Available online at www.ijpab.com

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **3 (2):** 317-320 (2015)

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

Research Article

OPEN ACCESS

Genetic and Phylogenetic Assesment of Sexually Dimorphic Species, *Diplacodes trivalis* (Odonata: Libellulidae) using Cytochrome Oxidase I Gene

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ABSTRACT

Sexual dimorphism is a characteristic phenomenon exhibited by Libellulidae and Aeshnidae family of Odonates. Diplacodes trivalis is a sexually dimorphic Libellulidae species and the present study was carried out to check whether any genetic change had occurred in both the sexes and how it is phylogenetically related with other Odonate members. DNA barcoding using CO I gene offers the opportunity for a standard system of species identification based on the analysis of small fragment of DNA. The PCR amplification of partial cytochrome oxidase I gene of Diplacodes trivalis yielded a product of 466bp length (GenBank Accession: KP 835512).The length and nucleotide sequence of DNA and was found to be similar in both sexes. BLASTn program showed 99% sequence similarity to the same species reported from Mizoram, TamilNadu and Japan. The result indicated that this sexually dimorphic species does not have any genetic changes with respect to their morphological differentiation.

***Key words**: Odonates, Diplacodes trivalis, DNA barcoding, Cytochrome oxidase I gene*

INTRODUCTION

In the invertebrate world, Odonates are always attracting the human beings for their variety of colour, powerful flight and extraordinary sense of vision. The prey of the adults consists mostly of the insects that are harmful to crops, orchards and forest and thus has a regulatory impact on Agroforestry[1]. They are valuable as indicators of aquatic and terrestrial ecosystem and play a vital role as prey and predators to maintain the balance of tropic level of food chain[2]. Approximately 6500 extant species in over 600 genera and 28 families are known all over the world[3]. About 474 species in 142 genera and 18 families are identified from India[4] out of which 154 species are from Kerala[5]. The order Odonata is divided into two main suborders: Zygoptera (damselflies) and Anisoptera (dragonflies).The most species-rich and widespread family is mainly the tropical Libellulidae (Anisoptera), comprised of 140 genera and about 962 species.

Diplacodes trivalis is one of the commonest libellulid dragonflies in garden, paddy fields, and playground known as 'Ground skimmer' or 'Blue percher', widely distributed in Oriental region and Pacific islands. This species shows an extreme case of sexual dimorphism with clear distinct morphological differences in both the sexes. Male has beautiful blue eyes, pale azure blue face with reddish brown coloured eye above and pale bluish or yellowish colour below. Thorax is greenish yellow or olivaceous. The dorso-lateral area is violet brown and is speckled with minute dots. Legs are greenish yellow marked with black and the wings are transparent. Abdominal segments 1-7 are greenish yellow with middorsal and subdorsal black stripes and the remaining segments are black in colour. They usually breed in muddy puddles, tanks and pond edges. Females resembles to young or sub adult male.

DNA barcoding is a taxonomic system structured on sequence information from short stretches of DNA sequence. A region of approximately 648-bp of the mitochondrial cytochrome oxidase I (COI) gene was initially proposed as the barcode source to identify and delimit all animal species. The methodology involves the sequencing of that portion of DNA, followed by a comparison with other sequences previously deposited in a database. Species were identified by matching the obtained sequence with sequences of known identity already in the database[6]. The cytochrome oxidase I subunit gene was chosen for animal barcoding because insertions and deletions are rare and the also the robust primers for COI enabling the sequencing of representatives of all animal phyla. This gene possess a great range of phylogenetic signal showing fast rates of nucleotide substitution that not only enable the discrimination of cryptic species but also can reveal phylogeographic structures within a species. Molecular phylogenetic analysis were extensively carried out using COI gene sequences in various group of insects like dipterans[7, 8], lepidopterans[9, 10], heteropterans[11] and hymenopterans[12]. The present study is the phylogenetic assessment of sexually dimorphic *D. trivalis* on the basis of nucleotide comparison of partial sequence of COI gene among other related odonate species.

METHODOLOGY

Specimens of both sexes of *Diplacodes trivalis* species were collected by from Malappuram, Kerala by hand sweep netting method and taxonomic identification was done by available keys, identification guides and expert consultation. Additional information regarding date of collection, location etc. about each specimen were also recorded. Each specimen was then placed in a separate collecting bottle, assigned a code number and stored in 70% ethanol as voucher specimen. DNA was extracted from one of the thoracic legs of both male and female species. The tissue was homogenized and DNA in the homogenate was isolated using Ultrapure Genomic DNA Pre Kit. About 2 ng of DNA was PCR amplified for mitochondrial cytochrome oxidase subunit I (COI) gene using forward primer having base sequence 5'-GGAACAGCATTAAGAGTTTTAATTCGA-3' and reverse primer with sequence 5'-GATCTCCACCTGCCGGGTC-3'. The PCR was performed in a 30 µl reaction volume which contained approximately 2 µl of 2ng of DNA, 3 µl of 10x buffer, 3 µl 25nm $Mgcl_2$, 0.3 µl dNTP, 0.3 µl Taq polymerase enzyme, 0.5 µl primer and 20.9 µl of distilled water. The COI barcode region was subsequently amplified under the thermal conditions of initial denaturation step for 5 min at 95°C, followed by 30 cycles of 10s at 95°C, 10s at 50°C and 1 min at 72°C and ending with a final phase of 72°C for 3 min and held at 4^0C. The PCR products were visualized on a 2% agarose gel and it was column purified using Mo Bio UltraClean PCR Clean-up Kit (Mol Bio Laboratories, Inc. California). The purified PCR product was sequenced by Sanger's sequencing method[13]. The DNA sequences were aligned using the multiple sequence alignment tool ClustalW and the consensus sequence was taken for the analysis. The final sequence was searched for its similarity using BLAST of NCBI (www.ncbi.nlm.nih.gov/). The phylogenetic tree was plotted using neighbour joining method with MEGA6 software[14].

RESULTS

Morphological difference between male and female *D. trivalis* can be externally identified (Figure 1a and 1b) using authentic reference guides[4,15] and taxonomically confirmed by expert consultation. The PCR amplification of mitochondrial cytochrome oxidase I gene from both sexes of *D. trivalis* yielded a product of 466bp length (Gen Bank Accession: KP 835512). Phylogenetic tree constructed by Neighbour – Joining method (Figure 2) and the percentage of nucleotide divergence was calculated (Table 1).

Fig. 1: Sexual dimorphism in *Diplacodes trivalis* (a) Male (b) Female

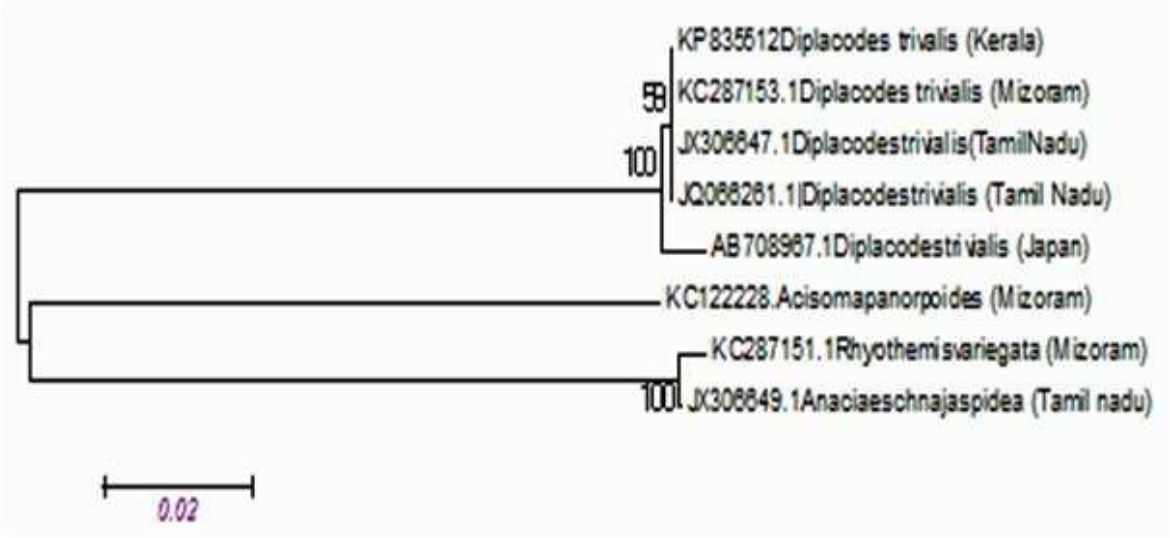

Fig. 2: Phylogenetic tree of *Diplacodes trivalis* by Neighbour-joining method

Table 1: The Percentage of nucleotide divergence of *Diplacodes trivalis* from Kerala with other Odonata species

Species Name	GenBank Accession Number	Percentage of Divergence
Diplacodes trivalis	KP 835512 (KERALA)	0
Diplacodes trivalis	KC 287153 (MIZORAM)	1%
Diplacodes trivalis	JX 306647 (TAMIL NADU)	1%
Diplacodes trivalis	AB 708967 (JAPAN)	1%
Diplacodes trivalis	JQ 066261 (TAMIL NADU)	1%
Acisoma panorpoides	KC 122228 (MIZORAM)	14%
Anaciaeschna jaspidea	JX 306649 (TAMIL NADU)	15%
Rhyothemis variegata	KC 287151 (MIZORAM)	15%

DISCUSSION

DNA barcoding using COI gene sequence has proven to be an effective approach to species identification and thus has been used successfully in groups such as insects, birds and fish[16]. Nucleotide divergence has been a primary criterion for delimiting species and detecting cryptic species in initiatives such as DNA taxonomy. Thus analysis of molecular data has proven to be important for understanding deep phylogenetic relationship, examining population structure within a species and thereby assigning unknown specimens[17].

The length and nucleotide sequence of partially amplified cytochrome oxidase I gene of *D. trivalis* in the present study was found to be similar in both sexes. Even though sexual dimorphism exhibited differences in morphological features, DNA sequence analysis confirmed its unambiguous taxonomic position. BLASTn analysis showed 99% sequence similarity with the same species reported from Mizoram, Tamil Nadu and Japan. Phylogenetic tree promptly interprets the genetic relatedness of *D. trivalis* from different geographical areas and the existence of a common ancestor which is represented as the clade with bootstrap value 100. In their evolutionary history, due to continental shifting and other geographical barriers, they shifted into two geographically isolated areas and phylogenetically diverged as two sister clades: one representing those species in India (Kerala, Mizoram and Tamil Nadu) and the other in Japan. This joined clade is also sister to other Libellulidae members such as *Acisoma panorpoides*, *Rhyothemis variegate* and *Anaciaeschna jaspidea*.

Acknowledgement

The financial assistance from Kerala State Council for Science Technology and Environment, Thiruvananthapuram under Science Research Scheme is gratefully acknowledged.

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