Research & Reviews: Journal of Zoological Sciences

e-ISSN:2321-6190
p-ISSN:2347-2294

Genetic Characterization of Three Species of the Genus *Cornudiscoides* Kulkarni, 1969 (Monogenoidea: Dactylogyridae), Parasitizing Long Whiskered Cat Fish *Sperata aor* (Ham) Using Ribosomal and Mitochondrial DNA

Verma Jyoti, Rajvanshi Saroj and Agrawal Nirupama*

Department of Zoology, University of Lucknow, Lucknow, Uttar Pradesh, India

Research Article

Received date: 15/04/2018
Accepted date: 06/06/2018
Published date: 08/06/2018

***For Correspondence**

Agrawal Nirupama, Department of Zoology, University of Lucknow, Lucknow, Uttar Pradesh, India,
Tel: +915222740230.

E-mail: jyotiaug22@gmail.com

Keywords: *Cornudiscoides longicirrus*, *Cornudiscoides aori*, *Cornudiscoides mystusi*, *Sperata*, 28S and 18S ribosomal subunits, Protein structures, Polymerase chain reaction, Phylogeny

ABSTRACT

Three congeneric species of the genus *Cornudiscoides* Kulkarni, 1969 (*Monogenoidea: Dactylogyridae*) viz. *C. longicirrus*, *C. aori* and *C. mystusi*, parasitizing gills of *Sperata aor* were examined. These morphologically valid species were subjected to molecular analysis, using partial 28S, 18S rDNA and Mitochondrial COI gene to characterize them genetically. 28S and 18S ribosomal markers are reliable and useful tools to trace the evolutionary linkage at generic/specific level. Comparative study of 28S and 18S showed close relation of these species with other *Cornudiscoides sp*. While, mt COI based structural proteomics (1D/2D/3D) confirms species differentiation. Apart from molecular characterization, current study strengthens earlier morphological validation of these three species of *Cornudiscoides*

INTRODUCTION

Genus *Cornudiscoides* Kulkarni, 1969 (Monogenoidea: Dactylogyridae) was considered as oioxenus (strictly host specific/host specialist) monogenoid, parasitize only species of *Mystus Scopoli*. Transfer of some fish species of *Mystus* under the genus *Sperata* Holly, 1939 has changed status of the genus *Cornudiscoides* to metasteoxenous (sub-category) of category mesoxenous that parastize on gills of more than one genus of a family [1]. So far, sixteen nominal species of *Cornudiscoides* are reported in India [1] thirteen parasitizing on *Mystus spp*. and three on *Sperata sp*. (earlier under the genus *Mystus*). *S. aor* harbors three oioxenous congeneric species, *C. mystusi* Dubey et al. *C. longicirrus* Agrawal et al. and *C. aori* Agrawal et al. *Sperata aor* is commercially important freshwater cat fish [2] having significant food value [3,4]. The host harboring *Cornudiscoides* ranged from India, Nepal, Sri Lanka, Bangladesh, Myanmar Thailand, Indochina, Peninsular Malaysia, Singapore, Syria and East Indies to China [3] while the genus *Cornudiscoides* is reported only from India, Pakistan, Sri Lanka and Peninsular Malaysia [1,5].

In the present paper, we have used 28S and 18S ribosomal DNA to characterize the *Cornudiscoides* species and to evaluate relationship using phylogenetic analysis as ribosomal DNA is an effective molecular marker for molecular analysis due to availability of large number of copies and presence of highly conserved to variable regions [6,7]. Some pioneer workers have already reported importance of 28S ribosomal DNA in molecular taxonomy and systematic placement of helminthes parasites [8,9] while some reported, 18S ribosomal DNA is more instructive to explain relationship among them Mitochondrial COI based 1D, 2D and 3D (primary, secondary and tertiary) protein conformations have also been utilized herein for species differentiation. Beside

e-ISSN:2321-6190
p-ISSN:2347-2294

these, we are attempting for genetic validation of *Cornudiscoides* multi-species complex parasitizing *S. aor* that substantiate morphological study.

MATERIALS AND METHODS

Collection and Identification

Live fish were procured from river Gomati and local fish markets of Lucknow (26°8'N 80°9'E), Uttar Pradesh, India. Gills were placed in Petri dish with tap water and examined for monogenoideans under binocular. Parasites were placed in a drop of water on slide under the cover slip and identified using "An Encyclopedia of Indian *Monogenoidea*" by Pandey and Agrawal under Phase Contrast Microscope (Olympus BX-51, Tokyo, Japan). Measurements and drawings of sclerotized parts of query *Cornudiscoides* spp. (**Tables 1-4**) are taken from their original descriptions [1] and present study.

Table 1. Details of gene, primers and their sources.

Gene	Primer Name	Sequence 5' to 3'	Source
28S rDNA	Forward primer (Ancy 55)	GAGATTAGCCCATCACCGAAGG	Plaisance et al.
	Reverse primer (LSU 1200 R)	GCATAGTTCACCATCTTTCGG	
18S rDNA	Forward primer (Worm A)	ACGAATGGCTCATTAAATCAG	Plaisance et al.
	Reverse primer (Worm B)	TTGCTGCACTCTTCATC	
Mt COI	Forward primer (Asmit1)	TTTTTTGGGCATCCTGAGGTTTAT	Chaabane et al.
	Reverse primer (Asmit2)	TAAAGAAAGAACATAATGAAAATG	

Table 2. List of parasite (obtained/retrieved from GenBank), their hosts, base pair length and their accession number.

Monogenoideans	Host Species	Sequence Length (bp)			GenBank No.		COI
		28S	18S	COI	28S	18S	
C. longicirrus	*Sperata aor*	993	1030	393	KY009858	KY01892	Submitted
C. aori	*Sperata aor*	998	928	395	KY009859	KY018928	Submitted
C. mystusi	*Sperata aor*	1000	1109	395	KY091690	KY091691	Submitted
C. proximus	*M. vittatus*	362	600	-	GQ925913.1	KU235550	
Thaparocleidus susanae	-	324	-	-	-	KC962228.1	-
T. aori	-	328	-	-	-	KC962227.1	-
Euryhaliotrematoides sp.1	-	-	1752	-	-	EU836217.1	-
Euryhaliotrema sp. 3	-	-	1645	-	-	EU836215.1	-

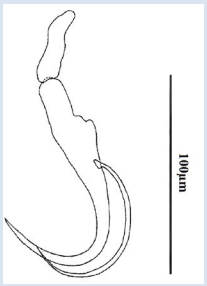
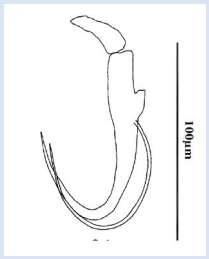
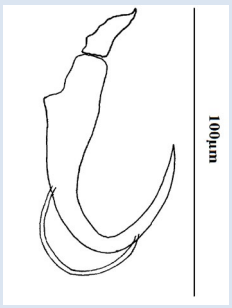
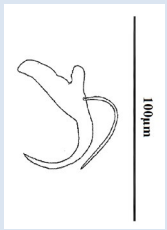
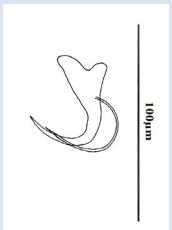
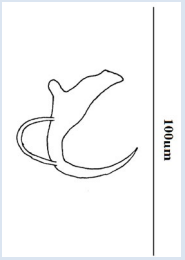
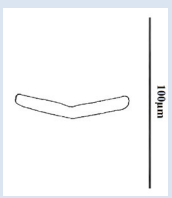
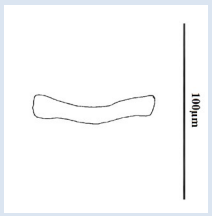
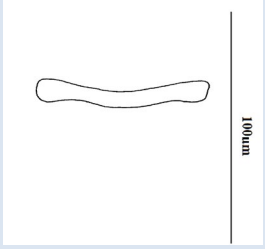
Table 3. Measurements of three species of *Cornudiscoides*, parasitizing *Sperta aor.*

Characters	*C. longicirrus*	*C. aori*	*C. mystusi*
	Agrawal et al. (µm)	Agrawal et al. (µm)	Present Study (µm)
Body			
Length	329-765	387-568	314-475
Width	112-147	82-110	60-74
Pharynx			
Diameter	30-45	23-29	25-30
Haptor			
Length	132-140	59-112	58-60
Width	94-99	89-105	55-65
Dorsal Anchor			
Total length	-	-	-
Inner length	44-48	36-38	34-38
Outer length	34-40	28-33	29-35
Recurve Point	23-28	32-26	21-24
Patch	20-24	12-15	11-14

e-ISSN:2321-6190
p-ISSN:2347-2294

Ventral Anchor			
Total length	-	-	-
Inner length	27-30	21-24	21-24
Outer length	22-25	12-19	22-25
Recurve Point	14-16	13-15	20-24
Dorsal bar	29-42	28-33	30-34
Ventral bar	68-114	76-112	31-34
Ovary			
Length	71-96	45-72	50-56
Width	55-70	30-46	55-60
Testes			
Length	79-96	51-61	71-74
Width	52-62	34-46	73-76
Hooks			
Large hooks	24-28	17-22	24-26
Small hooks	12-14	11-12	13-14
Copulatory complex			
Copulatory tube	71-96	48-45	86-89
Accessory piece	32-40	21-24	28-30
Vagina			
Length	31-42	31-42	40-50

Table 4. Hard parts of *C. longicirrus, C. aori* and *C. mystusi*, as drawn in original description.

Characteristics	*Cornudiscoides longicirrus*	*Cornudiscoides aori*	*Cornudiscoides mystusi*
Dorsal anchor			
Ventral anchor			
Dorsal bar			

e-ISSN:2321-6190
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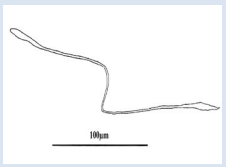
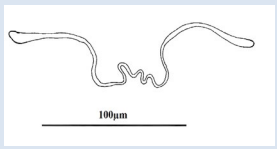
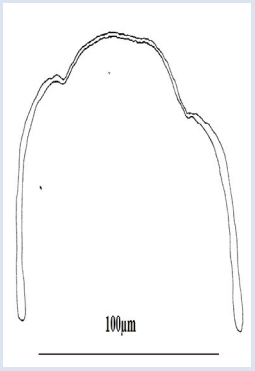
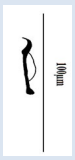
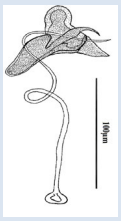
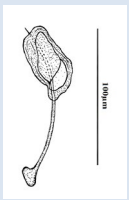
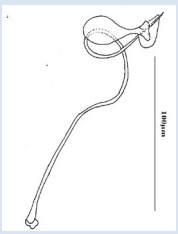
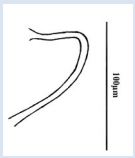
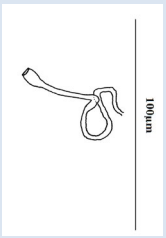
Ventral bar			
Large hook			
Small hook			
Copulatory complex			
Vaginal armature			

Table 5. Characteristic features of protein data set with template.

S. No.	Parasite Name (Target)	Number of Amino Acids	Molecular Weight	Positively/Negatively Charged Amino Acid	Best hit Template	Query/Confidence Coverage/Percent Identity RMSD value
1	*C. aori*	126	14848.31	9/4	d12asa	34%/16.4%/42%/0.000Å
2	*C. longicirrus*	120	14055.50	14/6	c2e5zA	17%/21.7%/30%/0.000Å
3	*C. mystusi*	125	15148.36	5/9	d1scfc	22%/24.4%/30%/0.000Å

Parasites were stored in absolute alcohol for molecular analyses. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per manufacturer's protocol. Partial 28S rDNA, 18S rDNA and Mt COI regions were amplified using primers mentioned in **Table 1.** The polymerase chain reaction (PCR) amplifications were performed in a final volume of 12.5 µl, containing 10× buffer (100 mM Tris, pH 9.0), 50 mM KCl, 15 mM $MgCl_2$, 2.5 U Taq polymerase enzyme, 10 mM of each deoxynucleotide triphosphate (dNTP's) and 3 µl DNA. For 28S and 18S, following thermo cycling profile was utilized: denaturation of DNA (94°C for 3 min.); 35 cycles of amplification (94°C for 30s, 52°C for 30s and 72°C for 2 min); 10 min extension and hold at 72°C. For COI, denaturation of DNA (95°C for 5 min.); 35 cycle of amplification (94°C for 1 min. 50°C for 1 min. and 72°C for 1 min.); 6 min extension and hold at 72°C. PCR products were visualized on 2% Agarose gel in TAE (Tris–acetic acid–EDTA) buffer,

e-ISSN:2321-6190
p-ISSN:2347-2294

stained with Ethidium Bromide (EtBr) under ultraviolet (UV) light. The purified PCR products were used for sequencing. Sequencing was done by Amnion Biosciences, Bangalore using an automated sequencer (Model Name 3130×1/3130x/GA-1203-019). The obtained nucleotide sequences (partial 28S and 18S rDNA) of *Cornudiscoides spp*. were used for phylogenetic analysis along with published sequences, retrieved from GenBank (**Table 2**).

Sequence Analysis

Blastn was performed to search sequence similarity of newly obtained sequences of partial 28S and 18S rDNA regions. Number of nucleotide (ATGC) was calculated through https://www.sciencebuddies.org. A phylogenetic analysis was performed using MEGA 6.06 [10] and trees were generated through Neighbor-joining NJ and Minimum Evolution (ME) methods at 1000 bootstrap value. Translation of nucleotide sequences of COI gene was done by Emboss transeq tool version 6.6.0 of Expasy [11,12]. All asterisks were omitted from primary protein dataset (1D/amino acid string) of *C. longicirrus*, *C. aori* and *C. mystusi*. ExPASy ProtParam was used to predict statistical values of amino acid (**Table 5**) sequence [13]. 3D conformation of 1D data set was generated by Threading method (sequence that have query coverage below 80%) using HMM-HMM (Hidden Markov Model) that facilitate searching of homologous template for target protein [14]. The target-template complexes were predicted by Chimera 1.10.1 [15,16]. Target and template protein conformations were annotated with different colors (*C. aori*, *C. longicirrus* and *C. mystusi* were coded with red, green and magenta while template was cyan blue for all species). Root mean square values (RMSD) were calculated to estimate sequence divergence between target and template protein sequence [15,16].

RESULTS

Molecular Study

The amplicon length of different molecular markers for *C. longicirrus*, *C. aori* and *C. mystus*i were 993, 988 and 1000 base pairs for large ribosomal subunit; 1030, 928 and 1109 base pairs for small ribosomal subunit and 393, 395 and 419 for COI. Blastn search for large subunit and small subunit of ribosomal DNA of these three species reveals highest sequence similarity with *C. proximus*. The estimated G+C contents value of *C. longicirrus*, *C. aori* and *C. mystusi* was 45.9%, 45.3%, 46.7% for 28S rDNA; 46.9% 48.4% 46.7% for 18S rDNA and 34%, 31.3% and 32.6% for mt DNA respectively. Small ribosomal subunit has more variability in nucleotide composition than large subunit as it is less conserved in comparison to 28S rDNA region. Clustal Omega based alignment showed, 985 base pair (bp) alignment sites for 28S rDNA region, of which 137 sites are substitution (128 parsimony informative sites and 9 parsimony-uninformative sites) and 7 sites are insertions/deletions. 18S rDNA region showed 886 bp alignment sites having 76 substitutions (74 parsimony informative sites, 2 parsimony-uninformative) and 19 insertions/deletions.

Phylogenetic Analysis

Six newly obtained and other retrieved sequences (GenBank) were used to reconstruct the phylogenetic trees (NJ and ME methods) for two different regions (partial 28S and 18S rDNA) to evaluate genetic relativeness of these three species (present study) with other *Cornudiscoides* species (**Figures 1 and 2**). All phylogenetic trees depicted well-supported bootstrap values and similar evolutionary pattern for query species used in study (**Figures 1 and 2**). The *Cornudiscoides* species of *Sperata aor* clustered together, forming a sister group with *C. proximus* (parasite of *Mystus* species) and a separate clade with *Thaparocleidus sp*. (**Figure 1**) and Euryhaliotrematoides sp. (**Figure 2**). Where Thaparocleidus and Euryhaliotrematoides are treated as out-group. In 28S phylogenetic tree *C. aori* and *C. longicirrus* are more closely related than *C. mystusi*, forming a subgroup with *C. proximus*. However, 18S region based tree showed close liaison between *C. aori* and C. *mystus*i than *C. longicirrus*.

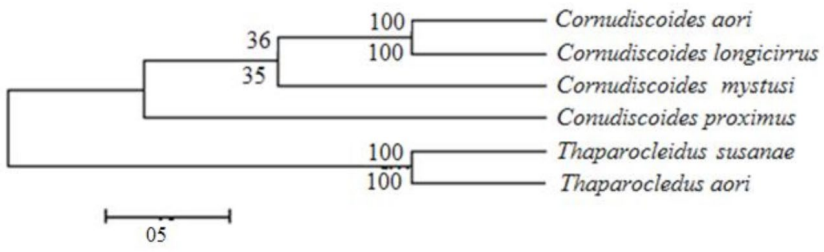


Figure 1

Figure 1. Tree topology found with partial 28S rDNA data using NJ/ME/UPGMA methods with bootstrap values for NJ and ME method (shown above and below branches).

e-ISSN:2321-6190
p-ISSN:2347-2294

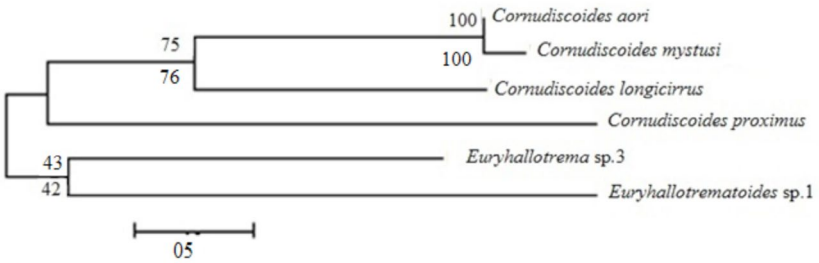


Figure 2

Figure 2. Tree topology found with partial 18S rDNA data using NJ/ME/UPGMA methods with bootstrap values for NJ and ME method (shown above and below branches).

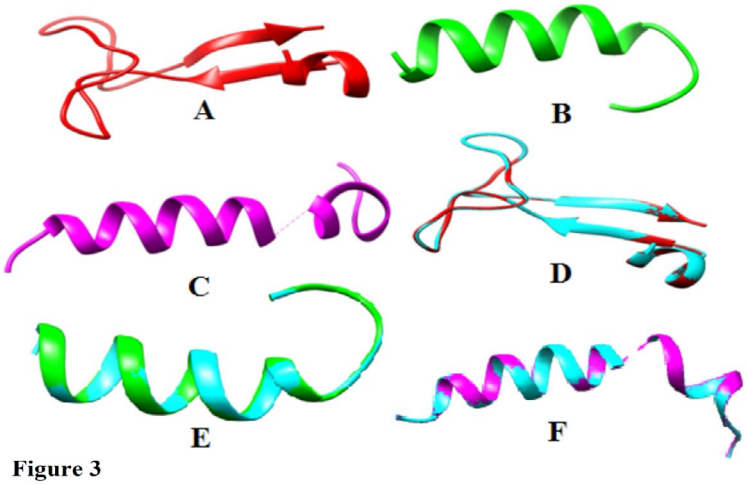


Figure 3

Figure 3. (A) 3D conformation of *C. aori* (B) *C. longicirrus* (C) *C. mystusi* (D) target-template complex of *C. aori* (E) *C. longicirrus* (F) *C. mystusi.*

3D Conformation of Protein of Cornudisoides spp.

Each query species comprises unique amino acid composition (primary data set) suggesting species variation at primary level (**Table 5**). However, main characteristic features of 2D conformation (disordered, a-helix, Beta-strand and TM helix) are also peculiar for all three query species. *C. aori* has 7%, 41%, 31% and 13%, disordered, a-helix, Beta-strand and TM helix which is 8%, 98%, 0% and 65% for C. *mystusi*. Howbeit, 2D conformation of *C. longicirrus* has only disordered (16%), alpha helix (45%) and beta strand (18%). Tertiary structures of all three *Cornudiscoides spp*. are also different from each other (**Figure 3A-3C**) evincing species differentiation. Nevertheless, species dissimilarity, the superimposed target-template complexes, predicted by structural alignment of target and template protein suggests structural identity/similarity between both protein sequences. Identical sequences of target protein get annotated with same colour as that of template. Similar/non-match sequences remain in their original colour (**Figure 3D and 3F**). *C. aori*, *C. longicirrus* and *C. mystusi*, all three have 0.00 Å RMSD value with template respectively (**Table 5**). While structural superimposition of all three *Cornudiscoides spp*. have 0.633 Å RMSD value insinuating sequence divergence among these three species. 3D proteomic structure based genomic linkage showed more or less similar conformation for *C. longicirrus* and *C. mystusi* while *C. aori* conforms into a different structure. These structural resemblances are also visible in sclerotized parts of *Cornudiscoides* species (**Table 4**). *C. longicirrus* and *C. mystusi* have almost same morphological features (reproductive parts long with coil/twist) rather than *C. aori* (short, simple). Ramachandran plot values exhibiting, 87.2% amino acid in favoured region, 5.1% in allowed and 7.7% in outlier region for *C. aori*, which is 100%, 0.0%, 0.0% and 94.4%, 5.6%, 0.0% for *C. mystus*i and *C. longicirrus* respectively. These values indicating that *C. mystusi* and *C. longicirrus* have stable and good conformation while *C. aori* has stable and average hypothetical protein structure.

e-ISSN:2321-6190
p-ISSN:2347-2294

DISCUSSION

Agrawal et al. morphologically validated three monogenoidean species collected from *Sperata* namely *C. longcirrus*, *C. aori* and *C. mystusi*, based on morphology of reproductive parts (key characters of species differentiation). Earlier parasitological documents have already demonstrated that reproductive parts of monogenoid provide species level identification [17] while haptoral parts are of generic importance. Here, molecular tools have been employed to complement morphology based species identification, genetic characterization and systematic placement of these three species of *Cornudiscoides*. The PCR-based DNA sequencing proves worthy for species identification of closely related congeneric parasites [18]. The ribosomal DNA of monogenoids is still being used expeditiously to evaluate phylogenetic relationship at family and subfamiy level [19-21]. Ribosomal DNA based analysis is truly useful for evidencing intra and inter-specific variations among *Cornudiscoides* species as it evolves with varying rates from highly conserved (18S, 5.8S and 28S) to variable (transcribed and non-transcribed or IGS) regions [22] and provide easy alignment for taxa [23] that is useful for genetic characterization of species. Present work evidences, comparative study of 28S and 18S regions of ribosomal DNA reveals considerable sequence similarity at genetic level and difference at specific level.

The Phylograms for 28S and 18S ribosomal DNA region depicted same topology and display clear-cut relationship among them. The evolutionary linkage of *Cornudiscoides* species suggests, evolution of *Cornudiscoides* spp. parasitizing *Mystus* is prior to those infesting *Sperata* as it was already shown in earlier documents [24]. Lakra et al. and it reveals co-speciation of host and parasite. If parasite demonstrates phenomenon of host switching, phylogenetic congruence between parasite-host will be imperfect and parasite based host speciation cannot be traced [25-31].

COI based protein conformations are efficient to unwind the complexity among the parasite community. The mitochondrial genome database has made it accessible across the world that facilitates global identification system and no doubt compensate with all deficits of morphology based species differentiation. The intra/interspecific biodiversity will be easily predictable using mitochondrial region, specifically for genome modifications under geographical changes, sibling species, hybridized forms, erroneous placements, misidentification and validation of taxonomic establishments. Thus, sequence based genetic identification system brings basic and modern biology together to substantiate taxonomic research which is a ground of all modern science and technology. Here, involvement of translated mitochondrial DNA sequence based biological identification is used that offers outstanding ways to identify and distinguish closely related species of multi-species complex as *Cornudiscoides* (validated by Agrawal et al.). The protein coding regions of mt COI gene chiefly affects morphological features of species [18]. These morphological differentiations are species specific. The proteomic Insilco studies are seemingly valuable for intra/inter specific differentiation and validation of species among parasitic community. Thus, computer based study of mitochondrial sequences showing expeditious ways to predict genetic variations at varying level. Primary information (1D dataset/amino acid sequence) suggests genetic variability among these three *Cornudiscoides sp*. These variations express into completely different 2D/3D protein conformations. The obtained results of 1D/2D/3D conformations assisting genetic validation of three query species of multi-species complex. The 3D protein conformations of all species have completely different set of characters, explaining species variation and offering instant view to differentiate congeneric species. The information rich data based structural proteomics of query species suggests 100% correct identification and differentiation. Our observations support earlier placement of these three query species under *Cornudiscoides* as they are genetically allied to each other which is also reflected by sclerotized parts of monogenoids.

CONCLUSIONS

Molecular biology seemingly corroborates morphological identification of three query species as *C. longicirrus*, *C. aori* and *C. mystusi* (consanguineous of other *Cornudiscoides spp*.) and suggesting genetic distinction. The use of COI additionally strengthens genetic linkage and differentiation of query species. We are at the opinion, the value and robustness of any classification scheme, lower to higher taxonomic categories; rely upon basic concept of species identification.

ACKNOWLEDGEMENTS

This work was financially supported by University Grant Commission, India to Jyoti Verma [F25-1/2013-14 (BSR)/7-109/2007] and Nirupama Agrawal [F-410/2010] and [F.6-6/2017-18/Emeritus-2017-18-Gen-9293/ (SA-II)].

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e-ISSN:2321-6190
p-ISSN:2347-2294

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