IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

PROGRESS IN USE OF MOLECULAR MARKER CYTOCHROME OXIDASE I GENE FOR SPECIES IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF NEUROTHEMIS FULVIA (ODONATA: LIBELLULIDAE)

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Abstract

Neurothemis fulvia,commonly known as 'Fulvous forest skimmer'is one of the dominant libellulidae member distributed in Asian countries. It always seen associated with agroecosystem as well as other aquatic habitats. The present study was done to find out and develop a molecular barcode for easily identifying the species and thereby infer its phylogeny.The PCR amplification yielded a product having 600bp length.The sequence deposited in nucleic acid databases for public accession having NCBI GenBank Accession No. KP835515 and Barcode of Life Data System BIN Cluster ID – BOLD: ACD6379.The phylogenetic tree constructed by Neighbour-Joining method clearly showed that this species doesn't have any sequence divergence as time progressed. Phylogenetically this species is very close to close to *N. intermedia*, *N. fluctans* and *N. tullia* with respective the divergence of 17.6%, 18.10% and 18.55% respectively.

Keywords:Odonata,Libellulidae,Mitochondrial marker

INTRODUCTION

The order Odonata, consisting of dragonflies and damselflies, are the most popular insects in the public figure. They are known to be existed in the carboniferous period along with mayflies and well known as the enchanting "Charismatic fauna" of the insect world due to the existence of variety of colours. They are the living representatives of primitive winged insects found in all biological realms except Antarctica. Historical studies proved that most of the species exists today are truly the descendants of proto-odonates and their fossils resembles to those existed in the Mesozoic era (Emiliyamma et al., 2005). As both life stages are tightly correlated with aquatic habitat, they are widely used for studying ecological, behavioural, biochemical and evolutionary aspects (Corbet, 1999).

Libellulidae represents the most cosmopolitan family of the Order Odonata including brightly coloured medium sized dragonflies having about 1000 species. The most diagonistic key of this group is the presence of foot shaped anal loop in the hind wing, notch found on the posterior side of compound eye and triangles in the wings dissimilar in size and orientation. They are usually seen associated with ponds, lakes and still waters with their highest peak seen in April to September.

Mitochondrial markers considered as promising instrument for Insect systematics (Cameron.,2014). It is a highly conserved 15-18 kbp long DNA span containing 37 functional genes comprising 13 protein coding genes, 2 rRNA genes and 22 tRNA genes (Boore et al., 1999). Among these techniques, the analysis of mitochondrial DNA is particularly useful in discriminating between closely related species

The fact that mitochondrial DNA has maternal inheritance, its high mutation rate due to limited repair system, high nucleotide substitution rate (5-10 times more rapidly than nuclear) and relatively simple conserved structure makes it suitable for examining population and subpopulation structures among related taxa (Brown et al., 1979)

Materials and methods

1. Sample Collection and Preservation

Dragonflies belongs to Libelluidae family were collected from Tirur,Malappuram.Collections were made by hand sweep netting and random field sampling method was done to cover the entire study area.Identification was done by observing wing venation, colour pattern and genitalia, described in available keys/identification guides. 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.

Figure 1: Dragonfly collecton site (Malappuram:Tirur (10.9146º N 75.9221ºE)

2. DNA extraction, amplification and sequencing

DNA from selected dragonflies was extracted from leg using 'Origin 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'ATTAGTGCCGTTAATACTTGGTGCTCC3') and reverse primer (5'AAAATTGGATCTCCTCCCCCTGC3') in Takara PCR thermocycler. The thermo cycler 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 Sangers method. Phylogenetic analysis done by MEGA software (Tamura et al., 2013).

3. Data Analysis

Mitochondrial COI sequence data for the selected dragonflies was sequenced and submitted in 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 clustal w.

RESULTS

Neurothemis fulvia is a rusty coloured dragonfly species commonly called "Fulvous forest skimmer" diagnosed by reddish brown coloured head, thorax and abdomen in males. Their wings are dark reddish and opaque having reddish brown pterostigma and also a transparent triangular area at the tip (Fig1). The female members are paler and rusty brown in colour with their wings are amber yellow in colour. This rusty coloured dragonfly commonly observed as large colonies in almost all dense vegetation areas.

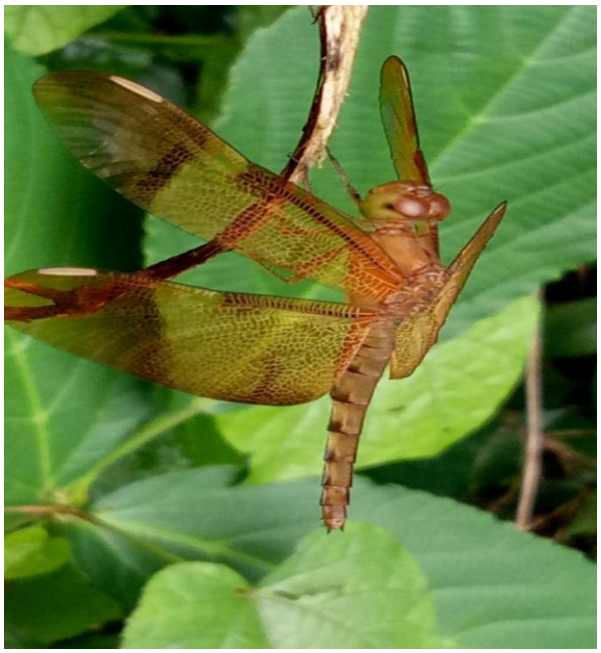
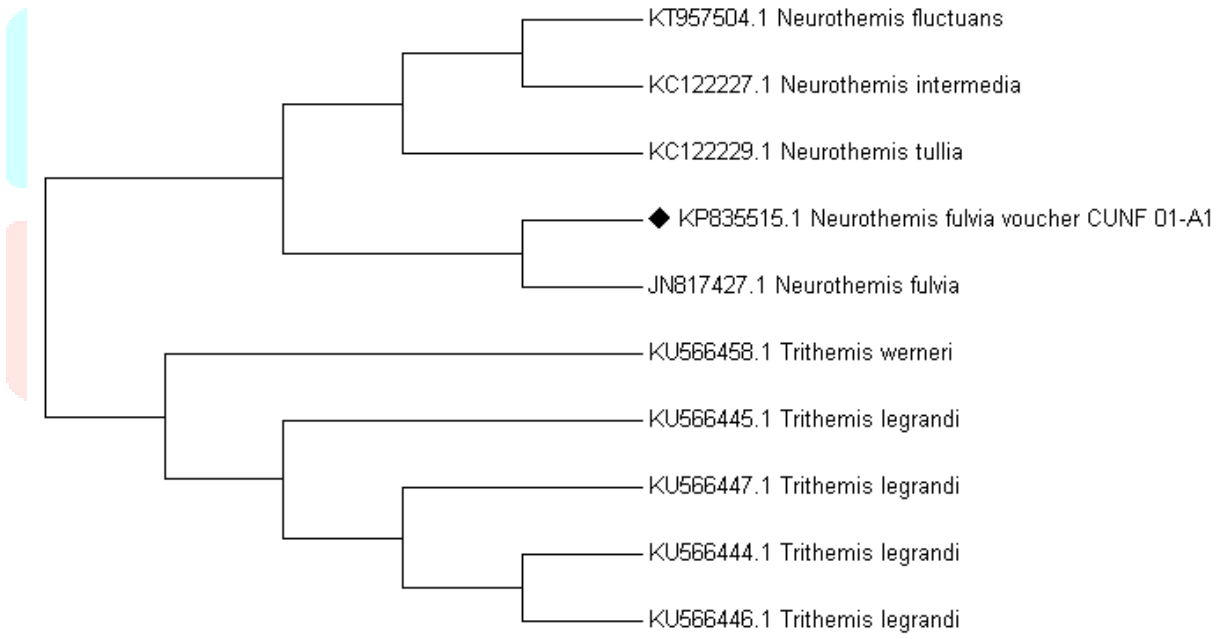


Figure 1 : *Neurothemis fulvia*

The partial coding sequence of mitochondrial COI gene of *Neurothemis fulvia* collected from Malappuram district (11.0300° N 760500° E) was PCR amplified using forward and reverse primer respectively as 5' – ACTGCCCACGCCTTTGTAATAATTTTC – 3' and 5' – GCTATTACTATACTATTAACTGA – 3'.The PCR amplification yielded a product having 600bp length.. The sequence was deposited in nucleic acid databases for public accession having NCBI GenBank Accession No. KP835515 and Barcode of Life Data System BIN Cluster ID – BOLD: ACD6379 with Specimen ID – GBMIN88796-17 .

The COI sequence of *Neurothemis fulvia* showed bias to nucleotide AT, with following composition of nucleotides T = 33.7%, C = 20.0%, A = 27.7% and G = 18.6% (Table:1). This high AT content of 61.4% over 38.6% of GC is mainly due to the mutational pressure on a single nuceotide substitution during the evolutionary period of time.

The 600bp sequence obtained by amplification process yielded 200 long translated amino acid sequence. Both nucleotide and protein BLAST analysis showed that this species is 100% sequence similarity to the same species reported from Mizoram (JN817427). The evolutionary history was inferred using the Neighbour-Joining method and the evolutionary history of the taxa was analyzed. The analysis involved 10 nucleotide sequences and the codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 583 positions in the final dataset after eliminating all positions containing gaps and missing data. The phylogenetic tree constructed by Neighbour-Joining method clearly showed that this species doesn't have any sequence divergence as time progressed. Phylogenetically this species is very close to close to *N. intermedia*, *N. fluctans* and *N. tullia* with respective the divergence of 17.6%, 18.10% and 18.55% respectively. The above result is confirmed by the divergence table plotted by Maximum Composite Likelihood model (Table:1).



Molecular phylogenetic tree of *Neurothemis fulvia* inferred by NJ tree method

Table 2: Nucleotide substitution table plotted by Maximum Composite Likelihood model

Domain: Data																
	T(U)	C	A	G	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C-3	A-3	G-3
KP835515.1 *Neurothemis fulvia* (KERALA)	33.7	20.0	27.7	18.6	21	20.0	27.2	31.8	43	26.7	14.9	15.4	37	13.3	41.0	8.7
JN817427.1 *Neurothemis fulvia*	33.7	20.0	27.7	18.6	21	20.0	27.2	31.8	43	26.7	14.9	15.4	37	13.3	41.0	8.7
KC122229.1 *Neurothemis tullia*	34.4	19.3	26.0	20.2	20	20.0	28.2	31.8	43	26.7	14.9	15.4	40	11.3	35.1	13.4
KT957504.1 *Neurothemis fluctuans*	34.2	18.8	27.4	19.7	22	17.9	28.2	31.8	43	26.7	14.9	15.4	37	11.8	39.0	11.8
KC122227.1 *Neurothemis sp*	34.2	18.6	27.5	19.7	22	17.9	28.2	31.8	43	26.7	14.9	15.4	37	11.3	39.5	11.8
KU566458.1 *Trithemis werneri*	36.2	15.4	31.5	16.9	24	16.4	28.2	31.8	43	26.7	14.9	15.4	42	3.1	51.3	3.6
KU566447.1 *Trithemis legrandi*	33.8	17.8	30.9	17.4	22	19.0	27.7	31.8	43	26.7	14.9	15.4	37	7.7	50.3	5.1
KU566445.1 *Trithemis legrandi*	33.8	17.8	30.9	17.4	22	19.0	27.7	31.8	43	26.7	14.9	15.4	37	7.7	50.3	5.1
KU566444.1 *Trithemis legrandi*	33.9	17.8	30.8	17.5	22	19.0	27.7	31.8	43	26.8	14.4	15.5	37	7.7	50.3	5.1
KU566446.1 *Trithemis legrandi*	34.0	17.6	30.9	17.4	22	19.0	27.7	31.8	43	26.7	14.9	15.4	37	7.2	50.3	5.1
Avg.	34.2	18.3	29.1	18.3	22	18.8	27.8	31.8	43	26.7	14.8	15.4	38	9.4	44.8	7.9

The BOLD database analysis showed 99.82-100% similarity sequences already reported in BOLD system. The line diagram of this species for the confirmation of above statement is shown in (Fig. 22 f). The close matching BIN of the species is found to be 3% and the most similar species is found to be reported from Mizoram having the accession number BOLD ACD6379. The average and maximum nucleotide distance to this species is found to be 0.43% (p-distance) and 1.61% (p-distance) respectively. The generic close neighbour was *Neurothemis tullia* having a divergence of 1% (BOLD: ADK3152).

Table 2 : Percentage of evolutionary divergence of *Neurothemis fulvia* with its closely related species accessible from NCBI GenBank

Sl. No.	Accession No	Organism	Percentage of divergence
1	KP835515	*Neurothemis fulvia (Kerala)*	
2	JN817427	*Neurothemis fulvia (Mizoram)*	0.00
3	KC12229	*Neurothemis tullia*	18.55
4	KT957504	*Neurothemis fluctans*	18.10
5	KC12227	*Neurothemis intermedia*	17.67
6	KU566458	*Trithemis werneri*	23.14
7	KU566447	*Trithemis legrandi*	23.92
8	KU566444	*Trithemis legrandi*	23.92
9	KU566446	*Trithemis legrandi*	23.92

DISCUSSION

Neurothemis fulvia is commonly known as 'Fulvous forest skimmer' and is geographically distributed in Asian countries (Mitra, 2010). Morphological identification was done using the authentic taxonomic keys (Emiliyamma et al., 2005). This species is always seen associated with agroecosystem and also in ponds and other aquatic habitat. This is a pioneer molecular work from Kerala and its sequence was found to be conserved during the evolutionary period of time. This sequence doesn't have any kind of sequence divergence to the same species.Phylogenetically this species is very close to Neurothemis tullia by NCBI and BOLD system. However all *Neurothemis* genus are having a monophyletic ancestry as all members were splitted from a single node (Jisha and Sebastian, 2015). Hence the barcode generated helped to easily spot the specimen very and also to infer phylogenetic status.

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