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MITOCHONDRIAL COI GENE BARCODING OF FOUR SPECIES OF GENUS *ISCHNURA* (ODONATA: COENAGRIONIDAE) FROM INDIA

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ABSTRACT: Taxonomically, identification of complex taxa based on external morphological characters may leads to wrong identification of unknown organism at species level because these characters may also be altered due to geographical and seasonal variations. To overcome this molecular taxonomy has been emerged. In India, out of 9 morphologically described species of genus *Ischnura* (Odonata: Coenagrionidae), *Ischnura senegalensis* is the only species, which has been barcoded on the basis of COI gene. During the present study, mitochondrial COI gene has been barcoded for four species of genus *Ischnura* (*Ischnura aurora*, *Ischnura forcipata*, *Ischnura rufostigma* and *Ischnura senegalensis*), collected from fresh water bodies from the states of Punjab and Himachal Pradesh (India). Across the final alignment of COI sequences of 352bp, there are 282 conserved sites (80.1%), which represents that COI gene is highly conserved. Conspecific species show <1% divergence range (0% to 0.6%) and interspecific divergence ranges from 7.5% to 15.6%. COI gene sequences of all the species shows 95 – 100% similarity with the sequences of conspecific species deposited in GeneBank. Mitochondrial COI gene based DNA barcoding has been done for *Ischnura aurora*, *Ischnura forcipata* and *Ischnura rufostigma* for the first time from India and for *Ischnura forcipata* throughout the world.

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KEYWORDS: Odonata; *Ischnura*; Mitochondrial COI gene; Barcoding; Genetic Divergence.

INTRODUCTION

DNA barcoding is a valuable method for identification of biological samples. Mitochondrial DNA is used as molecular marker due to its maternal inheritance and lack of recombination. In mitochondrial genome, cytochrome c oxidase subunit I gene is taken as DNA barcode because it encode highly conserved protein (cytochrome c oxidase, subunit 1, complex IV) in animals, if any change in this protein occurs, it would adversely affect the organism because this protein involved in oxidative respiration. This gene has been taken because of following advantages: - COI gene is easily amplified by using universal primers HCO 2198 and LCO 1490[10]. 658-bp region known as Folmer region of COI gene recognised as a perfect barcode[14]. CO1 gene has enough mutation rate in the nucleotide sequences, which is responsible for large variation between species but very little amount of variation occurs within species[22]. Average value of sequence divergence between conspecifics is 0.25% and between congeneric is 6.8% and for more distantly related taxa this value is high[14].

COI gene based DNA barcoding and phylogenetic relationships of approximately 211 species of family Coenagrionidae have been done worldwide[1,2,3,5,9,13,17,20,24,25,29,33,34].

However, out of 60 Indian coenagrionid species, COI gene based DNA barcoding have been done only for 5 species. These are *Ceriagrion cerinorubellum*, *Ceriagrion coromandalian* and *Ischnura senegalensis*[13] ,*Rhodischnura nursei*[5] and *Aciagrion occidentale*[18].

Damselflies of genus *Ischnura* of family Coenogrionidae are also known as forktails damselflies because males have a forked projection at the tip of the abdomen. Globally, genus *Ischnura* includes 72 species[30], while 9 species are present in India[32]. *Ischnura senegalensis* is the only Indian species, which has been studied on the basis of COI gene[13]. In the present study, mitochondrial COI gene based DNA barcoding of four species of genus- *Ischnura* (*Ischnura aurora, Ischnura forcipata, Ischnura rufostigma* and *Ischnura senegalensis*) have been carried out.

MATERIALS AND METHODS

For the present study, four species of genus *Ischnura* (*Ischnura aurora, Ischnura forcipata, Ischnura rufostigma* and *Ischnura senegalensis*) were collected from different localities of Punjab and Himachal Pradesh (India) areas (Table –1), by using sweep net from ponds vegetation and marshy areas of running water. Stretched samples were identified morphologically by consulting "The Fauna of British India including Ceylon and Burma" Volume I[11] and "Field guide about Dragonflies of India" by Subramanian[31].

Table 1: Collection data of species

S. No.	Name of species	Dates of collections	Places visited
1.	*Ischnura aurora* Brauer, 1865	06-04-2017	Village- Ugani Sahib, Rajpura, Patial (Punjab)
2.	*Ischnura forcipata* Morton, 1907	09-6-2017	Chamba (Himachal Pradesh)
3.	*Ischnura rufostigma* Selys, 1876	30-6-2017	Andhretta (Himachal Pradesh)
4.	*Ischnura senegalensis* (Rambur, 1842)	18-05-2017	Ranuka Ji (Himachal Pradesh)

Molecular Work

Extraction of Mitochondrial DNA, Amplification and Sequencing of COI gene segment:-Extraction of mitochondrial DNA is done by following the method given by Kambhampti and Rai[19] Agarose gel electrophoresis was used to check the integrity of extracted DNA. PCR

amplifications were done with a total volume of 25 µl, containing 8.5 µl PCR Water, 12.5ul Master Mix, 1ul each primer-LCO-1490 and HCO-2198[10], 1 µl BSA and 1µl DNA. Thermal cycling program of PCR procedure is- 1 cycle for initial denaturation at 95º C for 5 minutes; 35 cycles for denaturation at 95º C for 1 minute, annealing at 50º C for 1 minute, extension at 72º C for 90 seconds;1 cycle for final extension at 72º C for 7 minutes. All PCR products were visualized *via* 1% agarose gel electrophoresis with ethedium bromide (EtBr) staining, under UV light using Gel Documentation system to confirm successful DNA amplification. Amplified products were sequenced from Yaazh Genomics, Bangalore by using Sanger dideoxy sequencing method.

Sequence alignment, Accession numbers, Genetic distance analysis and Phylogenetic Analysis:- Corresponding COI sequences of conspecific specimens deposited by other workers were downloaded from Gene Bank by Blast search. All the sequences were aligned, edited and trimmed manually by using Clustal W in MEGA v7 software. Accession numbers for all the four species were attained. Interspecific and intraspecific genetic distances, Conserved, variable and parsimony informative sites of COI gene fragment of four species of genus *Ischnura* were recovered, using MEGA v7. Phylogenetic tree was prepared by using Neighbor-joining method based on K2P distances in MEGA7 for these four species and *Ceriagrion cerinorubellum* is taken as outgroup species.

RESULTS AND DISCUSSION

Sequence Length:-

Table- 2 Length of COI gene

S.No.	Species	Accession Number	Sequence Length
1.	*Ischnura aurora* Brauer, 1865	MG517558	657-bp
2.	*Ischnura forcipata* Morton, 1907	MH183146	575-bp
3.	*Ischnura rufostigma* Selys, 1876	MG517560	565-bp
4.	*Ischnura senegalensis* (Rambur, 1842)	MG517561	657-bp

COI gene sequence fragment has been compared with available GenBank-registered COI gene sequence fragment through Blast search to verify the accuracy of sequences.

Genetic divergence:-For distance-based threshold analyses, mean distances of CO1 sequences within and among species have been calculated, using the Kimura 2-parameter (K2P) substitution model in

MEGA 7[21].

Table- 3 Estimates of evolutionary divergence between COI gene sequences of *Ischnura* species

Species	1	2	3	4	5	6	7	8
1. MG517561 *Ischnura senegalensis*								
2. MF358817 *Ischnura senegalensis*	0.000							
3. MG517558 *Ischnura aurora*	0.117	0.117						
4. KT957493 *Ischnura aurora*	0.117	0.117	0.000					
5. MH183146 *Ischnura forcipata*	0.148	0.148	0.156	0.156				
6. LC198678 *Ischnura forcipata*	0.148	0.148	0.156	0.156	0.003			
7. MG517560 *Ischnura rufostigma*	0.075	0.075	0.114	0.114	0.148	0.148		
8. KX263696 *Ischnura rufostigma*	0.079	0.079	0.117	0.117	0.152	0.152	0.006	

i. **Intraspecific divergence of species** Conspecifics show <1% divergence and divergence ranges from 0% to 0.6%.

ii. **Interspecific divergence of species** Interspecific K2P divergence over COI sequences pairs ranges from 7.5% to 15.6%.

Nucleotide base composition

Percentage value of nucleotide bases of sequenced fragment of COI gene are:-

T = 35.8%, C = 17.0%, A = 30.7%, G = 16.5% for *Ischnura aurora*;

T = 34.9 %, C = 16.8 %, A = 31.0 %, G = 17.3 % for *Ischnura rufostigma*;

T = 33.2 %, C = 19.0%, A = 29.8%, G = 17.9% for *Ischnura forcipata*;

T = 35.2 %, C = 16.8 %, A = 30.4 %, G = 17.6% for *Ischnura senegalensis*.

Mean percentage value of nucleotides bases of sequenced fragment of COI gene are: - G=17.3%, C=17.4%, A=30.5% and T=34.8%.

Conserved, variable and parsimony informative sites

Across the final alignment of COI sequences of 352bp, there are 282 conserved sites (80.1%), 70 variable sites (19.8%) and 68 parsimony informative sites (19.3%) in the dataset, which confirms that COI gene is highly conserved.

Phylogenetic Analysis

Neighbor-joining tree based on K2P distances has been reconstructed for all the four species and for outgroup species *Ceriagrion cerinorubellum*. Data includes nine sequences of COI gene fragment including outgroup species. Final dataset contains 352 positions. It has been studied by bootstrap consensus, N-J tree inferred

from 1000 replicates.

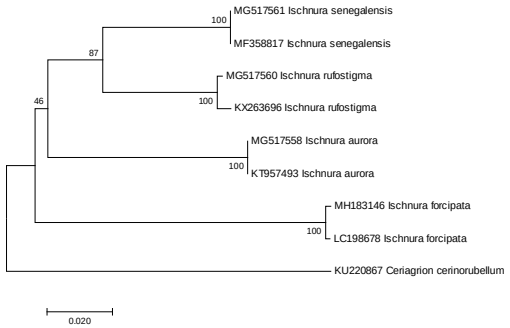

Figure 1. Phylogenetic tree based on COI gene fragment for four species of genus *Ischnura* by using Neighbor-joining method.

Presently, DNA barcoding of mitochondrial COI gene fragment of four species of genus *Ischnura* (*Ischnura aurora*, *Ischnura forcipata*, *Ischnura rufostigma*, *Ischnura senegalensis*) of the family Coenagrionidae from different areas of Punjab and Himachal Pradesh has been done. Conserved, variable and parsimony informative sites have been calculated for the first time for these species.

Length of COI gene barcode

COI sequences greater than 500-bp, considered as standard barcode according to the Barcode of Life Data System (BOLD)[27]. In present study, **657-bp** length of COI gene fragment of *Ischnura aurora* is used, as same base pair length has been analysed for the same species by Dijkstra *et al.*[5]; Ning *et al.*[24]; Ramage *et al.*[26], while only 451-bp length has been used by Futahashi[12].

565-bp length of COI gene fragment of *Ischnura rufostigma* is analysed during the present investigation, while for the same species, 451-bp has been used by Futahashi[12] and 550-bp by Ning *et al.*[24].

Presently, **657-bp** length of COI gene fragment of *Ischnura senegalensis* is used, as same base pair length studied for the same species by Dijkstra *et al.*[5]. Moreover many authors have investigated the different base pair length of COI gene for this species as 451-bp[12], 541-bp[1], 1147-bp[20] and 428-bp[16].

575-bp length of COI gene fragment of *Ischnura forcipata* is analysed for the first time during the present study.

Interspecific and Intraspecific divergence (Table- 3)

Out of these four *Ischnura* species, highest value of intraspeciûc divergence is observed for *Ischnura rufostigma* (0.6%). Interspecific divergence over COI sequences pairs, ranges from 7.5% to 15.6% among the *Ischnura* species. Moreover, lowest distance values are noted between *Ischnura rufostigma* and *Ischnura senegalensis* and highest distance values are recorded in *Ischnura aurora* and *Ischnura forcipata.* Interspecific and intraspecific genetic divergence based on COI gene fragment have been calculated for the first time for *Ischnura aurora*, *Ischnura rufostigma* and *Ischnura forcipata*, while results of *Ischnura senegalensis* are in accordance to the earlier report given on the same species from Namibia by Bergmann *et al.*[1].

Nucleotide composition

High percentage of A and T bases in

COI gene sequences are found in all the four species, which is the characteristic feature of insect mtDNA[23]. A nucleotide base content is found to be highest at first codon position and its average value is 51.1%. G content is being lowest in studied COI fragment at first codon position and its average value is 4.1%. Among the four species, *Ischnura aurora* is having G=1.7% only. T, C, A, G nucleotide contents at third position are same for all the samples, which are 44.4%, 28.2%, 11.1% and 16.2%, respectively. Nucleotide base composition of COI gene fragment has been calculated for the first time in all these species.

Phylogenetic analysis

Mostly, branches of tree are well supported with bootstrap values ranges from 87%-100% except 46% value for one node. The outgroup *Ceriagrion cerinorubellum* is significantly separated from all the four species of genus *Ischnura*.

Phylogenetic analysis based on tree represents that ***Ischnura aurora*** shares cluster with *Ischnura rufostigma* and *Ischnura senegalensis* but occurs at different node as earlier reported by Dumont *et al*.[7]; Dijkstra *et al*.[5] and Ning *et al*.[24] based on Bayesian analysis.

Ischnura rufostigma shares cluster with *Ischnura senegalensis* and *Ischnura aurora* and also shows close relationship to *Ischnura senegalensis* than to *Ischnura aurora* as observed by Ning *et al*.[24] based on Bayesian inference.

Ischnura senegalensis shares cluster with *Ischnura rufostigma* and *Ischnura aurora* and shows close relationship to *Ischnura rufostigma* than to *Ischnura aurora* as analysed by Dijkstra *et al*.[5] and Ning *et al*.[24] based on Bayesian analysis.

Ischnura forcipata is highly separated from other three *Ischnura* species (*Ischnura aurora*, *Ischnura senegalensis* and *Ischnura rufostigma*). Moreover, these results are in accordance with earlier study based on nuclear genes-ITS1, ITS2, 18S rRNA and 5.8S rRNA genes on the same species by Dumont *et al*.[7]. Relationship of *Ischnura forcipata* based on COI gene marker has been done for the first time.

All the presently studied species shows 95 – 100% similarity in the sequences of conspecifics species deposited in GeneBank by other workers in the world. This study determined that COI gene based DNA barcoding is most favorable tool for identification and phylogenetic analysis of species, genus and family.

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