International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-7, Issue-2, 2016, pp545-550
http://www.bipublication.com

Research Article

Analysis of Taxonomic Relationships and Species Divergence Of Libellulidae (Odonata: Anisoptera) Members Using Cytochrome Oxidase I Gene

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[Received-25/02/2016, Accepted-11/03/2016, Published-25/04/2016]

ABSTRACT

Libellulidae are commonly called 'skimmers' or 'perchers' representing the largest dragonfly family in the world. They are cosmopolitan in distribution and consist of 142 genera and 871 species. This family displays remarkable diversity in behaviour and morphology and consequently focused on studies of comparative population ecology, sexual selection, phylogeography and the evolution of mating behaviour. In the present study we deciphered the phylogenetic relationships of nine Libellulidae members by Neighbour-joining (NJ) and Maximum likelihood methods using partial cytochrome oxidase I gene as the marker. The phylogenetic tree inferred the sister clade relationship of the representing libellulidae members and confirmed the evolutionary divergence in relation with branch length. The averages A+T content of all these species are 62.03 % while G+C content is 37.97 % showing strong A+T bias. The transition/ transversion ratio are found to be 0.858 for purines and 2.533 for pyramidines indicating higher mutations are exhibited by the transition of Thymine, Uracil and Cytosine. The present study thus concluded that the mitochondrial cytochrome oxidase subunit I (COI) gene sequence of Libellulidae members demonstrated substantial variation; therefore it can be used for molecular taxonomy and for the phylogenetic studies.

Keywords: *Libellulidae, cytochrome oxidase I gene, molecular phylogeny*

[1] INTRODUCTION

The Libellulidae (skimmers) family is a large group of dragonflies, consisting of many large and colourful species often seen around water bodies. Most skimmers are rather robust bodied with fairly thick abdomen, patterned wings and bright colours on the thorax and abdomen.

Their body length varies from as small as 20mm to as long as 75mm and prefers to live in lentic water bodies like ponds and lakes. These are sexually dimorphic species with clear differences in size, colour and markings in both the sexes. Males of many species exhibit pruinosity with the development of a whitish blue, powdery appearance. Female skimmers oviposit on the surface of the water, dipping their abdomens and depositing eggs into the water as they hover on water surface.

Development undergoes incomplete metamorphosis through egg, naiad and adult. The main identification feature of Libellulidae members is the wing venation, the wing triangles of these members differ between the forewing and hind wing and also the anal loop in the hind wing is shaped like a boot.

Recent advances in molecular techniques have provided powerful tools for studies of intra or inter specific phylogeographic patterns. Odonata represent the most ancient insect branch and have received much attention in the evolutionary, ecological and conservational biology research [2]. DNA barcoding is a new genomic technology for recognizing and ordering species. The utility of mitochondrial genes for resolving genus level phylogenetic relationships are well known [11].

The principle objective of the present study is the use of mitochondrial cytochrome oxidase I gene to assess the phylogenetic descendance of different members of Libellulidae. Several phylogenetic studies were conducted among odonates related to evolutionary divergence, sexual dimorphism, confirmation of species identification etc. [4, 5]. The main advantage of molecular phylogeny is that it can be performed at low cost without the involvement expert taxonomist in a group. Also the changes in DNA form the basis for all other evolutionary changes such as changes in morphology, comparison of gene sequences which allows the study of evolution at most basic level.

The cytochrome oxidase I (COI) gene was chosen for insect DNA barcoding because insertions and deletions are rare which often leads to a shift in the reading frame.

The universal primers for COI are very robust, and also 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, but small intraspecific divergence meaning that species frequently form clearly distinguishable clusters on a distance based phylogenetic trees.

[2] MATERIALS AND METHODS

2.1 Sample Collection and Preservation

A total of 28 individuals belonging to 9 species of dragonflies under the family Libellulidae were collected from different districts of North Kerala, India by hand sweep netting and random field sampling methods. Identification was done by observing wing venation, colour pattern and genitalia, using available authentid taxonomic keys/ identification guides. Each specimen was preserved in separate collecting bottles, assigned a code number and stored in 70% ethanol as voucher specimen.

2.2 DNA extraction, amplification and sequencing

DNA from the tissues of the selected dragonflies was extracted from leg using available DNA extraction kit. The isolated DNA was confirmed using 1% Agarose gel. The isolated DNA was PCR amplified using appropriate forward and reverse primers. 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, 50°C for 1minute, 72°C for 45 seconds followed by a final step of 72°C for 3 minutes. The PCR product was confirmed using 2% Agarose gel electrophoresis and were sequenced with both primers using in automated sequencer ABI 3730XL by Sangers method.Phylogenetic analyses were done by MEGA6 software [13].

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 the ClustalW program.

[3] RESULTS

The mitochondrial cytochrome oxidase I gene of all the nine libellulidae members were amplified using PCR. The databases revealed definite identity matches in the range of 98%-100%. All the members are performed for similarity searches such as Blast and confirmed species are deposited in Gen Bank for future reference.

Gen bank accession numbers and their base pair lengths are described in Table 1 and Table 2.

Table -1: GenBank Accession numbers and percentage of identity match in database of different species of Libellulidae members used in the study

Sl. No.	Organism	Common Name	Amplified gene size	GenBank Accession No.	% of identity match
1.	*Orthetrum sabina*	Green Marsh Hawk	500 bp	KP 938529	100
2.	*Rhyothemis variegata*	Common picture wing	450 bp	KP 938530	100
3.	*Diplacodes trivalis*	Ground skimmer	466 bp	KP 835512	100
4.	*Acisoma panorpoides*	Grizzled pintail	479 bp	KT 222947	100
5.	*Bradinopyga geminata*	Granite ghost	468bp	KM 096995	99
6.	*Pantala flavescence*	Wandering glider	444 bp	KR 011198	99
7.	*Neurothemis tullia*	Pied paddy skimmer	351 bp	KP 835513	99
8.	*Brachythemis contaminata*	Ditch jewel	383 bp	KP 938531	98
9.	*Neurothemis intermedia*	Paddy field parasol	370 bp	KP 835514	98

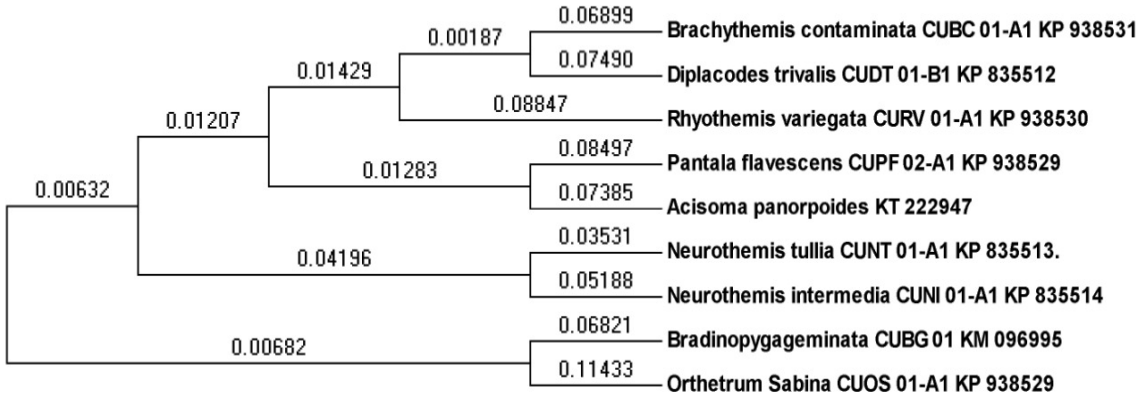

Fig -: 1 Evolutionary relationship of the selected dragonfly species using NJ method

The evolutionary history was inferred using the Neighbor-Joining method [10]. The optimal tree with the sum of branch length = 0.83881675 is shown. The evolutionary distances were computed using the Jukes-Cantor method [6] and are in the units of the number of base substitutions per site (Fig 2).

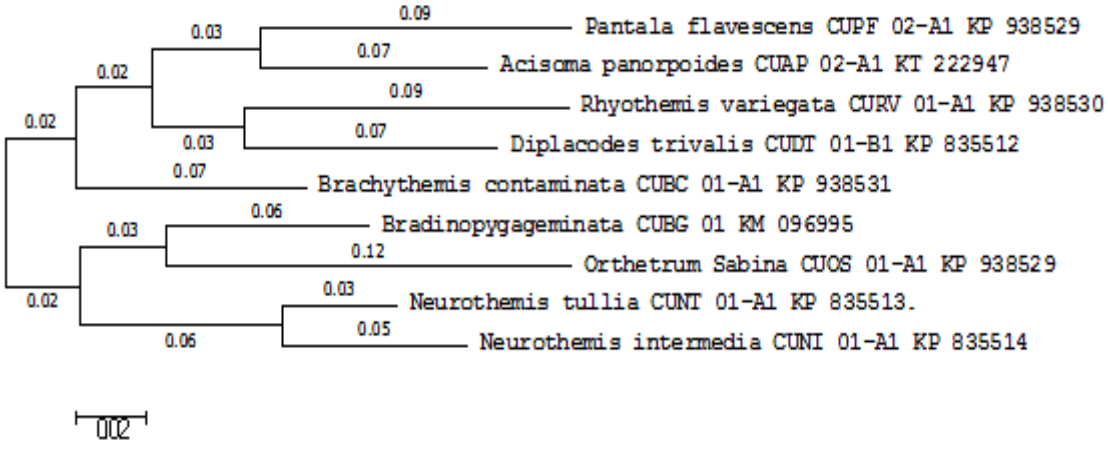

Fig 2 - : Evolutionary relationships of different Dragonflies

The number of base substitution per site between sequences shows standard error estimate shown above the diagonal. Analyses were conducted using the maximum composite likelihood model. The difference in the composition bias among the sequences was considered in the evolutionary comparison (Table 3).

Table 2-: Estimates of Evolutionary Divergence between Sequences

Sl. No.	Organism	Evolutionary Divergence between species							
		1	2	3	4	5	6	7	8
1	*B. geminate*	0							
2	*P. flavescence*	0.19							
3	*B. contaminata*	0.15	0.15						
4	*R. variegate*	0.16	0.18	0.14					
5	*O. sabina*	0.16	0.18	0.21	0.19				
6	*N. tullia*	0.14	0.16	0.15	0.16	0.18			
7	*D. trivalis*	0.14	0.17	0.13	0.15	0.21	0.16		
8	*N. intermedia*	0.16	0.16	0.18	0.18	0.18	0.08	0.18	
9	*A. panorpoides*	0.16	0.14	0.15	0.17	0.20	0.15	0.14	0.16

Each entry is the probability of substitution (r) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei (1993) model [12]. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 100. The nucleotide frequencies are A = 29.02%, T/U = 33.01%, C = 19.28%, and G = 18.69%. The maximum Log likelihood for this computation was -850.007 (Table 4).

Table 3 -: Maximum Likelihood Estimate of Substitution Matrix

	A	T/U	C	G
A	-	9.03	5.27	3.80
T/U	7.94	-	13.13	5.11
C	7.94	22.48	-	5.11
G	5.90	9.03	5.27	-

The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences [7]. The estimates of the disparity index per site are shown for each sequence pair above the diagonal (Table 4).

Table -4: Test of the Homogeneity of Substitution Patterns between Sequences

	1	2	3	4	5	6	7	8	9
B. geminate		0.00	0.02	0.00	0.00	0.00	0.00	0.04	0.00
P. flavescence	1.00		0.16	0.23	0.00	0.00	0.15	0.00	0.24
B. contaminata	0.33	0.12		0.00	0.00	0.11	0.00	0.00	0.17
R. variegate	1.00	0.07	1.00		0.02	0.09	0.00	0.29	0.00
O. Sabina	1.00	1.00	1.00	0.31		0.00	0.00	0.00	0.08
N. tullia	1.00	1.00	0.16	0.20	1.00		0.04	0.15	0.14
D. trivalis	1.00	0.12	1.00	1.00	1.00	0.34		0.03	0.10
N. intermedia	0.31	1.00	1.00	0.05	1.00	0.05	0.35		0.46
A. panorpoides	1.00	0.12	0.12	1.00	0.27	0.13	0.15	0.00	

The probability of rejecting the null hypothesis of strict-neutrality (dN = dS) (below diagonal) is shown. Values of P less than 0.05 are considered significant at the 5% level and are highlighted. The test statistic (dN - dS) is shown above the diagonal. dS and dN are the numbers of synonymous and no synonymous substitutions per site, respectively. The variance of the difference was computed using the analytical method. Analyses were conducted using the Nei-Gojobori method [8] (Table 5).

Table - 5: Codon-based test of Neutrality for analysis between sequences.

	1	2	3	4	5	6	7	8	9
B. geminate		0.9	0.4	1.1	-0.9	1.2	0.4	2.3	0.9
P. flavescence	0.4		-1.1	-0.5	1.7	0.6	1.6	1.4	0.8
B. contaminata	0.7	0.3		0.2	-0.7	-0.2	-0.3	-0.4	-1.0
R. variegate	0.3	0.6	0.9		0.0	-0.9	-0.7	-0.2	-0.2
O. Sabina	0.4	0.1	0.5	1.0		1.8	0.2	2.7	0.1
N. tullia	0.2	0.5	0.8	0.4	0.1		-1.1	1.7	-0.3
D. trivalis	0.7	0.1	0.8	0.5	0.8	0.3		-0.2	0.1
N. intermedia	0.0	0.2	0.7	0.8	0.0	0.1	0.8		0.5
A. panorpoides	0.3	0.4	0.3	0.9	0.9	0.8	0.9	0.6	

[4] DISCUSSION AND CONCLUSION

DNA barcoding technique for identifying already identified organism at any taxonomic level is a powerful application to biodiversity related studies. The cytochrome oxidase I gene of mitochondrial genome is a common molecular marker in several DNA barcoding studies [3]. Among odonates, identification keys based on morphological characters often do not exceed the family level and some genera do not form monophyletic groups [1]. Generally the Suborder Anisoptera formed of 3 clades namely Libelluloides, Macromiids and Corduliids. The Libelluloides have recently been subjected to a careful analysis using selected portion of the nuclear 28S and the mitochondrial 16S genes [14]. Nitrogen dehydrogenase (NDI) gene was also used as an alternative molecular marker of cytochrome oxidase I gene. It is evident that about 54 species of Odonates coming under 22 genera could be discriminated through the unique combination of character states based on this gene (ND1 gene) [9]. Here we constructed a phylogenetic relationship of 9 libellulidae members using COI gene and the confirmed sequences are deposited in NCBI for future references (Table 1). All these species showed 98-100 % sequence similarity to the same species deposited in Gen Bank. The GenBank results showed maximum identity of 100% for *Diplacodes trivialis, Ortherum Sabina, Rhyothemis variegate, Acisoma panorpoides* and 99 % for *Bradinopygea geminata, Pantala flavescence, Neurothemis tullia* and 98 % for *Neurothemis intermedia* and *Brachytmemis contaminate* (Table 2). The evolutionary tree constructed by Neighbour joining method confirmed the sister clade relationship of the representing members. The tree showed that the maximum divergence was found in the clade containing *Orthetrum sabina* and *Bradinopyga geminate* (Figure 1) indicating maximum divergence. The optimal tree with the sum of branch length = 0.83881675 is shown in Figure 2. From this diagram it is confirmed that maximum nucleotide substitutions was found in *Orthetrum sabina* than other members. Comparing the two taxonomically closely related species such as *Neurothemis tullia* and *Neurothemis inermedia*, maximum divergence was found to be in the latter one with respect to the branch length. But these two species are originating from a single clade and thereby confirmed the genus level taxonomy. The nucleotides of A, T, C and G are in the following concentration A = 29.02%, T/U = 33.01%, C = 19.28%, and G = 18.69% (Table 4) which indicates high AT content compared to GC content. The transition/ transversion ratio are found to be 0.858 for purines and 2.533 for pyramidines, indicating mutations are exhibited by Thymine, Uracil and Cytosine. The shift to AT compositional bias with low 'G' in the sense and 'C' in the template strand may have arisen through directed mutational pressure [15]. The sister clades are *Brachytemis contaminate* and *Diplacodes trivalis, Pantala flavescence* and

Acisoma panorpoides, Neurothemis tullia and *Neurothemis intermedia* with most recently orginated clade as *Bradinopyga geminate* and *Orthetrum sabina*. Therefore the present study is used for confirming molecular level taxonomic identification, their taxonomic relationships of the representing members and its evolutionary divergence.

ACKNOWLEDGMENT

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

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