrating morphological and molecular information. It help us to understand their origin, diversification, developmental information and thus providing basis to establish relationships between taxa (Singh, 2012). Mitochondrial genes is the superior markers gene than nuclear genes because of their abundance, lack of introns, limited exposure to recombination and haploid mode of inheritance (Saccone *et al.*, 1999). The D loop region of the Mt DNA represents the non-coding contracting region having high mutation rate and hence widely used in evolutionary studies.

A short DNA sequence of 600 bp in the mitochondrial gene encoding cytochrome oxidase I (CO I) gene has been accepted as practical standardised species level DNA barcode for many group of animals, and it used as a reliable method for the identification of species in a variety of both invertebrate and vertebrate taxa (Hebert *et al.* 2003). This gene has been widely used in systematic studies because it represents the largest gene compared to other cytochrome oxidase subunit and has high deletion insertion events. It also has high nucleotide substitution rate and hence helping for discriminating cryptic

species (Surajit *et al.*, 2014). There were many reports on the prompt application of mitochondrial CO I gene based DNA barcoding for the accurate identification of various odonate species from present authors (Jisha and Sebastian, 2016a; 2016b; 2015a; 2015b; 2015c; 2015d; 2015e).

The insect Order Odonata representing dragonflies and damselflies are the beneficial insects of mankind. This group incorporate rich phenotypic and ecological diversity within one single insect order and hence constitute excellent candidate for ecological and evolutionary studies (Cordoba, 2008). Gomphidae represents the second largest family of this group reporting 958 species world-wide, out of which 90 species known from India (Subramanian, 2009). These are medium sized dragonflies with males characteristically having a club at their abdominal segments and females possessing a vestigial ovipositor (Jessica *et al.*, 2017). They have small and widely separated green or blue coloured eyes, black stripes could be seen on their yellow or green coloured thorax and lack bright colour patterns. Their cryptic coloured body thus helps to avoid detection from predators and also they possess little difference in their sexes. Most of the members are habitat specialists and hence their abundance is correlated with the indicators of clean and stable water streams. They are ecologically important as

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predators of other insects including bees and wasps and sometimes certain dragonflies also.

Onychogomphus malabarensis is an endemic species known to be reported only from the Palakkad district of Kerala, India (Subramanian *et al.*, 2011). The main objective of the present study aims to provide a molecular barcode for this species and to analyze its phylogenetic relationships.

MATERIALS AND METHODS

Sample collection and preservation

The Gomphidae species *Onychogomphus malabarensis* was collected from Palakkad district of Kerala, India. It was collected by hand sweep netting and random field sampling method was used to cover the entire study area. Identification was done by observing wing venation, colour pattern and genitalia, described in available keys/ identification guides (Emiliyamma *et al.*, 2005). Additional information regarding date of collection, locality etc. about each specimen was also recorded. Each specimen was then placed in a separate collecting bottle, assigned a code number and stored in 70% ethanol until further use. One or more legs were removed for DNA isolation and kept in ethanol until further use.

DNA extraction, amplification and sequencing

DNA from selected dragonflies was extracted from leg using commercially available DNA extraction kit. The obtained DNA was confirmed using 1% agarose gel. About 2ng of DNA was PCR amplified for mitochondrial cytochrome oxidase subunit I (COI) gene using forward primer (5'-TCGGTGCATGAGCAGGTATAGTAGGTAC-3') and reverse primer (5'-AATAGGATCTCCTCCACCTGCTG-3'). The thermocycler conditions were slightly modified as follows: 1 initial cycle of 5 minute at 95°C followed by 30 cycles of 95°C for 10 seconds and 50°C for 1 minute, 72°C for 45 seconds. This is followed by a final step of 72°C for 3 minutes. The obtained PCR product was checked using 2% agarose gel electrophoresis and were sequenced with both the forward and reverse primers using an automated sequencer ABI 3730XL by Sanger method (Sanger and Coulson, 1975). Phylogenetic analysis was done by MEGA7 software (Tamura *et al.*, 2013) and phylogenetic tree was constructed by Neighbour joining method (Tamura *et al.*, 2004).

Data Analysis

Mitochondrial COI sequence data for the selected dragonflies was sequenced and submitted in NCBI GenBank. The aligned sequences were used for species identification using BLAST. The sequences from GenBank were retrieved and sequences of each species generated from this study were compared and aligned using ClustalW.

RESULTS AND DISCUSSION

Onychogomphus malabarensis under the family Gomphidae was morphologically identified from identifying guides and taxonomic expert (Figure 1). The genus *Onychogomphus* are commonly known as 'Pincertails'. This species are usually distributed in terrestrial and freshwater habitats and forest ecosystem. The partial coding sequence of mitochondrial COI

gene of *Onychogomphus malabarensis* was PCR amplified using suitable primer mentioned elsewhere.



Figure 1 *Onychogomphus malabarensis*

The PCR amplification of partial COI sequence of *Onychogomphus malabarensis* isolated from Kerala, India yielded a product having 602 bp. The phylogenetic tree (Figure 2), sequence divergence table (Table 1) and nucleotide substitution table (Table 2) were presented herewith. The sequence was deposited in the NCBI GenBank having the accession number KX503058 for world-wide accession.

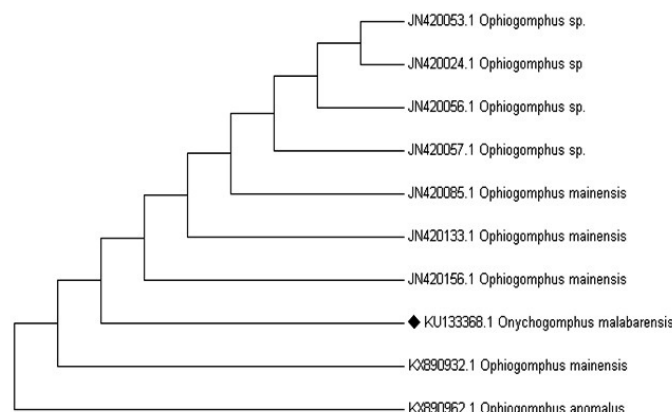


Figure 2 Phylogenetic tree constructed by Neighbour joining method

Table 1 Percentage of evolutionary divergence of *Onychogomphus malabarensis* with closely related species

Species name	% of divergence
KU133368. <i>Onychogomphus malabarensis</i> (Kerala)	0.00%
KX890962 <i>Ophiogomphus anomalus</i> (America)	0.00%
KX890932 <i>Ophiogomphus mainensis</i>	0.00%
JN420156 <i>Ophiogomphus mainensis</i>	0.00%
JN420133 <i>Ophiogomphus mainensis</i>	0.00%
JN420085 <i>Ophiogomphus mainensis</i>	0.00%
JN420057 <i>Ophiogomphus sp</i>	0.00%
JN420056 <i>Ophiogomphus sp</i>	0.00%
JN420053 <i>Ophiogomphus sp</i>	0.00%
JN420024 <i>Ophiogomphus sp</i>	0.00%

Table 2 Table showing maximum likelihood estimate of nucleotide substitution matrix

	A	T/U	C	G
A	-	11.52	5.87	5.37
T/U	10.58	-	5.87	5.37
C	10.58	11.51	-	5.37
G	10.57	11.52	5.87	-

The evolutionary history was inferred using Neighbour joining method (Saitou *et al.*, 1987). The evolutionary distance was computed using Maximum Likelihood method (Tamura *et al.*, 2004). The analysis involved 10 nucleotide sequences and the

codon positions containing gaps and the codon positions include 1st, 2nd and 3rd and missing data were eliminated. There were a total of 602 positions in the final dataset and the evolutionary analyses were conducted in MEGA 7 (Kumar *et al.*, 2016). The BLAST analysis showed that this species is 100% sequence similar to the *Ophiogomphus anomalus* reported from USA (GenBank accession: KX890962). The Percentage of evolutionary distance was also calculated using Maximum composite likelihood method and it showed that it doesn't have major sequence divergence from other Gomphidae members.

The insect order Odonata helps to integrate evolutionary genomics due to its complex life cycle, flight behaviour, diversity in ecological niches and their sensitivity to human activities and thus provide a promising tool for genomic studies. As they are sister taxon to the order Ephemeroptera (mayflies), it can be easily trace out the origin of winged insects and also its relationship with other Neopteran insects. Since each species in this order has their own habitat specificity and courtship behaviour, large interspecific variations can be easily assessed by analysing their genomic sequence and ecological observations. Thus their phylogenetic positions and several evolutionary innovations make them an attractive model to bridge between ecology and evolutionary genomics (Bybee *et al.*, 2016). Molecular barcoding and phylogenetic studies of several Odonata species were also done using different marker genes such as cytochrome oxidase I (COI), COII and 12S rRNA (Artiss *et al.*, 2001). Most of the studies concluded a monophyletic origin to every species. The nucleotide BLAST, Protein BLAST and percentage of divergence clearly demarcated that this was strictly a Gomphidae member because it doesn't have major changes in the nucleotide sequences. The average nucleotide frequencies are A = 31.73%, T = 34.55%, C = 17.61% and G = 16.11% indicating high AT content. This was supported by the reports of Chippendale *et al.* (1999) who stated that the overall A + T content were high among the order Odonata.

The present study of this endemic species is a new report to NCBI. Thus it is pioneer work of this species and we have specifically analyzed the phylogenetic position of this species based on DNA sequences.

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