



Analysis of Phylogenetic Status of Different *Neurothemis* (Odonata:libellulidae) Species Using Cytochrome Oxidase I Gene Sequence

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ABSTRACT

For taxonomists *Neurothemis* is a confusing genus under Order Odonata, because of the morphological variants existing within the species. Here we have analysed the phylogenetic relationships of three different species of *Neurothemis* by the partial sequencing of mitochondrial cytochrome oxidase subunit I (COI) gene. Phylogenetic tree constructed by Neighbour joining method proved that *Neurothemis tullia* and *Neurothemis intermedia* are taxonomically more closer and they together formed a single clade in the tree. *Neurothemis fulvia* is sister to this clade but it represents the most diverged species in terms of branch length and nucleotide substitution. Comparison with the retrieved sequences confirmed that it strictly belong to Libellulidae family. The tree also depicted that *Neurothemis* genus is more close to *Orthetrum sabina* than *Diplacodes trivalis*. Hence the study concluded that DNA barcoding is an invaluable tool for confirming the species identification and to assess the proper phylogenetic relationships.

KEYWORDS : Odonates, Molecular phylogenetics, Cytochrome oxidase I gene, *Neurothemis* sp, Female polymorphism

1. Introduction

Kerala represents a mega diversity hotspot in the world due to its unique physical features. The average rainfall of 3107mm and 44 rivers flowing through this state makes it a highly habitable place for Odonates. Globally 6000 extant species of Odonates have been reported and out of which 474 species in 142 genera and 18 families are known from India and 154 species from Kerala (Tsuda 1991; Subramanian, 2014; Kiran and Raju, 2013).

Generally Odonates shows an enormous amount of variation both in their life histories and their activities (Lyons, 1999). The wings alone are one of the important taxonomic characters used for understanding and resolving the phylogenetic relationships with respect to morphological feature (Fraser, 1957). Various molecular phylogenetic studies were reported for the taxonomic authentication and evolutionary relationships among Odonates (Jisha Krishnan and Sebastian, 2015^a).

Molecular phylogenetics utilizes the combined analysis of molecular and statistical techniques for predicting the evolutionary relationships among genes or organisms. Phylogenetic data consist of hundreds of different species, each of which may have varying mutation rates and patterns that influence evolutionary change.

The introduction of DNA barcoding became a fast identification method for assessing biodiversity as well as unknown species identification (Hebert & Gregory, 2005). Here we have utilized the cytochrome oxidase I gene as molecular marker for phylogenetic analysis. Cytochrome oxidase I gene act as a identification marker in insects due to lack of introns, simple alignment and limited exposure to recombination. It 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. Sequence variation in this region generally shows large interspecific and small intraspecific divergences.

Neurothemis commonly called 'Red dragonflies' is a Libellulidae member commonly found in drains, ditches, shallow streams, paddy fields etc. There are about 18 species known to exist and out of which 3 species are commonly found in Kerala. Most of the species looks similar in terms of their appearance, behaviour and other notable characteristics, but in a close look and detailed study, they all are found to be reproductively isolated (Dow and Clausnitzer, 2012). Female polymorphism is one of the phenomena exhibited by the *Neurothemis tullia* species (Kante *et al.*, 2013). Genetic analysis of this polymorphic

species showed that there is no considerable variation among male, female and andromorphic females (Jisha Krishnan and Sebastian C.D, 2015^b).

Most of the females members of *Neurothemis* especially the species *Neurothemis tullia* exhibits female-limited polymorphism with a clear difference in the wing and body coloration (Schorr *et al.*, 2013). Sharon *et al.*, (2014) concluded their study that wing characters along with the shape of synthorax play a great role in the differentiation of one morphotype to another. This variation in the morphology is strictly exhibited by female species. Chesalmah *et al.*, (2000) showed that female species of *Neurothemis tullia* are more active; feed more heavily with high body weight compared to males and are considered to be an important predator of rice pest. The main objective of the present study was to assess the taxonomic relationship of 3 different species of *Neurothemis* and to conclude how it is related with other Odonate members.

2. Materials and Methods

2.1 Sample Collection and Preservation

Three different species of *Neurothemis* genus under the family Libellulidae were collected from Northern Kerala. Dragonflies were 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. 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.

2.2 DNA extraction, amplification and sequencing

DNA from selected dragonflies was extracted from leg using 'Origen 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'ATTAGTCCGTTAATACTTGGTCTCC3') and reverse primer (5'AAAATTGATCTCTCCCCCTGC3') 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 by

Sangers method. Phylogenetic analysis done by MEGA software (Tamura *et al.*, 2013).

2.3 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.

3. Results

Morphological identification from taxonomic experts confirmed the representing members of *Neurothemis* species as *Neurothemis tullia*, *Neurothemis intermedia* and *Neurothemis fulvia* (Fig: 1). Phylogenetic tree, Maximum Composite Likelihood and Composition of Nucleotide composition are given below (Fig: 2; Table 1 and 2).



Figure 1: (a) *Neurothemis fulvia*; (b) *Neurothemis tullia*; (c) *Neurothemis intermedia*

<i>Diplacodes trivalis</i>	22%	26%	23%	26%	-
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Table 2 : Composition of Nucleotide composition of sequences

	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 <i>N. fulvia</i>	32.5	19.0	29.8	18.6	40	30.3	13.1	16.2	35	8.2	49.0	8.2	22	18.4	27.6	31.6
KT222948 <i>N. tullia</i>	33.9	17.6	28.5	20.0	40	30.3	13.1	16.2	40	5.1	42.9	12.2	21	17.3	29.6	31.6
KP83551 <i>N. intermedia</i>	32.5	19.3	28.5	19.7	40	30.3	13.1	16.2	34	12.2	42.9	11.2	23	15.3	29.6	31.6

KP835512 <i>D. trivalis</i>	33.2	18.0	30.8	18.0	40	31.3	12.1	16.2	37	5.1	52.0	6.1	22	17.3	28.6	31.6
KP938529 <i>O. sabina</i>	34.6	17.6	29.8	18.0	40	30.3	13.1	16.2	40	7.1	46.9	6.1	23	15.3	29.6	31.6

4. DISCUSSION & CONCLUSION

Neurothemis genus comprised about 18 different species and out of which 3 species are reported from Kerala (Emiliyemma). In the present study 3 different species of *Neurothemis* were taxonomically identified from experts as *Neurothemis tullia*, *Neurothemis intermedia* and *Neurothemis fulvia*. Here we have PCR amplified the cytochrome oxidase I gene to predict their phylogenetic relationships. The amplified products were sequenced and deposited in Gen Bank with their accession numbers KP 835514, KP 835513 and KP835515 for *Neurothemis intermedia*, *Neurothemis tullia* and *Neurothemis fulvia* respectively. Female limited polymorphism as well as wing colour morph variants existing in males is making difficulty for identifying these species. Many studies have shown that wings of many insect species including that of intraspecific groups or populations and subspecies can be identified based on wing morphology alone (Tofilski, 2004). Cesar *et al.*, (2011) discriminated the sibling species of *Neurothemis* using the geometric morphometric analysis of the landmark data from the forewings and hindwings. But the similarities in the shape of the wing and its markings do not necessarily reflect close relationships and cannot help define the taxa more clearly (Wecker *et al.*, 2001). Hence in the present study we have used the molecular phylogenetic tools to assess the taxonomic identity and phylogenetic relationship by using the molecular marker cytochrome oxidase I gene. Jisha and Sebastian (2015b) concluded their study that the male, female and andromorphic female of *Neurothemis tullia* species are not having major differences in their COI gene. In the present study the phylogenetic tree constructed by Neighbour joining method clearly indicates that *Neurothemis tullia* and *Neuro-*

Neurothemis intermedia

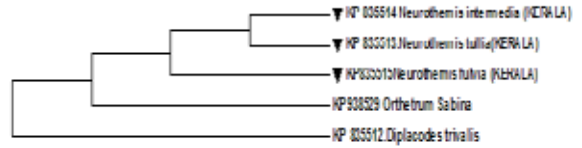


Figure 2: Phylogenetic tree constructed by Neighbour joining method

Table 1: Table showing analysis of percentage of evolutionary divergence between sequences

	<i>Neurothemis tullia</i>	<i>Neurothemis intermedia</i>	<i>Neurothemis fulvia</i>	<i>Orthetrum sabina</i>	<i>Diplacodes trivalis</i>
<i>Neurothemis tullia</i>	-	14 %	17 %	21%	22%
<i>Neurothemis intermedia</i>	14%	-	19 %	24%	26%
<i>Neurothemis fulvia</i>	17%	19%	-	26%	23%
<i>Orthetrum sabina</i>	21%	24%	26%	-	26%

themis intermedia were originated from a single clade and are having a sister clade relationship. Phylogenetically these two species are very close together than *Neurothemis fulvia*. *Neurothemis fulvia* represents the most diverged species in relation with nucleotide substitution and branch length. Comparing with the two outgroups species of Dragonflies from NCBI as *Orthetrum sabina* and *Diplacodes trivalis*, *Neurothemis* genus are having monophyletic ancestry. Taxonomically *Orthetrum sabina* is more close to *Neurothemis* genus than *Diplacodes trivalis*. To analyse the evolutionary divergence between *Neurothemis* species nucleotide substitution analysis was done. The average nucleotide composition frequencies are A=29.49%, T=33.36%, C=18.31% and G=18.85%, showing high A+T content (62.85 %) compared to G+C content (37.16 %). This is supported by the view of general agreement that A+T content is high among Odonates (Chippindale *et al.*, 1999). The nucleotide composition analysis showed that there occurs a second codon position change among all these sequences. This view reflects the higher evolutionary divergence between species. So the overall results shows that analysis based on mitochondrial gene is a successful tool and can be used for unraveling phylogenetic relationships among closely related species.

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