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Molecular phylogeny based on COI gene and 16S rRNA gene of the genus *Ictinogomphus* (Gomphidae: Anisoptera: Odonata) from India

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Abstract
The order Odonata, encompassing dragonflies (Anisoptera) and damselflies (Zygoptera), represents a highly diverse taxonomic group. This order is one of the most primitive among insects, includes the earliest winged insects. The term "Odonata" originates from the Greek word "odon," referring to their distinctive toothed mandibles. These insects are among the fastest fliers and inhabit freshwater ecosystems. Within dragonflies, the family Gomphidae is the second largest following Libellulidae. Traditionally, odonate identification relies on wing venation patterns and anal appendages, but morphological identification requires extensive taxonomic expertise and can be labor-intensive. To streamline this process, the mitochondrial COI gene and 16S rRNA gene have been utilized as reliable DNA barcodes, providing rapid and accurate species identification. Sequences from this study were submitted to NCBI and demonstrated 95% to 100% similarity with existing conspecific sequences in GenBank. Phylogenetic analysis of the genus *Ictinogomphus* was conducted using these mitochondrial genes and maximum likelihood and neighbor-joining methods were used to construct phylogenetic trees with the Kimura 2-parameter model. The genes were aligned using ClustalW, trimmed, and concatenated, resulting in sequences of 865 bp, with 615 bp as conserved sites and 187 bp as variable sites. The genus *Ictinogomphus* was found to be monophyletic, with *Microgomphus sauteri* (from the same family, Gomphidae) as an outgroup. Interspecific divergence ranged from 2.5% between *I. angulosus* and *I. decoratus* to 11.3% between *I. decoratus* and *I. rapax*, while intraspecific divergence ranged from 0.6% between *I. perinax* and *I. pertinax* which were collected from Japan to 2.4% between *I. rapax* from Punjab and *I. rapax* from America. This study reports, for the first time in India, the molecular analysis of *Ictinogomphus angulosus* and *Ictinogomphus rapax* for both COI and 16S rRNA genes, with *I. angulosus* analyzed at a global level.

Keywords: Gomphidae, *Ictinogomphus*, COI gene, 16S rRNA gene, phylogeny

Introduction

The family Gomphidae is the second largest family of suborder Anisoptera of order Odonata (Carle *et al.*, 2015; Ware *et al.*, 2017) [3, 23]. The members of this family are called gomphids because of the club like structure at the end of the abdomen. These dragonflies found in the freshwater habitat (Ware *et al.*2017) [23]. The classification of dragonflies is mainly based on the wing venation, but over time the similar characters evolve, the close relationship based on it, is difficult to do the correct identification (Dijkstra *et al*, 2013) [5]. The other morphological characteristics like genitalia and larvae may help to some extant but it could not give clear picture in case of closely related species (Carle, 1982; Pfau, 1991; Cranston and Gullan, 2009) [1, 2, 18]. To overcome the problem in morphological based identification, mtDNA sequence of COI gene is used as DNA barcode (Shearer and Coffroth, 2008; Pilgrim and von Dohlen, 2012; Hinojosa *et al.*, 2017) [10, 19, 21]. Mitochondrial COI gene is maternally inherited gene which is highly conserved gene due to lack of DNA recombination. This gene is a good choice to use as DNA barcode because it encodes highly conserved protein (COI) yet carries high mutation rate, which is helpful to differentiate the species but the difference occurred within species is very less (Saccone *et al.*, 1999) [20]. Moreover, this gene can easily be amplified by universal primers LCO-1490 and HCO-2198 (Folmer *et al.*, 1994) [8]. Along with COI gene, 16s rRNA gene is used to reinforce the results as this gene shows sufficient interspecific polymorphism and helps in evaluating the phylogeny (Mohanty *et al*. 2011) [17]. The conspecifics divergence ranges from 11.8% to 0.6%, while average congeneric divergence is 4.49%.

Materials and Methods

The mature specimens were captured from various locations across Indian states (Table 1). These specimens were collected with a sweeping net from the shores of freshwater bodies and green marshes. The gomphid species belonging to subfamily Ictininae were collected and identified using "The Fauna of British India including Ceylon and Burma" Volume I and "Field guide on Dragonflies of India" (Subramanian, 2019) [22].

Table 1: List of the gomphid specimens collected from various locations in India

S. No	Name of Species	Location	Longitude/ Latitude
1.	*Ictinogomphus rapax* (Rambur, 1842)	Maharashtra (Zilpi Lake)	21.0658° N/ 78.8666° E
		Punjab (Harike Wetland)	31.1012° N/75. 1200° E
2.	*Ictinogomphus angulosus* (Selys, 1854)	Jammu (Surinsar Lake)	32.7299° N/ 75.0587° E
3.	*Microgomphus souteri* (Fraser, 1924) []	Kerela (Kolayad)	11.8463°N/ 75.7008°E

Subfamily Ictininae is the smallest subfamily of the family Gomphidae, specimens are the largest in size among the species of this family. The species of this family have the close reticulation in their wings which is the characteristic feature. In fore wing and hind wing, the discoidal cells are being different in shape (Taylor and Francis, 1934). The stretched species have been preserved and photographed (Figure1). These specimens then preserved in alcohol at -20^0 C for further molecular studies.

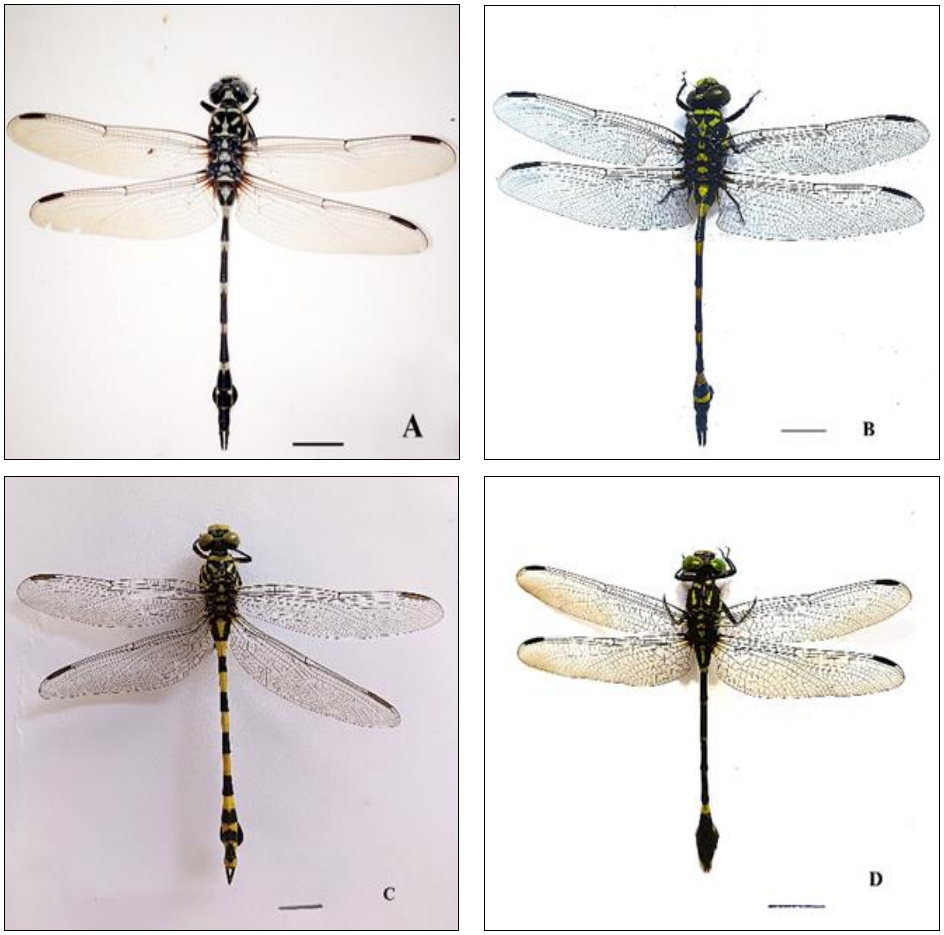

Fig 1: These are the illustration of collected species (A) *Ictinogomphus angulosus*, (B) *Ictinogomphus rapax* Punjab, (C) *Ictinogomphus rapax* Maharashtra and (D) *Microgomphus suateri* (Outgroup)

Molecular Work

Extraction, amplification and sequencing: DNA was extracted from specimens stored in alcohol at -20º C by HI-media DNA extraction kit. Gel electrophoresis was used to evaluate the DNA extraction. The PCR cocktail of 30 µl for COI and 16s DNA genes amplification includes 8.5 µl of PCR water, 1.5 µl of primers (Table2), 15 µl of mastermix, 1.5 µl of BSA, and 2 µl of extracted DNA. PCR mixture is then ready for Thermal cycler where PCR cycles were set as: - One cycle of initial denaturation at 95ºC for 5 minutes, followed by 35 cycles of denaturation at 95ºC for 1 minute, annealing at 50ºC for 1 minute, extension at 72ºC for 90 seconds, and a final extension at 72ºC for 7 minutes. To validate effective DNA amplification, all PCR products were seen using 1% agarose gel electrophoresis and EtBr staining under UV light using the Gel Documentation system. Amplified products were sequenced by Biologia lab at Karnal using the Sanger dideoxy technique.

Table 2: List of Primer used

Locus	Primer name	Primer sequence	Length (bp)	Annealing temp.	References
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	655–658	46◦C	Folmer *et al*., 1994 [8]
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA			
16S rRNA	ODO_12852F	AGAAACCGACCTGGCTTAAA	384–388	46◦C	Dijkstra *et al*., 2014 [6]
	ODO_13393R	CGCCTGTTTATCAAAAACAT			

Results

The sequences of species were uploaded to the NCBI GenBank and accession numbers were obtained from the GenBank. The conspecific sequences and sequences of other species were also download from NCBI GenBank. All these sequences were aligned and trimmed using Clustal W in MEGA XI software. The COI and 16s rRNA gene sequences were concatenated to find out the conspecific and interspecific divergences for genus *Ictinogomphus*. Phylogenetic trees were constructed using Neighbor joining and Maximum likelihood method based on K2P distance in MEGA XI for the genus *Ictinogomphus* and *Microgomphus sauteri* is taken as outgroup.

Table 3: List of accession numbers for COI and 16S rRNA sequences with their length and place

Sr. No	Name of Species	Country	Accession Number (COI gene)	Accession Number (16S rRNA gene)
1	*Ictinogomphus angulosus* (Selys, 1854)	India	OP933827 (795 bp)	OR371733 (540 bp)
2	*Ictinogomphus rapax* (Rambur, 1842)	America	KX891024 (680 bp)	KX890780 (523 bp)
		India	OR122451 (667 bp)	OR122456 (539 bp)
			PQ443877 (620 bp)	PQ443874(518 bp)
3	*Ictinogomphus pertinax* (Selys, 1854)	Japan	AB708703 (451 bp)	AB707759 (451 bp)
		Japan	AB708702 (451 bp)	AB707758 (451 bp)
4	*Ictinogomphus decorates* (Selys, 1854)	Japan	AB860040 (451 bp)	AB707757 (451 bp)
		Republic of Korea	AB708701 (451 bp)	AB860094 (451 bp)
5	*Microgomphus souteri* (Fraser, 1924)	India	OR395439 (736 bp)	OR921705 (490 bp)

Genetic divergence: COI gene and 16S rRNA gene have been used to find out the distance using the Kimura 2 parameter substitution model using MEGA XI.

Intraspecific divergence of species

The concatenated result of COI gene and 16S rRNA gene show that distance of conspecifics divergence ranges from 0.6% to 2.4%.

Interspecific divergence of species

The interspecific divergence over concatenated result of COI gene and 16S rRNA gene ranges from 2.5% to 11.3%.

Nucleotide base composition

Ictinogomphus rapax Maharashtra T= 34.0%, C= 16.8%, A= 32.0%, G=17.3%
Ictinogomphus rapax Punjab T= 34.0%, C= 16.9%, A= 31.3%, G= 17.9%
Ictinogomphus rapax America T= 34.7%, C= 16.5%, A= 31.7%, G= 17.2%
Ictinogomphus pertinax Japan T= 34.2%, C= 17.3%, A= 31.3%, G= 17.2%
Ictinogomphus pertinax Japan T= 34.0%, C= 17.5%, A= 30.9%, G= 17.6%
Ictinogomphus decoratus Japan T= 33.2%, C= 18.5%, A= 31.6%, G= 16.7%
Ictinogomphus decoratus Republic of Korea T= 33.6%, C= 18.0%, A= 31.3%, G= 17.0%
Ictinogomphus angulosus T= 33.7%, C= 17.7%, A= 30.8%, G= 17.7%
Mean percentage values of nucleotide T= 34.3%, C= 17.1%, A= 31.5%, G= 17.1%

Conserved, variable and parsimony informative sites

The sequences of both the genes after final alignment and trimming, were concatenated thus there were total 865 sites. The conserved sites were 615 (71%), variable sites were 187 (21.6%) and parsimony informative sites were 91 (10%). This confirms that these genes are highly conserved.

Phylogenetic analysis

The sequences of COI gene and 16S rRNA gene were concatenated to construct trees using Neighbor joining and Maximum likelihood method, *Microgomphus sauteri* was used as outgroup which is the member of same family as that of genus *Ictinogomphus*. The trees were constructed using nine sequences of COI gene and nine sequences 16S rRNA gene including outgroup. The trees were constructed by inferring 1000 replicates.

In the present study, the phylogenetic analysis using COI gene and 16S rRNA gene for four species of the genus *Ictinogomphus* (*I. rapax, I. angulosus, I. pertinax* and *I. decorates*). The intraspecific and interspecific divergence of these species has been calculated to find out divergence within the species, conspecifics have been collected from different regions of Indian subcontinent and also compared to species of another continent. The comparison of these species with species of other continent based on conserved, variable and parsimony informative sites have been done for the first time.

In both trees, *Microgomphus suateri* emerges on the separate branches out of the trees as it is an outgroup. In ML and NJ trees genus *Ictinogomphus* has been seen as the monophyletic group with high bootstrap values. All the conspecifics have been clustered together sharing single node. The species *Ictinogomphus rapax* was collected from two different regions of India, that is western ghats which is hotspot area of biodiversity lies in the tropical region and Harike Wetland which is a famous Ramsar site of Punjab lies in the temperate region. The sequences for *Ictinogomphus rapax* also download from the NCBI are of American origin. *Ictinogomphus pertinax* and *Ictinogomphus decorates* have seen to share same nodes with their respective conspecifics, both these species are of Japan origin. The COI gene and 16S rRNA gene sequencing of the *Ictinogomphus angulosus* which is collected from Jammu, India has been for the first time and its sequences have been uploaded for the first time on NCBI database. As for *Ictinogomphus pertinax* sampling was done from Meghalaya was not successfully amplified for COI gene and 16S rRnNA gene.

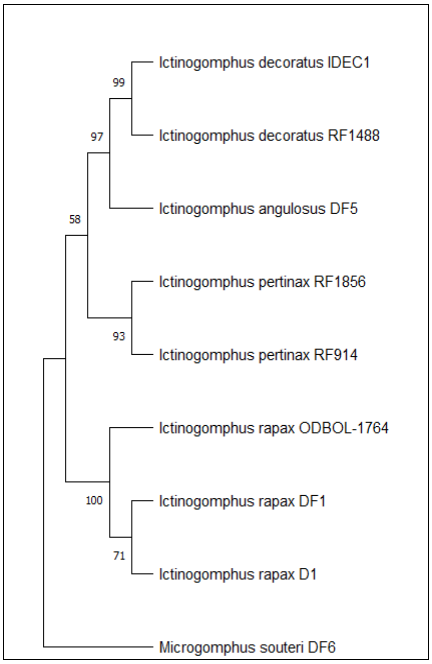

Fig 1: Maximum Likelihood Method Tree

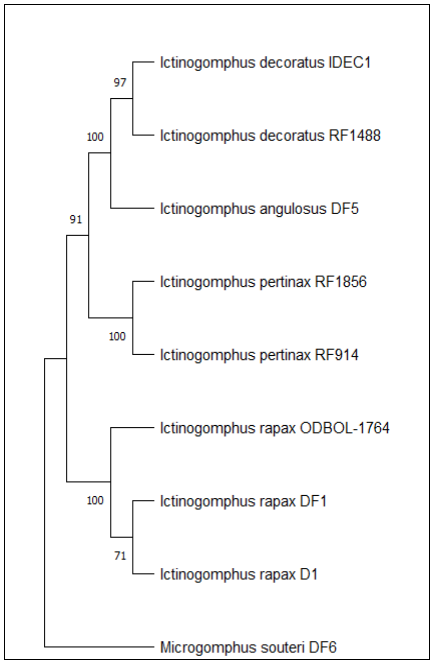

Fig 2: Neighbor joining method tree

Intraspecific and Interspecific divergences

Out of four species the highest intraspecific divergence 2.4% has been observed in genus *Ictinogomphus rapax* among the samples of species from Punjab and America (NCBI). The lowest intraspecific divergence 0.06%. has been found in the samples of *I. pertinax*. The interspecific divergence ranges from 2.5% among *I. angulosus* and *I. decoratus* to 11.8% between *I. rapax* a sample from Maharashtra and *I. decorates.*

Table 4: Intraspecific and Interspecific genetic divergence (%) within and among the species of genus *Ictinogomphus*

Name of Species (Voucher names)	1	2	3	4	5	6	7	8	9
Microgomphus souteri (DF6)									
Ictinogomphus rapax (DF1)	0.190								
Ictinogomphus rapax (D1)	0.193	0.015							
Ictinogomphus rapax (ODBOL-1764)	0.188	0.023	0.024						
Ictinogomphus pertinax (RF1856)	0.186	0.091	0.091	0.094					
Ictinogomphus pertinax (RF914)	0.179	0.084	0.087	0.087	0.006				
Ictinogomphus decoratus (IDEC1)	0.190	0.118	0.102	0.113	0.063	0.067			
Ictinogomphus decoratus (RF1488)	0.186	0.111	0.096	0.102	0.060	0.060	0.007		
Ictinogomphus angulosus (DF5)	0.188	0.098	0.078	0.093	0.054	0.054	0.032	0.025	

Discussion

In Odonata, genetic variability among different populations of species based on mitochondrial COI (Fleck *et al.*, 2006; Damm *et al.*, 2010; Low *et al.*, 2017) [4, 7, 15], COII and 16S have been done (Kiyoshi and Sota, 2006; Lee *et al.*, 2010) [13, 14]. Among the conspecifics (Punjab and Maharashtra) and the conspecifics (Punjab and America) the intraspecific divergence is 1.5% and 2.4% respectively for the species *Ictinogomphus rapax*. The high intraspecific divergence between the conspecific *I. rapax* from America with the conspecific of Indian origin shows the geographical isolation and non-overlapping of the range of gene flow (Laurenzeno *et al*. 2016). Among the conspecifics of *I. pertinax* and *I. decoratus* the intraspecific divergence is 0.6% and 0.7%, respectively as both the conspecific of the respective species seen to share same geographical area which led to continuous flow of the genes among the populations. The highest interspecific divergence found between *I. rapax* and *I. decoratus* which is 11.8%, while the lowest 2.5%. found between *I. angulosus* and *I. decoratus*. The mean interspecific divergence found is 13.2% which shows similarity with the interspecific genetic divergence found by Kim *et al*. (2014) [12]; Islam *et al.* (2018) [11]. Species formed distinct clades with their conspecifics with 100% bootstrap value in both the phylogenetic trees, a similar result have been found by Islam *et al.* (2018) [11].

Conclusion

The present study of the genetic variability in the genus *Ictinogomphus* has shown that the different geographical area impeded the gene flow among the populations of species *I. rapax* which led to inheritance of the distinct mitochondrial haplotype through distinct maternal lineage, while the overlapping of the range of the geneflow in case of *I. pertinax and I. decoratus* have shown least genetic variability. Thus, mitochondrial genes COI and 16S rRNA have shown to be good DNA barcode to study the intraspecific and interspecific genetic variability.

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