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A molecular phylogeny of Zygopterans (Insecta, Odonata) of Kerala, India

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ABSTRACT. Molecular phylogenetic reconstruction of the suborder Zygoptera based on sequences of the nuclear ribosomal gene 18S and mitochondrial gene COI was carried out using species collected from India. Sequence samples of 19 species belonging to 7 families of Zygoptera were used for the analysis. All the existing family levels in Zygoptera were confirmed as monophyletic clades in both analyses. While the 18S analysis resolved deep relations well, the COI analyses supported recently diverged clades. The analysis based on the COI gene showed the monophyly of families Coenagrionidae, Calopterygidae, Lestidae, Chlorocyphidae, and Platycnemididae and was found as a distinct clade. The remaining families Platystictidae and Euphaeidae were polyphyletic to the former clade showing more genetic divergence. In the 18S analysis, from the common ancestor, a monophyletic clade of Coenagrionidae, Platycnemididae, Lestidae and Chlorocyphidae evolved. Euphaeidae, Platystictidae and Calopterygidae were polyphyletic.

Keywords: Damselflies, monophyletic, 18S, COI, polyphyletic, taxonomy

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INTRODUCTION

Odonata is the order of primitive winged insects, dragonflies and damselflies, dating from the Permian period. It comprises 3 suborders: Anisoptera (dragonflies), Zygoptera (damselflies) and Anisozygoptera. Anisozygoptera is considered a living fossil and has only three species globally. The phylogeny of Anisoptera has been reasonably well studied and settled (Ware et al., 2007; Fleck et al., 2008). However phylogenetic studies of Zygoptera remain unfinished (Dijkstra et al., 2014). Especially in India, such kinds of studies are very rare. Conventionally morphological information has remained as the basis of odonate taxonomy. Especially wing venation was the focal point of most taxonomic works (Polhemus, 1997; Trueman, 1996; Carle & Kjer, 2002; Rehn, 2003; Bybee et al., 2008). Till the recent past, wing venation was a popular tool for odonate classification, and priority was given to morphological features more than any other sources of data (Fraser, 1957; Hennig et al., 1981; Pfau, 1991; Trueman, 1996). Homoplasy is the main drawback of these characters. The reliability of plesiomorphic traits in classification is not sufficient (Vick, 2000; Dijkstra & Vick, 2006). For this reasons, recent years have reliably followed the results from both morphological and molecular observation with special inferences on the misidentifications triggered by homoplastic traits. The application of molecular techniques in systematics evolved as additional information to increase the accuracy of traditional methods (Kjer et al., 2006; Dijkstra et al., 2007; Ware et al., 2007; Bybee et al., 2008; Carle et al., 2008; Ballare & Ware, 2011).

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The molecular strategies address the limitations allied with the conventional morphological strategies by unveiling the evolutionary relationships between insect taxa. Various molecular markers are used for this according to the nature of the study. Studies conducted by using both nuclear and mitochondrial genes revealed the peculiarities of the former one, such as higher resolution, lesser homoplasy and better bootstrap support than the latter (Brady, 2002; Danforth et al., 2003; Leys et al., 2000, 2002; Morris et al., 2002; Reed & Sperling, 1999). Further studies also supported that nuclear genes are advantageous over mitochondrial genes (Baker et al., 2001; Caterino et al., 2000; Lin & Danforth, 2004). Nuclear genes evolve at a slower rate than mitochondrial genes. Slowly evolving nuclear genes are ideal for the resolution of deeper branches (Hasegawa & Kasuya, 2006; Dumont et al., 2010). The phylogenetic study by combining both nuclear and mitochondrial data has become an ordinary process recently. These two genes have different evolutionary histories and are unlinked too. By comparing the nuclear and mitochondrial sequences, it is possible to study the substitution patterns of both (Lin & Danforth, 2004). However, in certain instances, to follow and maintain the integrity of research, a separate analysis has been implemented for various marker genes by various authors (Otto & Wilson, 2001). Cytochrome oxidase subunit I (COI) gene, is a crucial protein-coding gene in mitochondrial DNA and it is one of the most accepted marker genes for animal species identification for barcoding studies, molecular evolution studies and in analysing inter and intraspecific diversity (Tallei et al., 2017; Caterino et al., 2000; Rodrigues et al., 2017). Even the closely related species can be easily differentiated by the CO1 sequence divergence (Hebert et al., 2003). The nuclear gene 28S and 18S rRNAs are apt for deep branch resolution because of their highly conserved sequences and are also not suitable for species-level discrimination.

Dumont et al. (2005) produced a well-resolved phylogenetic hypothesis of the calopterygoid on a combination of molecular phylogeny using the ribosomal 18S and 5.8S genes and internal transcribed spacers (ITS1, ITS2), geographic analysis and fossil data. The authors tried to find out the phylogenetic relationships and correlate them with geographical and geological data. The study resulted in a strongly supported phylogenetic reconstruction which partially supported traditional taxonomy and denoted patterns of distribution. The odonate family level relationships were well scrutinized by Carle et al. (2008) inferred the families Lestidae and Synlestedae as sisters to other Zygopteran families. Dumont et al. (2010) documented odonate phylogeny using the nuclear ribosomal genes 5.8S, 18S and intergenic spacers ITS1 and ITS2. 18S analysis helped in the resolution of deep relations and has brought Zygoptera and Epiprocta as monophyletic. Hämäläinen et al. (2015) used molecular and morphological methods for the revision of genus *Dysphaea* Selys, 1853. Phylogenetic analysis was done by using three marker genes COI, 16S and 28S rRNA genes. Dijkstra et al. (2014) carried out a vast phylogenetic reconstruction of damselflies including 59% of all the known genera and all families except Hemiphlebiidae by using 16S and COI mitochondrial and 28S nuclear marker genes. A comparative study of traditional and molecular methods of phylogeny was conducted by Huang et al. (2020) to scrutinise the compatibility between the two methods. The mitochondrial COI gene and the nuclear genes 18S, 28S rRNA and ITS were used for the molecular phylogeny of 10 Libellulid species (Banos et al., 2018; Gillespie et al., 2006). The present study focuses on the phylogenetic relationships of seven families of Zygoptera based on COI and 18S rRNA gene sequences.

MATERIAL AND METHODS

Samples of odonates were collected from different habitats of five districts of Kerala, which include Wayanad, Palakkad, Thrissur, Ernakulam and Idukki (Table 1) (Fig. 1). As the odonates can be easily found near water bodies the observations were mainly concentrated in the vicinity of water bodies including forest streams, rivers, ponds, paddy fields, lakes, canals, ditches and estuaries. The field study was continued in all seasons and the locations were randomly selected. Most of the observations were done between 9 AM and 1 PM because the majority of odonates were active during this period. A limited number of observations were done after 5 PM to observe the crepuscular species. The samples were collected using hand-sweeping nets and kept in collection bottles. The samples were identified with the help of photographs, keys and descriptions given in the literature (Fraser, 1933, 1934, 1936; Kiran & Raju, 2013). After identification, the samples were kept in storage vials having 70% ethanol at 0°C temperature in the freezer. The vials were labelled with the scientific name of the species, gender, date and location of the collection.

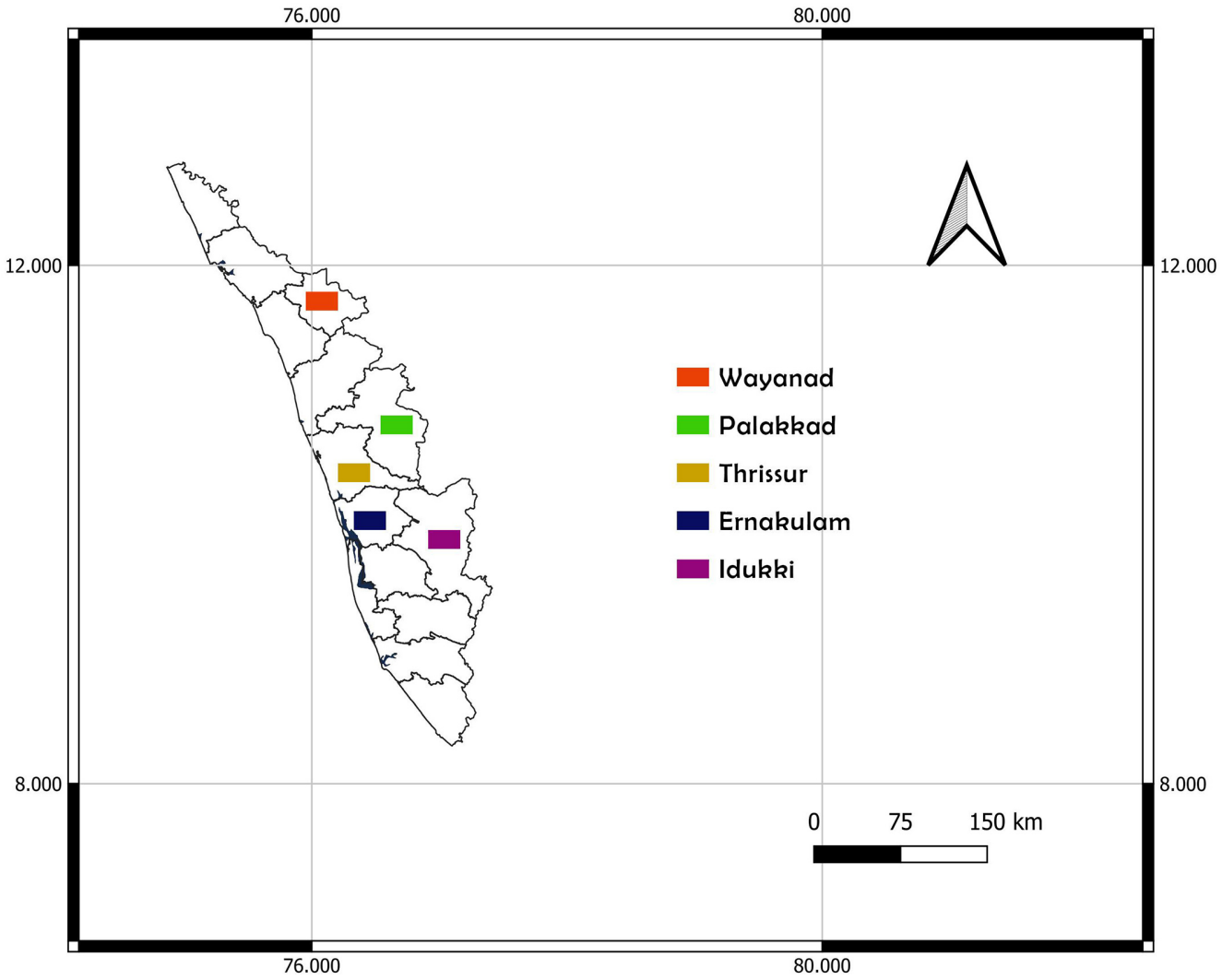

Figure 1. Study sites – Located in five districts (Wayanad, Palakkad, Thrissur, Ernakulam, Idukki) of Kerala state.

Specimens. Nuclear (18S rRNA gene) and mitochondrial (cytochrome c oxidase subunit I, COI) DNA fragments from a total of 19 individuals of suborder Zygoptera were sequenced for this study. Two Anisopteran species were selected as outgroups (Table 2). 3–4 thoracic legs of each specimen of damselfly were collected using forceps. Samples collected from each species were ground separately using mortar and pestle and used for DNA isolation and PCR amplification.

COI and 18S sequencing. DNA was extracted, amplified and purified using standard protocols. Genomic DNA was extracted from legs using the NucleoSpin® Tissue Kit (Macherey-Nagel). The COI gene amplification of the specimens was done using primer LCO (Forward: 5′ GGTCAACAAATCATAAAGA TATTGG 3′) and HCO (Reverse: 5′ TAAACTTCAGGGTGACCAAAAAATCA 3′) (Folmer et al., 1994). Conditions were: first denatured at 98°C for 30s, then 98°C for 5s, 45°C for 10s and 72°C for 15s in 10 cycles and another 30 cycles in which the annealing temperature was 50°C with the final extension step at 72°C for 60s. 18S gene was amplified using the primer pair 1F (Forward: 5′ TACCTGGTTGATCCTGC CAGTAG 3′) and 4R (Reverse: 5′ GAATTACCGCGGCTGCTGG 3′) as described by Giribet et al. (1996) under the following conditions: first denatured at 98°C for 30 s, then 98°C for 5s, 54°C for 10 s and 72°C for 15s for 40 cycles and the final extension step at 72°C for 60s. The PCR products were sequenced commercially (Rajiv Gandhi Centre for Biotechnology, Trivandrum.) by Sangers sequencing technique using an automated DNA sequencer.

Table 1. Study locations with details of coordinates, altitude and types of habitats. The districts they belong to are given in parentheses.

	Localities	Coordinates	Altitude (m a.s.l.)	Habitat type
1.	Chembuthara (Thrissur)	10°33'24.84"N, 76°19'00.84"E	28.956	Rocky stream
2.	Poomala (Thrissur)	10°36'34.56"N, 76°14'02.40"E	110	Dam reservoir
3.	Kanimangalam (Thrissur)	10°29'09.96"N, 76°12'31.68"E	11.00	Pond with vegetation
4.	Kodannur (Thrissur)	10°27'59.40"N, 76°11'03.12"E	12.999	Kole field
5.	Nellayi (Thrissur)	10°23'38.76"N, 76°17'10.68"E	39.93	Pond near the paddy field
6.	Kodungallur (Thrissur)	10°13'39.72"N, 76°11'49.56"E	5.99	Paddy field
7.	Thoomanam (Thrissur)	10°40'04.08"N, 76°16'14.88"E	12.80	Waterfalls
8.	Varantharappilly (Thrissur)	10°25'31.80"N, 76°19'49.44"E	10.97	Stream near rubber plantation
9.	Chettuva (Thrissur)	10°31'27.12"N, 76°02'52.44"E	7.01	Mangrove
10.	Moothakunnam (Ernakulam)	10°11'24.72"N, 76°12'00.72"E	5.23	Ditch near estuary
11.	North Paravur (Ernakulam)	10°08'40.56"N, 76°13'38.28"E	8.05	Pond with shoreline vegetation
12.	Malayattur (Ernakulam)	10°11'43.80"N, 76°29'48.48"E	14.94	River with shoreline plants
13.	Kuruppampady (Ernakulam)	10°06'40.32"N, 76°30'40.32"E	20.11	Vegetated small stream
14.	Cholamala (Wayanad)	11°32'22.92"N, 76°07'00.12"E	800.10	Rocky river
15.	Mundakai (Wayanad)	11°29'15.00"N, 76°09'20.16"E	999.74	Waterfalls
16.	Karapuzha (Wayanad)	11°37'05.16"N, 76°10'51.24"E	800.13	Dam reservoir
17.	Kalladi (Wayanad)	11°30'43.20"N, 76°07'58.80"E	999.84	Rocky stream
18.	Ambalavayal (Wayanad)	11°38'09.60"N, 76°12'13.32"E	973.84	Pond with emergent vegetation
19.	Choondale (Wayanad)	11°34'19.56"N, 76°03'28.80"E	749.81	River with grassy shore
20.	Moopainad (Wayanad)	11°32'09.24"N, 76°10'15.96"E	937.87	Ditch with shoreline plants
21.	Panamaram (Wayanad)	11°44'17.16"N, 76°04'26.40"E	716.89	Paddy field
22.	Kollamkodu (Palakkad)	10°47'42.72"N, 76°39'46.80"E	112.78	Pond
23.	Ottappalam (Palakkad)	10°46'36.12"N, 76°22'33.24"E	53.94	Pond
24.	Puthuppariyaram (Palakkad)	10°51'34.92"N, 76°37'22.44"E	85.95	Waterfalls
25.	Thodupuzha (Idukki)	09°53'42.36"N, 76°43'25.32"E	39.93	Banana plantation
26.	Kappithottam (Idukki)	09°53'21.12"N, 76°43'25.32"E	18.89	Paddy field

Data analysis. After sequencing, the obtained sequences were processed using various bioinformatics tools. The reverse complement of the reverse sequence was generated using the Reverse complement bioinformatic tool. The reverse sequence was used along with the forward sequence in the Emboss merger, which merged two overlapping nucleic acids into one (Bell & Kramvis, 2013). The NCBI Basic Local Alignment Search Tool [BLAST] (Johnson et al., 2008) was used to check the sequence similarity of the resultant sequence with other sequences in the database. The COI sequences generated in this study were translated into amino acid sequences with the aid of the ExPASy (Expert Protein Analysis System) translate tool of the Swiss Institute of Bioinformatics to recognize and identify any premature stop codons lead by sequencing errors. The edited sequences were submitted to GenBank through the submission portal and received accession numbers (Table 2). The 18S ribosomal RNA were aligned using ClustalW (Ouvrard et al., 2000) Gap opening penalty, Gap extension penalty and Delay divergent cutoff were set to 10, 0.5 and 30% respectively. The obtained alignment was manually analysed to ensure accuracy followed by consistency.

Phylogenetic analysis. Multiple sequences were aligned using the tool ClustalW under default parameters. The construction of trees was carried out using the Molecular Evolutionary Genetics Analysis version 11 (MEGA 11) software (Tamura et al. 2021). Model selection was done prior to the tree construction. The model with the lowest BIC (Bayesian Information Criterion) value was considered for tree construction. The tree was constructed based on the Maximum likelihood method (Hasegawa et al. 1991) and the best-fit model by bootstrap analyses over 500 replicates (Felsenstein, 1985).

Table 2. Details of the studied specimens including scientific names and accession numbers of respective sequences in GenBank.

No.	Scientific Name	Accession Numbers	
		COI	18S rRNA
1.	*Lestes praemorsus* Hagen [in Selys], 1862	MZ074000.1	MZ068299.1
2.	*Protosticta gravelyi* Laidlaw, 1915	MN974377.1	MZ882296.1
3.	*Neurobasis chinensis* Linnaeus, 1758	MW931875	MW931850.1
4.	*Heliocypha bisignata* Hagen [in Selys], 1853	MW940786.1	MW940775.1
5.	*Libellago indica* Fraser, 1928	MW309318.1	MZ098271.1
6.	*Dysphaea ethela* Fraser, 1924	MN882704.1	MZ817954.1
7.	*Copera vittata* Selys, 1863	MZ895506.1	MZ895795.1
8.	*Prodasineura verticalis* Selys, 1860	MZ081640.1	MZ081546.1
9.	*Aciagrion approximans krishna* Fraser, 1921	MW246065	MZ098107.1
10.	*Agriocnemis pieris* Laidlaw, 1919	MN850440	OK083599.1
11.	*Agriocnemis splendidissima* Laidlaw, 1919	MN850441	MZ803194.1
12.	*Archibasis oscillans* Selys, 1877	MW309421.1	MZ127377.1
13.	*Ceriagrion cerinorubellum* Brauer, 1865	MZ882339.1	MZ882369.1
14.	*Ceriagrion rubiae* Laidlaw, 1916	OK148120.1	OK105141.1
15.	*Ischnura rubilio* Selys, 1876	MN850442.1	MZ809355.1
16.	*Paracercion calamorum* Ris, 1916	MW940750.1	MZ220521.1
17.	*Paracercion malayanum* Selys, 1876	MZ700177.1	MZ882306.1
18.	*Pseudagrion decorum* Rambur, 1842	MZ254912.1	MZ220525.1
19.	*Pseudagrion indicum* Fraser, 1924	MN882703.1	MZ817953.1
20.	*Orthetrum luzonicum* Brauer, 1868	MZ092847.1	MZ092846.1
21.	*Palpopleura sexmaculata* Fabricius, 1787	OK083552.1	MZ092848.1

RESULTS

Phylogeny of the species belonging to the suborder Zygoptera based on partial COI and 18S rRNA gene sequences were resolved. The analysis involved 19 Zygopteran sequences generated during the present study and a species of suborder Anisoptera as an outgroup. A total of 20 sequences were involved in the analysis.

The analysis based on the COI gene (Fig. 2) showed the monophyly of families Coenagrionidae, Calopterygidae, Lestidae, Chlorocyphidae, and Platycnemididae and was found as a distinct clade. The remaining families Platystictidae and Euphaeidae were polyphyletic to the former clade showing more genetic divergence. Family Coenagrionidae was monophyletic (bootstrap: 95%) and Calopterygidae shared common ancestry with Coenagrionidae but genetically diverged. Chlorocyphidae and Platycnemididae were sister clades and Lestidae was paraphyletic to them. Genera such as *Agriocnemis, Paracercion* and *Ceriagrion* were formed in separate clusters with a bootstrap value of 100. In the 18S analysis result, all the species were grouped into distinct clusters according to the family they belong to (Fig. 3). Species of the family Euphaeidae were found as highly diverged from the common ancestor followed by the family Platystictidae (*Protosticta gravelyi*) and Calopterygidae (*Neurobasis chinensis*). From the common ancestor, a monophyletic clade of Coenagrionidae, Platycnemididae, Lestidae and Chlorocyphidae evolved. Euphaeidae, Platystictidae and Calopterygidae were polyphyletic.

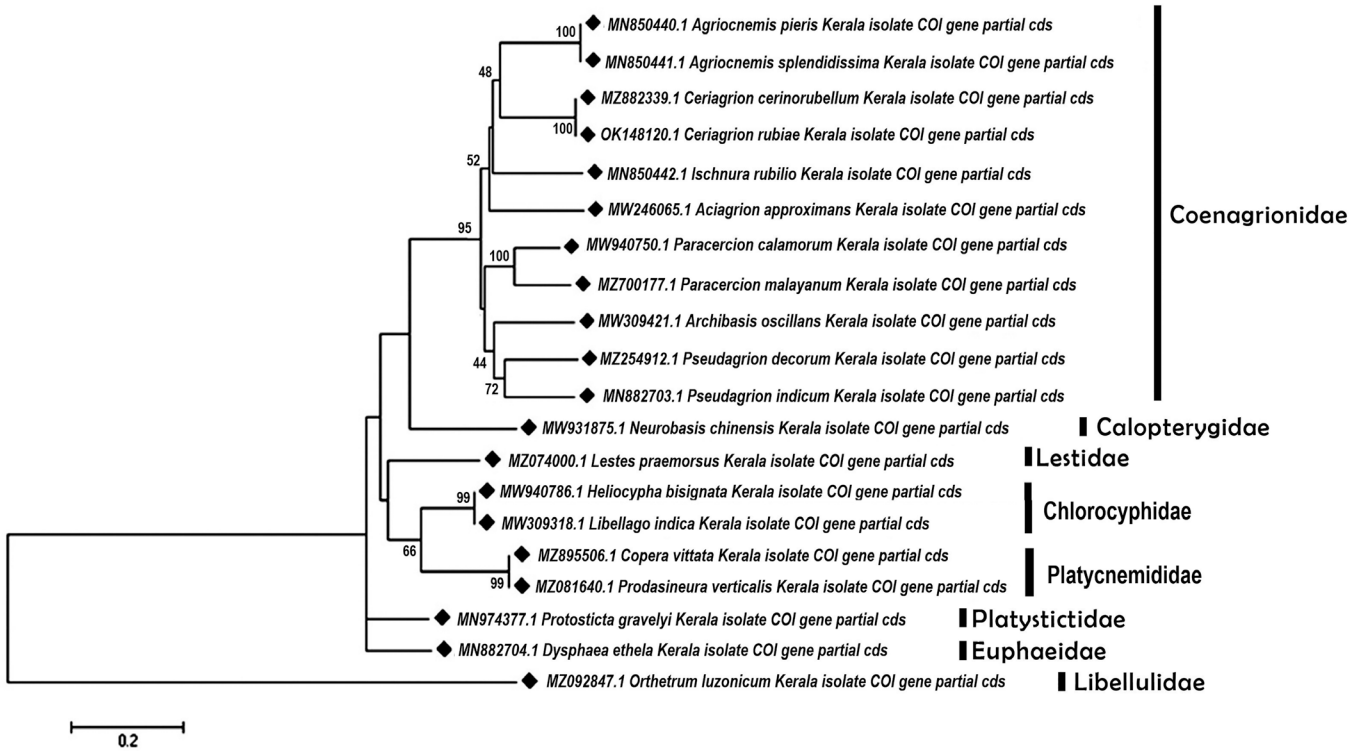

Figure 2. Phylogenetic reconstruction for 19 specimens of Zygoptera and an outgroup taxa based on COI gene sequences. The best Maximum Likelihood tree is shown. Bootstrap values are also displayed.

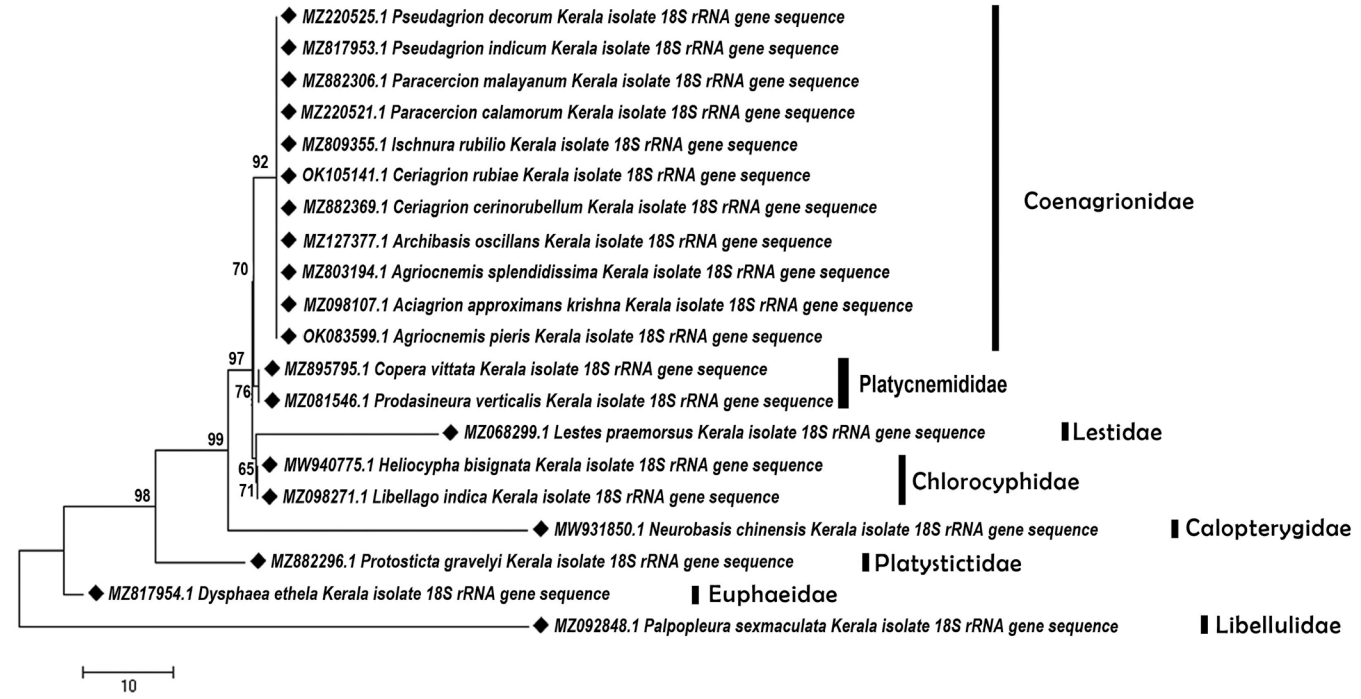

Figure 3. Phylogenetic reconstruction for 19 specimens of Zygoptera and an outgroup taxa based on 18S rRNA gene sequences. The best Maximum Likelihood tree is shown. Bootstrap values are also displayed.

DISCUSSION

The result of the phylogenetic analysis of Zygopteran members strongly supported the monophyly of the family Coenagrionidae by both marker genes (COI - 95% bootstrap and 18S rRNA - 92% bootstrap). The species of the family Platycnemididae clustered together to form a monophyletic clade with 99% (COI) and 76% (18S rDNA) bootstrap support. Both analyses supported the monophyly of Coenagrionidae, Calopterygidae, Lestidae, Chlorocyphidae and Platycnemididae and the polyphyly of Platystictidae and Euphaeidae. In the COI analysis result, the family Platycnemididae and family Chlorocyphidae are sister clades (Bootstrap: 66). In 18S rRNA analysis Chlorocyphidae formed a sister clade with the family Lestidae (Bootstrap: 65) and, Platycnemididae formed a sister clade with Coenagrionidae (97%).

A number of studies pointed out the sister group relationship of the family Lestidae with all other Zygopteran families (Bybee et al., 2008; Carle et al., 2008; Davis et al., 2011; Dumont et al., 2010, Dijkstra et al., 2013). Such a relationship was not observed in the present work. Platystictidae is sister to the remaining families (Bybee et al., 2008, Davis et al., 2011, Van tol et al., 2009, Dijkstra, 2013) however the present result showed that Platystictidae was sister to all other Zygopteran families except Euphaeidae. Both COI and 18S analyses were congruent with the above findings. The monophyly of Calopterygidae, Chlorocyphidae, and Euphaeidae (Bybee et al., 2008; Dumont et al., 2010, Rehn, 2003) was also observed in both analyses. Coenagrionidae was found to be monophyletic. Although Bybee et al. (2008) found this family as non-monophyletic it is because of non-Indian species were included in that study. The genera selected for the current study were found to be monophyletic in Bybee's work too. After a few years, the monophyly of Coenagrionidae was confirmed by Kim et al. (2014) with the help of concatenated mitochondrial and nuclear genes. Both COI and 18S analyses results were congruent in most of the relationships and supported the current taxonomy of Zygoptera which substantiated the efficiency of both in discriminating families level relationships.

AUTHOR'S CONTRIBUTION

The authors confirm their contribution to the paper as follows: N. Bose C.: Data collection, data analysis and interpretation, drafting the manuscript editing and revising; A. Bosewell: Phylogenetic analysis; F.K. Kakkassery: Conception or design of the work, critical revision of article, and editing. All authors read and approved the final version of the manuscript. Unfortunately, Dr. Francy K. Kakkassery passed away during the publishing period.

FUNDING

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AVAILABILITY OF DATA AND MATERIAL

The voucher specimens listed in this study are deposited at the Department of Zoology, St. Thomas' College (Autonomous), Thrissur and are available from the curator, upon request. All the DNA COI and 18S rRNA sequences used in this study are deposited in Genbank.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study only included arthropod material, and all required ethical guidelines for the treatment and use of animals were strictly adhered to in accordance with international, national, and institutional regulations. No human participants were involved in any studies conducted by the authors for this article.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

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تبارشناسی مولکولی سنجاقک‌ها (Insecta, Odonata) کرالا، هند

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چکیـده: تبارشناسی زیرراسته سنجاقک‌ها بر اساس توالی‌های ژن ریبوزومی هسته‌ای 18S و ژن میتوکندریایی COI بررسی شد. نمونه‌های توالی ۱۹ گونه متعلق به ۷ خانواده از سنجاقک‌ها برای تحلیل استفاده شد. تمامی سطوح خانواده موجود در این زیرراسته به عنوان کلادهای تک‌نیایی در هر دو تحلیل تأیید شدند. در حالی که تحلیل 18S روابط عمیق را به خوبی حل کرد، تحلیل‌های بدست آمده از توالی COI وجود کلادهای به تازگی فرگشت‌یافته را تایید کرد. تحلیل مبتنی بر ژن COI تک‌نیایی بودن خانواده‌های Coenagrionidae، Calopterygidae، Lestidae، Chlorocyphidae و Platycnemididae را نشان داده و آنها را به عنوان یک کلاد متمایز مشخص نمود. خانواده‌های باقی‌مانده شامل Platystictidae و Euphaeidae نسبت به کلاد قبلی، چندنیایی بودند و تنوع ژنتیکی بیشتری را نشان دادند. بر اساس تحلیل 18S، از جد مشترک، یک کلاد تک‌نیایی شامل Coenagrionidae، Platycnemididae، Lestidae و Chlorocyphidae فرگشت یافته است. خانواده‌های Euphaeidae، Platystictidae و Calopterygidae چندنیایی بودند.

واژگـان کلیدی: سنجاقک‌ها، تک‌نیایی، 18S، COI، چندنیایی، تاکسونومی