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Phylogenetic Analysis Based on Mitochondrial COI, ND1 and 16S rRNA Genes of Subfamily Ischnurinae Fraser, 1957 (Coenagrionidae: Zygoptera: Odonata)

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Abstract: Flight is the primary mode of locomotion in odonates which requires an adequate source of energy delivered by mitochondria and therefore mitochondrial genes of odonates might have been the target of different evolutionary constraints. Presently, molecular data based on mitochondrial COI, ND1 and 16S rRNA genes of 7 species of subfamily Ischnurinae (Coenagrionidae: Zygoptera: Odonata) have been generated. Phylogenetic analysis of 30 species of subfamily Ischnurinae based on concatenated sequences of COI and 16S rRNA genes in Maximum Likelihood phylogenetic treemethod have been done. The subfamily divided into 8 clades and shows ((((*Ischnura pamelae* + *Ischnura aurora* + *Ischnura pruinescens*) + *Ischnura nursei* + *Ischnura rufostigma* + *Ischnura senegalensis*) + *Ischnura kellicotti* + *Ischnura perparva* + *Ischnura asiatica* + *Ischnura ezoin*) + *Ischnura elegans* + *Ischnura forcipata* + *Ischnura intermedia*) + (*Enallagma circulatum*) + ((*Africallagma elongatum* + *Africallagma vaginale* + *Aciagrion brosseti* + *Azuragrion buchholzi* + *Coenagriocnemis insulare* + *Coenagriocnemis rufipes*) + *Aciagrion pallidum* + *Aciagrion approximans* + *Aciagrion migratum* + *Aciagrion hisopa* + *Aciagrion occidentale* + *Aciagrion borneense* + *Amphiallagma parvum*) + (*Acanthagrion rubrifrons* + *Mesoleptobasis cantralli*) + (*Thaumatagrion funereum*) relationships suggested by high bootstrap values which confirm the deep phylogenetic split. Genetic distances of the species within each clade are lower than the species of the opposite clade which indicate close relationship in the species of same clade than the species of other clades. Combined COI, ND1 and 16S rRNA genes analysis seems to be an effective tool for phylogenetic analysis and delineation of the species.

Keywords: Coenagrionidae, Ischnurinae, Mitochondrial genes, Concatenated sequences, Phylogeny, Genetic divergence

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Introduction

Odonates are one of the first insects to develop flight and looking at how flight works in odonates, the rest of flights can be morph out (Bybee, 2016). Family Coenagrionidae (suborder- Zygoptera) is

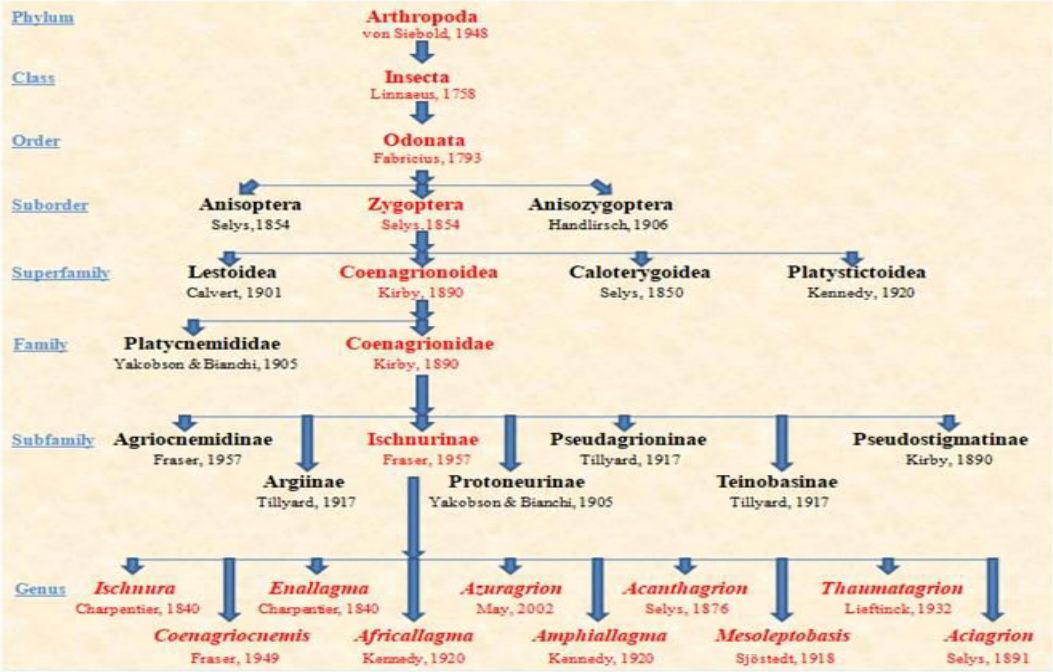

Fig. 1: Classification of subfamily Ischnurinae.

the most abundant among all the damselflies families and occupies a worldwide distribution with 1385 species under 126 genera (Paulson *et al*., 2023) and 61 species under 12 genera are available in India (Subramanian and Babu, 2017; Walia and Dhillon, 2023). This family consists of seven subfamilies Agriocnemidinae Fraser, 1957; Argiinae Tillyard, 1917; Ischnurinae Fraser, 1957; Protoneurinae Yakobson and Bianchi, 1905; Pseudagrioninae Tillyard, 1917; Pseudostigmatinae Kirby, 1890 and Teinobasinae Tillyard, 1917 (Dijkstra *et al*., 2014). Among them, subfamily Ischnurinae constitutes maximum number of genera (41) having 337 species worldwide (Fig. 1). In India, 4 subfamilies (Ischnurinae, Fraser, 1957; Agriocnemidinae Fraser, 1957; Pseudagrioninae Tillyard, 1917 and Teinobasinae Tillyard, 1917) of family Coenagrionidae are present and subfamily Ischnurinae contains 20 species under 4 genera (*Aciagrion* Selys, 1891; *Amphiallagma* Kennedy, 1920; *Enallagma* Charpentier, 1840 and *Ischnura* Charpentier, 1840).

As flight is the primary mode of locomotion in odonates, which requires an adequate source of energy delivered by mitochondria and therefore mitochondrial genes of odonates might have been the target of different evolutionary constraints (Katewa and Ballard, 2007; Yang *et al.*, 2014; Kvist *et al.*, 2015; Salin *et al.*, 2015; Newell *et al.*, 2016). The mitochondrial genome is widely used as a molecular marker for species identification and evolutionary studies due to its small size, maternal inheritance pattern, high evolutionary rate, easy amplification, conserved gene content and lack of recombination (Harrison, 1989). Despite the maximum research based on the mitochondrial CO1 gene, while relatively very less research on concatenated gene sequences for damselflies has been reported. Two protein coding genes (COI and ND1 gene) and one rRNA coding gene (16S rRNA gene) have been selected for the present study.CO1 gene is recognized as perfect barcode for identification of unknown species (Herbert, 2003). ND1 gene also shows better performance in resolving phylogenetic relationships especially in insects such as in Hawaiian drosophilids (Baker and DeSalle, 1997), in aphids (Lin and Danforth, 2004) and odonates (Dijkstra *et al.*, 2007; Rach *et*

al., 2008). Bergmann *et al.* (2013) suggested that combination of COI and ND1 fragments formed a better identification tool for odonate identification than single region alone. Combined analysis of sequences of COI and 16S rRNA genes along with morphological information also resolves the deep phylogenetic relationships for some odonate groups at various taxonomic levels (Brown *et al.*, 2000; Misof *et al.*, 2001, 2002; Ballare and Ware, 2011; Dijkstra *et al.*, 2014; Torres-Pachon *et al.*, 2017).

Molecular analysis based on COI gene of 85 species, ND1 gene of 6 species and 16S rRNA gene of 62 species of subfamily Ischnurinae has been done worldwide. 10 species based on COI gene, 5 species based on ND1 gene and 6 species based on 16S rRNA gene have been reported from India (Futahashi, 2011; Bergmann *et al.*, 2013; Dijkstra *et al.*, 2014, Futahashi, 2014; 2015; Ning *et al.*, 2016: Koroiva *et al.*, 2017; Sikes *et al.*, 2017; Willink *et al.*, 2019; Walia and Dhillon, 2019; 2023). Due to the scarcity of molecular data on the Indian species of subfamily Ischnurinae, the present study has been done for the identification and phylogenetic analysis based on mitochondrial CO1, ND1 and 16S rRNA genes.

Materials and Methods

38 species of family Coenagrionidae were examined: 30 species of the subfamily Ischnurinae and 8 species as outgroup from other subfamilies of family Coenagrionidae (Table 1). Out of 30 species, 6 species were of the present study and rest of the published nucleotide sequences species were downloaded from the GenBank databases. Presently, these steps were followed:

Collection, stretching and preservation of specimens: Specimens of subfamily Ischnurinae were collected by using aerial net from marshy areas, vegetation, lakes, ponds, rivers and streams. Collection of specimens was done in the seasons of pre-monsoon, monsoon, post-monsoon and also from the starting of winters (May to November) from different states of India (Himachal Pradesh, Maharashtra, Punjab, Tamil Nadu). Stretched samples were identified morphologically by consulting "The Fauna of British India" (Fraser, 1933) and "Field guide about Dragonflies of India" (Subramanian, 2009). Specimens of each species were preserved in glass vials containing absolute alcohol in the field and stored in deep freezer at -20 °C in the molecular laboratory of the department until DNA extraction.

Mitochondrial DNA extraction and gel electrophoresis: Extraction of mitochondrial DNA of specimens was done using the methodology given by Kambhampati and Rai (1991). Extracted mitochondrial DNA samples were analysed by gel electrophoresis and bands were visualised under the Gel Documentation Unit.

Amplification and gel electrophoresis: The extracted mitochondrial DNA was used to amplify the regions of three mitochondrial COI, 16S rRNA and ND1 genes by polymerase chain reaction (PCR) in the thermo cycler machine. COI gene was amplified by using forward primer LCO 1490 (5'GGTCAACAAATCATAAAGATATTGG3') and reverse primer HCO 2198 (5'TAAACTTCA GGGTGACCAAAAAATCA3') (Folmer *et al.*, 1994) following PCR thermal profile of initial denaturing at 97°C (5 min), 35 cycles of 1 min denaturing at 95°C, 1 min annealing at 45°C, 1 min extension at 72 °C and final extension 10 min at 72°C. 16S rRNA gene was amplified by using forward primer ODO_12852**(**5'AGAAACCGACCTGGCTTAAA3'**)** and reverse primer ODO_13393 (5'CGCCTGTTTAT CAAAAACAT3') **(**Dijkstra *et al.*, 2014) following PCR thermal profile of initial denaturing at 97°C (3 min), 35 cycles of 1 min denaturing at 94°C, 2 min annealing at 60°C, 40 sec extension at 72 °C and final extension 6 min at 72°C. ND1 gene was amplified by using forward primer CB-J-11545 (5'ACATGAATTGGAGCTCGACCAGT3') and reverse primer N1-N12051 (5'GATTTTGCTGAAGGT GAATCAGA3') (Sanchez-Guillen*et al.* 2011) following PCR thermal profile of initial denaturing at 97°C (5 min), 35 cycles of 1 min. denaturing at 95°C, 1 min annealing at 50°C, 2 min extension at 72 °C and final extension 8 min at 72°C. Amplified

PCR products were run in 1% agarose gel stained and visualized under UV light in BioRad Gel Documentation System.

Sequencing and accession numbers: Amplified regions of three genes were sequenced by Sanger dideoxy sequencing method from Molecular Lab Biologia, New Delhi. The sequences with high-quality electropherograms were chosen for analysis. The sequences were manually aligned and trimmed using MEGA v11's Clustal W algorithm. By reversing the complement of the reverse sequence, the forward and reverse sequences were combined into a contig by aligning their overlapping regions. These sequences of COI, ND1 and 16S rRNA genes of species were sent to the GenBank database to get accession numbers (Table 1).

Genetic variations and Phylogenetic analysis: Mitochondrial sequences of different genes linked together into a series by supergene alignment to make concatenated gene sequences. Two types of gene concatenation-one of three genes (COI, ND1, 16S rRNA) and other of two genes (COI, 16S rRNA) were generated. The sequences of CO1 and 16S rRNA genes of the same individual of particular species were downloaded from NCBI for the preparing concatenated sequences in MEGA v11 (Table I) because there is no sufficient data on ND1 gene for comparison. For this, total 45 concatenated sequences (made from 90 sequences) of 36 species of family Coenagrionidae were aligned properly, which also includes *Argia oculata*, *Argiocnemis rubescens*, *Bromeliagrion rehni*, *Protoneura paucinervis*, *Pseudagrion hamoni*, *Teinobasis cryptica* as outgroup species. Total 13 concatenated sequences (made from 39 sequences) of 13 species of family Coenagrionidae were aligned in the analysis based on three genes (COI, ND1, 16S rRNA) which also includes *Agriocnemis femina* and *Ceriagrion fallax* as outgroup species. For distance-based threshold analyses, genetic divergence within and between clades were analyzed by calculating interspecific and intergeneric genetic distances based on concatenated sequences of CO1 and 16S rRNA genes, using the Kimura 2-parameter (K2P) substitution model in MEGA v11 (Kumar *et al*. 2004).

Results and Discussion

Molecular data: In total 32 accession numbers of 7 species of subfamily Ischnurinae were obtained for COI gene, ND1 and 16S rRNA genes (Table 1). For COI gene 6 species [*Ischnura aurora* (from Harike, Punjab), *Ischnura elegans* (from Dalhousie, Himachal Pradesh), *Ischnura forcipata* (from Andreta, Himachal Pradesh), *Ischnura nursei* (from Harike, Punjab and Nagpur, Maharashtra), *Ischnura rufostigma* (from Andhretta, Himachal Pradesh) and *Ischnura senegalensis* (from Coimbatore, Tamil Nadu)] of genus *Ischnura* were barcoded. Earlier, 5 species of genus *Ischnura* were barcoded from India as *Ischnura aurora* from Patiala, Punjab (Walia and Dhillon, 2019), *Ischnura forcipata* from Chamba, Himachal Pradesh (Dumont, 2013; Dijkstra *et al*., 2014; Walia and Dhillon, 2019), *Ischnura nursei* (Dumont, 2013; Dijkstra *et al*., 2014), *Ischnura rufostigma* from Andhretta, Himachal Pradesh (Walia and Dhillon, 2019) and *Ischnura senegalensis* (Guan *et al*., 2013; Walia and Dhillon, 2019). One species of genus *Amphiallagma parvum* was barcoded from Nagpur, Maharashtra, Earlier, Walia and Dhillon (2023) did the DNA barcoding of *Amphiallagma parvum* (Table 1). For 16S rRNA gene total 11 accession numbers pertaining to 6 species of *Ischnura* genus and one species of *Amphiallagma* were obtained (Table 1). *Ischnura forcipata* is analysed for the first time based on 16S rRNA gene globally. 5 species were analysed for the first time from India and *Ischnura nursei* from Maharashtra species was analysed by Dijkstra *et al*. (2014) based on 16S rRNA gene. For ND1 gene total 7 accession numbers pertaining 5 species of *Ischnura* genus were obtained. *Ischnura forcipata* and *Ischnura nursei* are new to Genbank globally (Table 1). No earlier report is found from India.

Genetic divergence and phylogenetic analysis: The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1890). Initial tree(s)

Table 1: List of 30 species of the subfamily Ischnurinae and 8 species as outgroup from other subfamilies of family Coenagrionidae along with their voucher names, accession numbers based on mitochondrial CO1, ND1 and16S rRNA genes

S. No.	Name of the species	Voucher names	GenBank accession numbers			Locations	References
			CO1 gene	16S rRNA gene	ND1 gene		
1	*Acanthagrion rubrifrons* Leonard, 1977	RMNH.INS.504755	KF369274	KF369592	-	Suriname	Dijkstra *et al.* (2014)
2	*Aciagrion approximans* (Selys, 1876)	261	ON564852	ON740911	ON855034	India	Walia and Dhillon (2023)
3	*Aciagrion borneense* Ris, 1911	RMNH.INS.503596	KF369275	KF369593	-	Malaysia	Dijkstra *et al.* (2014)
4	*Aciagrion brosseti Legrand, 1982*	RMNH.INS.502280	KF369276	KF369594	-	Democratic Republic of the Congo: Orientale	Dijkstra *et al.* (2014)
5	*Aciagrion hisopa* (Selys, 1876)	202	ON564776	ON565820	ON647433	India	Walia and Dhillon (2023)
6	*Aciagrion migratum* (Selys, 1876)	523	ON679599	ON679517	ON855033	India	Walia and Dhillon 2023
		021	MW812349	ON159503	OL743241	India	Walia and Dhillon (2023)
7	*Aciagrion occidentale* Laidlaw, 1919	M6	ON632037	ON678609	ON855032	India	Walia and Dhillon (2023)
8	*Aciagrion pallidum* Selys, 1891	16A	ON748078	ON775347	ON855035	India	Walia and Dhillon (2023)
9	*Aciagrion approximans* (Selys, 1876)	261	ON564852	ON740911	ON855034	India	Walia and Dhillon (2023)
10	*Africallagma elongatum* (Martin, 1907)	RMNH.INS.504230	KF369279	KF369597	-	Tanzania	Dijkstra *et al.* (2014)
11	*Africallagma vaginale* (Sjöstedt, 1917)	RMNH.INS.502448	KF369280	KF369598	-	Gabon	Dijkstra *et al.* (2014)
12	*Amphiallagma parvum* (Selys, 1876)	73	ON545983	ON556411	ON615334	India	Walia and Dhillon (2023)
		205	MT250344	ON678610	-	India	Present study

13	*Azuragrion buchholzi* (Pinhey, 1971)	RMNH.INS.502620	KF369322	KF369644	-	Gabon	Dijkstra *et al*. (2014)
14	*Coenagrion emisrufipes* (Rambur, 1842)	RMNH.INS.504475	KF369348	KF369676	-	Mauritius	Dijkstra *et al*. (2014)
15	*Coenagrion caerulescens* (Fonscolombe, 1838)	Df972_MAR	KP272593	KP272397	-	Portugal	Ferreira *et al.* (2016)
16	*Enallagma circulatum* Selys, 1883	RF887	AB708491	AB707547	-	Japan	Futahashi 2011
17	*Ischnura asiatica* (Brauer, 1865)	RF919	AB708497	AB707553	-	Japan	Futahashi (2011)
18	*Ischnura aurora* (Brauer, 1865)	RF1253	AB708498	AB707554	-	Japan	Futahashi (2011)
		RMNH.INS.504855	KF369414	KF369749	-	Australia	Dijkstra *et al.* (2014)
		BEA548	MK818649	MK874550	-	Sweden	Willink *et al.* (2019)
		dm 9	MG517558	-	-	India	Walia and Dhillon (2019)
			-	MZ148478	-	India	Present Study
		IA	ON679598	ON678550	ON015930	India	Present Study
19	*Ischnura elegans* (Vander Linden, 1820)	ISc2	ON740943	ON740914	ON615329	India	Present Study
20	*Ischnura ezoin* (Asahina, 1952)	RF240	AB708467	AB707523	-	Japan	Futahashi (2011)
21	*Ischnura forcipata* Morton, 1907	dm 52	MT131486	OL657027	OL743242	India	Present Study
		dm 62	ON545814	ON679520	ON615328	India	Present Study
22	*Ischnura intermedia* Dumont, 1974	BEA676	MK818663	MK874566	-	Costa Rica	Willink *et al.* (2019)
23	*Ischnura kellicotti* Williamson, 1898	BEA105	MK818651	MK874565	-	Costa Rica	Willink *et al.* (2019)
24	*Ischnura nursei* Morton, 1907	IN	ON679605	OK560693	-	India	Present Study
		RN	MT516411	ON678549		India	Present Study

25	*Ischnura pamelae* Vick and Davies, 1988	BEA451	MK818650	MK874551	-	Costa Rica	Willink *et al.* (2019)
26	*Ischnura perparva* Selys, 1876	BEA084	MK818654	MK874557	-	Costa Rica	Willink *et al.* (2019)
27	*Ischnura pruinescens* (Tillyard, 1906)	BEA353	MK818635	MK874554	-	Costa Rica	Willink *et al.* (2019)
28	*Ischnura rufostigma* Selys, 1876	RF1875	AB708508	AB707564	-	Japan	Futahashi (2011)
		BEA465	MK818639	MK874538	-	Costa Rica	Willink *et al.* (2019)
		dm 40	ON545812	ON564511	ON647431	India	Present Study
29	*Ischnura senegalensis* (Rambur, 1842)	IS	ON682934	MZ149268	MZ711194	India	Present Study
		MS	MG517561	-	-	India	Walia and Dhillon (2019)
			-	ON565422	ON615327	India	Present Study
30	*Thaumatagrion funereum* Lieftinck, 1932	RMNH.INS.501979	KF369571	KF369932	-	Papua New Guinea	Dijkstra *et al.* (2014)
Outgroup species							
31	*Argia oculata* Hagen in Selys, 1865	RMNH.INS.504761	KF369306	KF369627	-	Suriname	Dijkstra *et al.* (2014)
32	*Agriocnemis femina* (Brauer, 1868)	74	ON545979	ON556413	ON647434	India	Present study
33	*Agriocnemis rubescens* Selys, 1877	RMNH.INS.500068	KF369308	KF369629	-	Malaysia	Dijkstra *et al.* (2014)
34	*Bromelia grionrehni* Garrison, 2005	RMNH.INS.501856	KF369326	KF369648	-	Peru	Dijkstra *et al.* (2014)
35	*Ceriagrion fallax* Ris, 1914		ON564850	ON678552	ON615332		
36	*Mesoleptobasis cantralli* Santos, 1961	RMNH.INS. 501848	KF369440	KF369778	-	Peru	Dijkstra *et al.* (2014)
37	*Protoneura paucinervis* Selys, 1886	RMNH.INS.501963	KF369519	KF369871	-	Peru	Dijkstra *et al.* (2014)
38	*Pseudagrion hamoni* Fraser, 1955	RMNH.INS.500376	KF369524	KF369877	-	South Africa	Dijkstra *et al.* (2014)

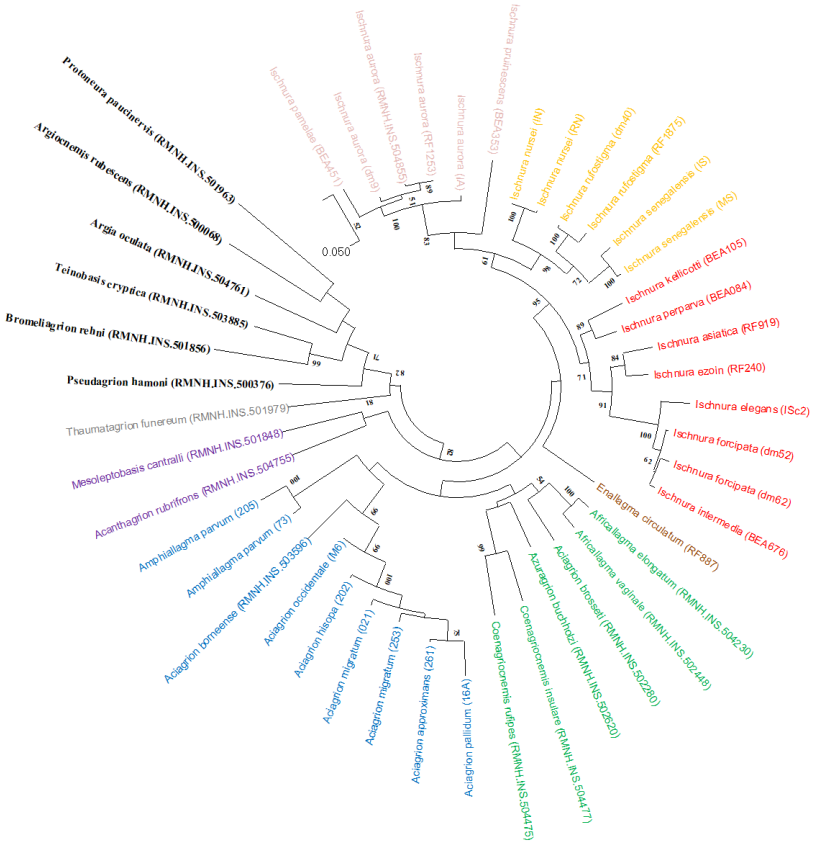

Fig. 2: Phylogenetic tree based on concatenated sequences of CO1 and 16S rRNA genes (with final dataset of 865 bp) for Ischnurinae species by using Maximum Likelihood method. The tree with the highest log likelihood (-11684.65) is shown. Species names with black colour represent outgroup species and species names with different colour represent different clades: clade 1, clade 2, clade 3, clade 4, clade 5, clade 6, clade 7 and clade 8.

for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair-wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) shown above the branches (Felsenstein,1985). Final dataset for phylogenetic trees based on two genes analysis was of 865 bp and based on three genes analysis was of 1315 bp (Figs. 2, 3). In both the phylogenetic trees outgroup species from different subfamilies were significantly separated from all the ingroup species of subfamily Ischnurinae. Conspecific species are recovered at same node. In two genes based analysis, 30 species of subfamily Ischnurinae get clustered into 8 clades comprising 10 genera: *Thaumatagrion* Lieftinck, 1932, *Ischnura* Charpentier, 1840, *Coenagriocnemis* Fraser, 1949, *Enallagma* Charpentier, 1840, *Azuragrion* May, 2002, *Africallagma* Kennedy, 1920, *Aciagrion* Selys, 1891, *Amphiallagma* Kennedy, 1920, *Mesoleptobasis* Sjöstedt, 1918 and *Acanthagrion* Selys, 1876. These 8 clades were-- Clade I comprises- *Ischnura pamelae*, *Ischnura aurora* and *Ischnura pruinescens*; clade II comprises- *Ischnura nursei*, *Ischnura rufostigma* and *Ischnura senegalensis*; clade III comprises- *Ischnura kellicotti*, *Ischnura perparva*, *Ischnura asiatica* and *Ischnura ezoin*; clade IV comprises- *Ischnura elegans*, *Ischnura forcipata* and *Ischnura intermedia*; clade V comprises- *Enallagma circulatum*; clade VI comprises- *Africallagma elongatum*, *Africallagma vaginale, Aciagrion brosseti, Azuragrion buchholzi*, *Coenagriocnemis insulare*, and *Coenagriocnemis rufipes*; clade VII comprises- *Aciagrion pallidum*, *Aciagrion approximans*, *Aciagrion migratum, Aciagrion*

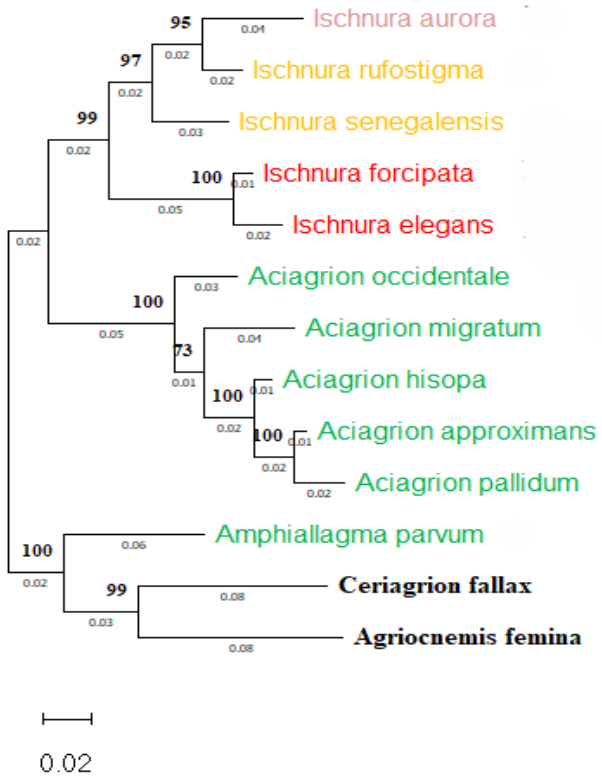

Fig. 3: Phylogenetic tree based on concatenated sequences of COI, ND1 and 16S rRNA genes (with final dataset of 1315 b p) for Ischnurinae species by using Maximum Likelihood method. The tree with the highest log likelihood (-7053.12) is shown. Species names with black colour represent outgroup species and species names with different colour represent different clades: clade 1, clade 2, clade 3 and clade 5.

hisopa, *Aciagrion occidentale*, *Aciagrion borneense* and *Amphiallagma parvum*; clade VIII comprises- *Acanthagrion rubrifrons*, *Mesoleptobasis cantralli and Thaumatagrion funereum.* In subfamily Ischnurinae the overall mean genetic distance is found to be 12.9 % (Table 2). Lowest genetic distance (10.07%) was found between Clade 1 and Clade 2, highest genetic distance (18.25%) was found between Clade 1 and Clade 8. All these clades show highest genetic distance with outgroup species. Species within same clade show low genetic distances which indicate that they are closely related and have a recent common ancestor with high genetic divergence between species of different clades that reflect distant relationships.

All species lies adjacent to their congeneric species except genus *Aciagrion brosseti* which is present in the clade VI closer to the species of genus *Africallagma* than the other species of genus *Aciagrion* (Fig. 2). Interspecific genetic distance of this species shows highest value (14.5%) among the species of genus *Aciagrion.* Sequences of *Aciagrion brosseti* was taken from the GenBank submitted by Dijkstra *et al.* (2014) and they found *Aciagrion brosseti* and species of genus *Africallagma* at same node in their phylogenetic tree based on combined sequences of nuclear (28S rRNA) and mitochondrial (16S rRNA, COI) genes. Therefore, *Aciagrion brosseti* was wrongly identified and this might be belongs to the other genus which needs reclassification. *Amphiallagma parvum* lies close to the species of genus *Aciagrion* in clade VII.

13 species of genus *Ischnura* get clustered into 3 clades with high bootstrap values- first clade is of *I. aurora*, *I. pruinescens* and *I. pamelae* (with 83% high bootstrap value); second is of *I. nursei*,

Table 2: Genetic distances (%) (interspecific and intergeneric genetic distances) among the clades (different groups of species) of subfamily Ischnurinae based on concatenated sequences of CO1 and 16S rRNA genes

Clades	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	Clade 7	Clade 8	Outgroup
Clade 1	**5.10**								
Clade 2	10.07	**5.59**							
Clade 3	11.01	10.53	**6.85**						
Clade 4	12.30	11.81	12.45	-					
Clade 5	13.91	13.62	13.90	12.02	**10.89**				
Clade 6	15.23	13.85	14.19	14.18	13.98	**9.55**			
Clade 7	18.24	15.77	15.91	15.1	15.69	16.31	**15**		
Clade 8	18.25	16.40	17.52	16.8	18.34	19.41	19.0	-	
Outgroup	19.01	18.88	19.59	19.7	19.45	20.36	20.1	20.3	**18.4**

I. rufostigma and *I. senegalensis* (with 98% high bootstrap value) and third is of *I. asiatica*, *I. ezoin*, *I. kellicotti*, *I. perparva*, *I. elegans*, *I. forcipata* and *I. intermedia* (with 71% high bootstrap value), based on combined analysis of two genes (COI, 16S rRNA) using Maximum Likelihood method (Fig. 2). Genetic divergence patterns of the species within each clade is lower (ranges from 5.10% to 6.85%) than the species of the opposite clades (ranges from 10.07 %to 18.3%) which indicate close relationship in the species of same clade than the species of other clades (Table 2). Combined analysis of three genes (COI, ND1, 16S rRNA) also support the position of *I. aurora*, *I. elegans*, *I. forcipata*, *I. rufostigma* and *I. senegalensis* in their respective clades with high bootstrap values (95%-100%) based on Maximum Parsimony methods (Fig. 3).

Earlier, Brown *et al*. (2000) reported close phylogenetic relationship of genera *Enallagma* and *Ischnura* of subfamily Ishnurinae, using mitochondrial markers (COI, COII, intervening Leu-tRNA) based on Maximum Parsimony method. Similarly, in the present study these genera are also found in adjacent clades under the same subfamily. It is found that among the species of genus *Ischnura, Enallagma* (clade IV) lies close to the species of clade III (Fig. 2). But according to genetic divergence values clade IV shows lower value with clade II (11.81%) than with clade III (12.45%).

Conclusion

Presently sequence data of species of subfamily Ischnurinae has been generated for studying the diversity, their identification and phylogenetic analysis. In phylogenetic trees, 30 species get clustered into 8 distinct clades with high bootstarp values based on maximum likelihood method. High bootstrap values in phylogenetic trees and

between clades divergences confirm the deep phylogenetic split, which is the strong indication of independent radiations and differences in radiation events may be related to different habitat adaptations of species in these clades. Combined COI, ND1 and 16S rRNA gene analysis seems to be an effective tool for phylogenetic analysis and delineation of the species.

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Conflict of Interest

The authors declare no conflicts of interest.

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