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Molecular and phylogenetic analysis of the genus *Orthetrum* (Odonata: Anisoptera: Libellulidae) using mitochondrial CO1 gene

Lalṭanpuii[1,2]*, N. Senthil Kumar[2] and Manu Thomas Mathai[1]

[1]*Department of Zoology, Madras Christian College, Tambaram, Chennai 600 059, India*
[2]*Department of Biotechnology, Mizoram University, Mizoram 796 004, India*

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ABSTRACT

Molecular phylogenetic relationships among members of the odonate genus *Orthetrum* (Odonata: Anisoptera: Libellulidae) were examined using 403 bp of mitochondrial COI. The support for monophyly of the *Orthetrum* was found in some studies with unresolved complexity. The *O. sabina, O. serapia* and *O. trincaria* formed a seperate and distinct group from the morphological analysis. We analysed the COI sequences of 22 species of *Orthetrum* using MEGA6. The p-distance between the members and the rate of transitional and transversional substitution was generated. The analysis indicated that the *Orthetrum* are monophyletic and *O. sabina* and *O. trinacria* formed a disticnt and a seperate group.

Key words: Molecular phylogeny; mitochondrial COI; monophyly; *Orthetrum*; p-distance.

INTRODUCTION

The genus *Orthetrum* includes about 60 species, of which one-half is distributed in tropical Africa and the rest extends across Eurasia to Australia. The genus is diverse in appearance in the Oriental region, such as red, boldly patterned and wingmarked species.[1] 14 species of *Orthetrum* are reported from India[2] and in Mizoram 4 species have been recorded. Morphological analyses indicated that *O. trinacria* forms a distinct group with *O. sabina* and *O. serapia.*[2] *O. sabina* and *O. trincaria* are well known for feeding on other odonate species, sometimes of greater size than their own.[3] It has been found that the African *O. trinacria* has spread out into Madagascar, Mesopotamia and Europe, while tropical Asia's most dominant species, *O. Sabina,* has migrated to northern Africa, Turkey and Europe.[1] Some support for the monophyly of *Orthetrum* have been found, but the inclusion of other species suggests some unresolved complexity.[4,5]

Corresponding author: Lalṭanpuii
Phone: +91-9436152033
E-mail: laltetei@yahoo.co.in

In the present study we infer a phylogeny for the genus *Orthetrum* utilising the cytocrome *c* oxydase subunit 1 (CO1) gene of the mitochondrial DNA. The mitochondrial DNA is a good choice for phylogenetic study because of its fast mutational rate which gives a significant variation between species, lack of introns, limited exposure to recombination and its haploid mode of inheritance. CO1 universal primer is very robust, enabling recovery of its 5' end from most of the animal phyla and it possesses a great phylogenetic signal.[6]

MATERIALS AND METHODS

Taxon sampling

The taxon sample for the present study included 18 *Orthetrum* species retrieved from GenBank (National Centre for Biotechnology Information) with the locality and the accession numbers. The 4 species generated from Mizoram were also included in the present study (Table 1). The gomphidus, *Davidus lunatus* was selected as outgroup.

MOLECULAR EVOLUTIONARY GENETIC ANALYSIS (MEGA)

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6.[7] All the 23 sequences of were aligned and edited using clustalW implimented in MEGA6.

Table 1. List of taxa for the present study.

Taxon name	Country	Genbank Accession no.
Davidius lunatus (Bartenev, 1914)	Republic of Korea	EU591677
O. pruinosum (Burmeister,1839)	Mizoram, India	KC122236
O. sabina (Drury, 1770)	Mizoram, India	KC122234
O. triangulare (Selys, 1878)	Mizoram, India	KC287152
O. glaucum (Brauer, 1865)	Mizoram, India	KC122232
O. triangulare (Selys, 1878)	Republic of Korea	KF257074
O. lineostigma (Selys, 1886)	Republic of Korea	KF257071
O. albistylum (Selys, 1848)	Republic of Korea	KF257070
O. japonicum (Uhler,1858)	Republic of Korea	KF257061
O. triangulare (Selys, 1878)	Japan	AB781568
O. sabina (Drury, 1770)	Japan	AB781554
O. pruinosum (Burmeister,1839)	Japan	AB781552
O. melania (Selys, 1883)	Japan	AB781551
O. luzonicum (Brauer, 1868)	Japan	AB781544
O. glaucum (Brauer, 1865)	Japan	AB781542
O. trinacria (Selys, 1841)	Germany	KC912286
O. julia falsum (Longfield, 1955)	Germany	KC912281
O. coerulescens (Fabricius, 1798)	Germany	KC912271
O. chrysostigma (Burmeister, 1839)	Germany	KC912262
O. brachiale (Palisot de Beauvois, 1805)	Germany	KC912258
O. poecilops (Ris, 1916)	Japan	AB709089
O. internum (McLachlan, 1894)	Japan	AB709025
O. japonicum (Uhler,1858)	Japan	AB709026

Relationships between individual taxa were assessed by maximum likelihood (ML) method with nucleotides distances (p-distance), transition/transversion rate ratios, and nucleotide diversity. The p-distance is the proportion (p) of nucleotide sites at which two sequences being compared are different. It is obtained by dividing the number of nucleotide differences by the total number of nucleotides compared considering the proportion of nucleotide sites that are different.[8]

The overall ts/tv bias (R) was calculated by the formula: $R = [A \times G\ k_1 + T \times C \times k_2] / [(A + G) \times (T + C)]$, where k_1 = purine and k_2 = pyrimidine. For simplicity, the sum of r values is made equal to 100.[9] The analysis involved 22 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 403 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.[7]

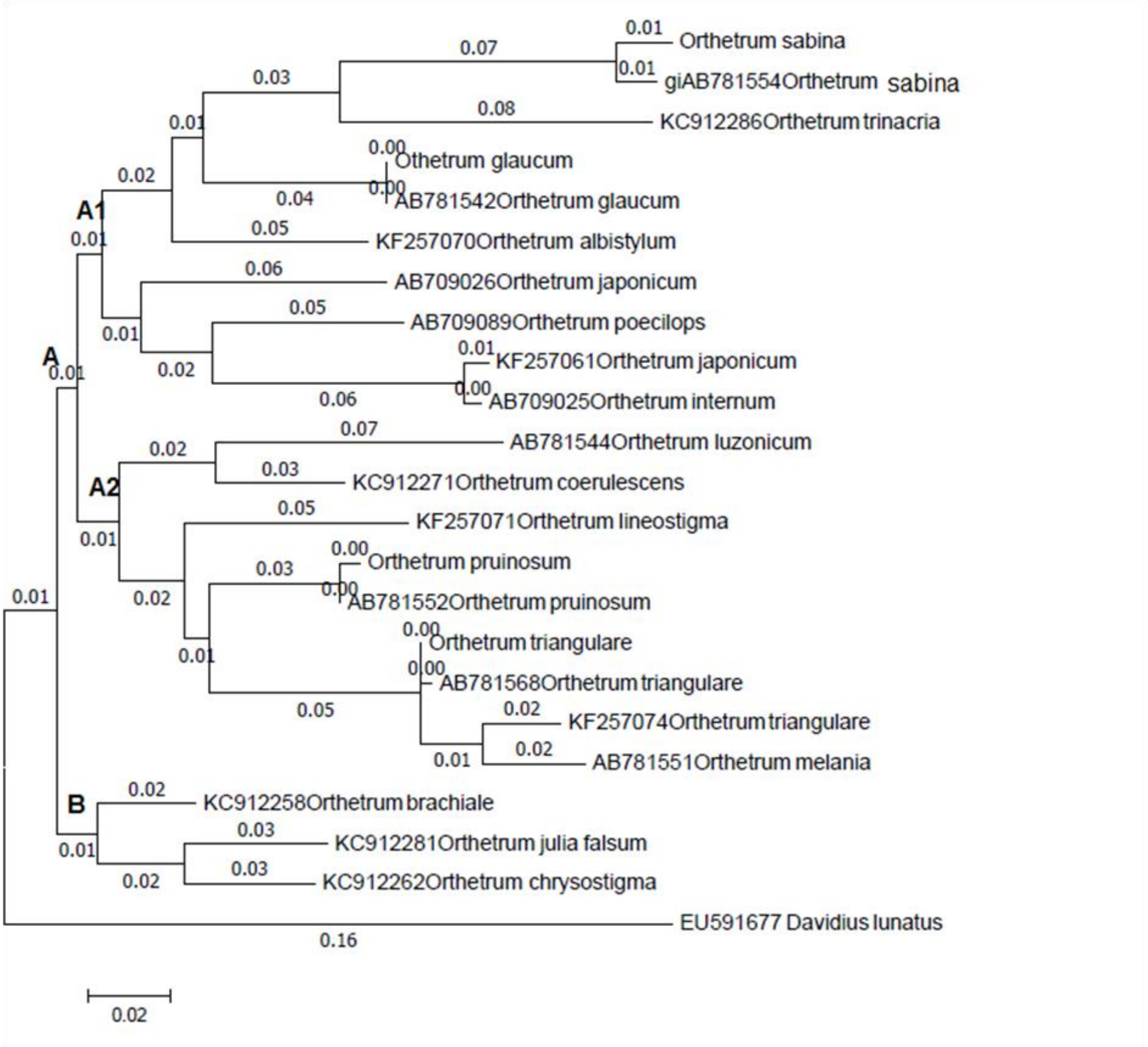

Figure 1. Molecular phylogenetic analysis by maximum likelihood method.

Table 2. The genetic distance (p-distance) between the *Orthetrum* species.

	Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
1	*Orthetrum_pruinosum*																					
2	*Orthetrum_sabina*	0.142																				
3	*Orthetrum_triangulare*	0.084	0.158																			
4	*Othetrum_glaucum*	0.102	0.132	0.112																		
5	*KF257074Orthetrum_triangulare*	0.092	0.177	0.033	0.136																	
6	*KF257071Orthetrum_lineostigma*	0.090	0.180	0.107	0.121	0.114																
7	*KF257070Orthetrum_albistylum*	0.111	0.138	0.120	0.094	0.138	0.134															
8	*KF257061Orthetrum_japonicum*	0.099	0.175	0.125	0.138	0.140	0.107	0.139														
9	*AB781568Orthetrum_triangulare*	0.087	0.161	0.002	0.112	0.035	0.110	0.117	0.128													
10	*giAB781554Orthetrum*	0.142	0.023	0.158	0.126	0.180	0.173	0.138	0.166	0.161												
11	*AB781552Orthetrum_pruinosum*	0.005	0.148	0.079	0.103	0.087	0.085	0.111	0.099	0.081	0.148											
12	*AB781551Orthetrum_melania*	0.096	0.183	0.038	0.151	0.043	0.125	0.144	0.116	0.041	0.186	0.095										
13	*AB781544Orthetrum_luzonicum*	0.116	0.170	0.121	0.114	0.136	0.135	0.116	0.119	0.124	0.164	0.122	0.139									
14	*AB781542Orthetrum_glaucum*	0.102	0.132	0.112	0.000	0.136	0.121	0.094	0.138	0.112	0.126	0.103	0.151	0.114								
15	*KC912286Orthetrum_trinacria*	0.114	0.147	0.147	0.136	0.169	0.153	0.154	0.149	0.150	0.147	0.114	0.178	0.160	0.136							
16	*KC912281Orthetrum_julia_falsum*	0.108	0.153	0.129	0.114	0.141	0.120	0.113	0.137	0.132	0.150	0.102	0.142	0.138	0.114	0.138						
17	*KC912271Orthetrum_coerulescens*	0.099	0.160	0.103	0.111	0.111	0.117	0.119	0.127	0.106	0.160	0.093	0.123	0.098	0.111	0.148	0.113					
18	*KC912262Orthetrum_chrysostigma*	0.116	0.159	0.129	0.117	0.147	0.117	0.096	0.122	0.132	0.156	0.111	0.142	0.135	0.117	0.153	0.065	0.102				
19	*KC912258Orthetrum_brachiale*	0.094	0.145	0.106	0.088	0.123	0.088	0.093	0.104	0.108	0.142	0.094	0.126	0.123	0.088	0.130	0.074	0.094	0.076			
20	*AB709089Orthetrum_poecilops*	0.119	0.150	0.105	0.114	0.113	0.123	0.107	0.107	0.108	0.141	0.113	0.119	0.145	0.114	0.172	0.128	0.116	0.113	0.098		
21	*AB709025Orthetrum_internum*	0.102	0.175	0.128	0.137	0.143	0.113	0.139	0.010	0.131	0.172	0.102	0.119	0.125	0.137	0.162	0.140	0.127	0.122	0.107	0.108	
22	*AB709026Orthetrum_japonicum*	0.117	0.176	0.118	0.129	0.127	0.133	0.122	0.133	0.121	0.179	0.111	0.127	0.145	0.129	0.174	0.118	0.108	0.117	0.108	0.110	0.133

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model.[10] Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 23 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 403 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.[7]

Results

The genetic distance between the 22 species of *Orthetrum* was generated (Table 2). The nucleotide distance (p-distance) between *O. lineostigma* and *O. sabina* was 0.180; O. *triangulare* and *O. sabina* was 0.180*; O. melania* and *O. sabin* was 0.183 and 0.186*; O. japonicum* and *O. sabina* was 0.179*; O. trinacria* and *O. melania* was 0.178 which were found to be highest p-distace. The p-distance between *O. pruinosum* from Mizoram and Japan was 0.005 and the p-distance between *O. trinagulare* from Mizoram against Korea and Japan was 0.033 and 0.002 and between Korea and Japan was found to be 0.035. The p-distance between *O. glaucum* from Japan and Mizoram was found to be zero.

The maximum likelihood estimate of transitional substitution matrix between A/G = 5.58, T/C = 14.44, C/T = 25.64 and G/A = 11.09 (Table 3). The nucleotide frequencies are A = 31.64%, T/U = 33.56%, C = 18.89%, and G = 15.91%. The transition/transversion rate ratios are k_1 = 1.621 (purines) and k_2 = 3.533 (pyrimidines). The estimated transition/ transversion bias (R) is 1.225.

The tree with the highest log likelihood (-2745.6358) is shown (Figure 1). The Maximum Likelihood tree generated shows the monophyly of *Orthetrum* with respect to the outgroup having 2 distinct groups A and B. The Clade B consisted of *O. brachiale*, *O. julia falsum* and *O. chysostygma*, and the rest of the taxa are included in clade A. Clade A can be divided into clade A1 and A2. Within clade A1 the 2 *O. sabina* and *O. trinacria* formed a sister clade with *O. glaucum* and *O. albistylum*; these two sister clades formed a sister clade with *O. poecilop*, *O. japonicum* and *O. internum.* In clade A2 *O. coerulescens* and *O. luzonicum* formed a sister clade with 2 *O. glaucum*, 3 *O. tringulare*, *O. lineostigma* and *O. melania*.

Table 3. Maximum composite likelihood estimate of the pattern of nucleotide substitution.

	A	T	C	G
A	-	7.26	4.09	**5.58**
T	6.84	-	**14.44**	3.44
C	6.84	**25.64**	-	3.44
G	**11.09**	7.26	4.09	-

NOTE: Each entry shows the probability of substitution (r) from one base (row) to another base (column). Rates of different transitional substitutions are shown in **bold** and those of transversional substitutions are shown in *italics*.

Discussions

The genus *Orthetrum* having more than 60 species are suggested to be monophyletic from the previous morphological and molecular studies but with a complex resolution. Morphological analyses indicated that *O. trinacria* forms a distinct group with *O. sabina* and *O. serapia*.[1,3,4] In the present study the COI gene for *O. serapia* is not available; we analysed 22 species of *Orthetrum* using the COI gene. The phylogenetic tree showed the *Orthetrum* are monophyletic in relation to the outgroup *Davidus lunatus*. The *O. sabina* and *O. trinacria* were found to be most distantly related to the rest of the *Orthetrum* analysed having a genetic distance of approximately 18%. In the phylogenetic analysis *O. sabina* from oriental region and *O. trinacria* from Europe were forming a separate and distinct sister clade eventhough the genetic distance between them was found to be 15%. The *O. sabina* and *O. trinacria* forming a sister clade and a distinct group from the rest of the *Orthetrum* analysed is in congruent with the behaviour and morphological characters indicated by Silsby[3] and Dijkstra and Kalkman.[1] The genetic distance of individuals of *O. pruinosum*, *O. glaucum, O. triangulare* and *O. sabina* sampled from different regions (all from oriental region) were found to be very low. The nucleotide composition of *Orthetrum* COI was A + T rich, which is typical for arthropods.[11] The rate of transitional substitution is higher than transversional substitution in the COI sequence analysis.

The present analysis indicated that the *Orthetrum* are monophyletic and *O. sabina* and *O. trinacria* formed a disticnt and a seperate group. The inclusion of more species, more molecular markers and other phylogenetical analysis methods will further resolve the complexity of the genus *Orthetrum*.

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