Molecular characterization of Indian species of the genus *Cornudiscoides* Kulkarni, 1969 (Monogenoidea: Dactylogyridae)

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**Abstract**

Molecular characterization and phylogenetic study based on partial sequences of 28S and 18S ribosomal DNA (rDNA) of sixteen Indian species of the genus *Cornudiscoides* (Monogenoidea: Dactylogyridae) were conducted to decode the genetic relationship between them and with other members of the family Dactylogyridae. Blastn searches disclosed the significant similarity among the species of the *Cornudiscoides* for large ribosomal subunits as well as for small ribosomal subunit showing genetic relatedness. The phylogenetic tree using neighbour-joining (NJ) and minimum evolution (ME) methods for 28S ribosomal subunit depicted that all *Cornudiscoides* species clustering in a single clade and forming sister clade with other members of the family Dactylogyridae and similar results were obtained from 18S ribosomal subunit. Thus, the present study demonstrated that both 28S and 18S ribosomal subunits are very helpful in discriminating *Cornudiscoides* species (intra or interspecific variation) and in the establishment of the evolutionary relationship among them.

**Keywords:** *Cornudiscoides*, Dactylogyridae, Large and small ribosomal subunit, Maximum likelihood methods, Neighbour-joining, Phylogenetic analysis

**INTRODUCTION**

Systematics is a branch of science that described the world’s biodiversity and its interrelationships (Van Steenberge et al., 2015). Traditionally, taxonomy-based only morphological characters, but, given the constraints of morphology-based strategies in discriminating between cryptic species or decoding the variations of intraspecific variations, an integrative approach of taxonomy has been proposed (Dayrat et al., 2005). According to Schlick-Steiner et al. (2010) at least three impartial datasets: morphology, nuclear DNA and other supporting proof from another discipline should be used.

Hard parts, i.e. haptor and male and female copulatory complex, are the main diagnostic features in monogenoideans taxonomy. Changes in the environmental conditions like locality and geographic distribution of host and age of parasites, the hard parts may exhibit variation in their structure (Agrawal et al., 2020). Thus, morphology is alone not sufficient in identifying species. Eukaryotic genes encoding ribosomal RNA (rRNA) is not only used as an effective taxonomic tool but recognized as a potential target for species identification, differentiation and phylogenetic analysis of helminths parasites. The 28S and 18S ribosomal DNA (rDNA) fragments particularly lend themselves to study as they provide sequences along with constant sites that permit multiple alignments among or between homologues, and variable sites that give phylogenetic information (Hillis and Dixon, 1991). In the recent era of molecular taxonomy, the ribosomal RNA (typical nucleic acid) frequently targeted for sequencing in eukaryotes and prokaryotes both (Olsen and Woese, 1993).

Kulkarni (1969) erected the genus *Cornudiscoides* at Hyderabad and established three species *C. heterotylus* Kulkarni, 1969 (type species), *C. microtylus* Kulkarni, 1969 and *C. megalorchis* Kulkarni, 1969 from *Mystus tengara*. To date, 16 species of the genus *Cornudiscoides* have been described and are distributed throughout South East Asia (Agrawal et al., 2016). Earlier reports showed that large ribosomal subunit...
(Mollaret et al., 1997 and 2000; Olson and Littlewood, 2002; and Verma et al., 2017) and small ribosomal (Matejusová et al., 2001, and Verma et al., 2017) useful to resolve relationships among monogeneoidean parasites. In this study, twelve known species namely C. heterotylus Kulkarni, 1996; C. mystusi (Rizvi, 1971) Dubey et al., 1992; C. proximus Gusev, 1976; C. geminus Gusev, 1976; C. agarwali Agrawal and Vishwakarma, 1996; C. bleekeri Agrawal and Vishwakarma, 1996; C. gussei Agrawal and Vishwakarma, 1996; C. susanai Agrawal and Vishwakarma, 1996; C. tukarami Agrawal and Vishwakarma, 1996; C. sclerosvaginals Devek and Pandey, 2007; C. longicirrus Agrawal et al., 2016; C. aori Agrawal et al., 2016 and four new species C. tripathi n. sp., C. speratai n. sp., C. indicus n. sp. and C. falcatum n. sp. were sequenced and partial sequences of the 28S and 18S ribosomal DNA were used to infer the relationships among the species of the genus Cornudiscoides and with the other members of the family Dactylogyridae.

MATERIALS AND METHODS

Collection of parasites

The hosts (commonly available freshwater food fishes for which ethical clearance is not required) were caught from River Gomati (Lucknow), Sai River (Lucknow), Manorama River (Basti), Betwa River (Jhansi), Amdhanpur Taal (Basti), Ramgarh Taal (Gorakhpur), and from the ponds of Mati (Barabanki) were examined Since January 2014 to January 2018. Collections were also made from the fish markets of Lucknow like Kaiserbagh, Daliganj, Dubbaga; Malihabad; Gonda, Sitapur, Hyderabad (Telangana) and Vizag (Andhra Pradesh). The live hosts were kept in plastic containers, aerated with battery operated aerators, brought to the laboratory, and maintained in glass aquaria. Fishes were identified with the help of Fish base (Froese and Pauly, 2014-2018), and Jayaram (1955). The gills of fishes were excised and were examined for the monogeneoids. Living worms were studied under a phase-contrast microscope (Olympus CX41, Tokyo, Japan). The monogeneoids were identified with the help of Pandey and Agrawal (2008). The gills infected with monogeneoid parasites were fixed in 3% formalin diluted with lukewarm water, temporary slides (glycerine mounts) and permanent slides were prepared for morphometric study. The method for staining, mounting and illustration of parasites done according to Kristisky et al. (1986). For the molecular study, the gills were stored in 100% ethanol.

Molecular analysis

DNA (genomic) of the parasite (Cornudiscoides) was extracted from ethanol-preserved specimens using DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to standard methods (Verma et al., 2018). A Partial region of 28S and 18S ribosomal DNA were amplified with the help of polymerase chain reaction (PCR) using the primers (Table 1) and reaction mixture prepared according to Verma et al., 2016 (Table 2). The thermal cycle started with 3 min at 94°C for initial denaturation; followed by 35 cycles of 30 s at 94°C, 30 s at 52°C for annealing, and 2min at 72°C (extension); and a final extension at 72°C for 10min followed by cooling at 4°C. C. PCR products were examined on 1% agarose gel, stained with ethidium bromide and visualized on a gel documentation system. Sequencing was carried out according to Verma et al. (2017).

Blastn (Basic Local Alignment Search Tool) was performed for partial sequences of both 28S and 18S rDNA to uncover the degree of resemblance between species of the genus Cornudiscoides. ATGC calculator was used to evaluating the nucleotide composition among different species of the genus Cornudiscoides in this study. Sequences of the different species of the genus Cornudiscoides for 28S and 18S ribosomal subunit were amplified and sequenced and analysed with neighbour-joining and maximum likelihood methods. Sequences were reconstructed using Bioedit prior to analysis. Molecular evolutionary genetics analysis MEGA7 version 7.0 (Kumar et al., 2016), is software designed to infer phylogenetic relationship and pattern of evolution of nucleic acid and protein.

RESULTS AND DISCUSSION

Molecular characterization

The partial sequences of 28S and 18S rDNA genes were used to assess the genetic differentiation of sixteen species of Cornudiscoides and their phylogenetic relationship among other groups of monogeneoidean parasites. The large and small ribosomal subunits are extremely useful to explain the phylogeny of monogeneoideans at the level of family and subfamily (Šimková et al., 2006) and the nucleotide sequences of monogeneoideans have sufficient phylogenetic information to decode the relationship among them (Cunningham et al., 2000).

Sixteen newly generated sequences ranged from 327-1041 base pair long for large ribosomal subunit and 483-1109 base pair long for small ribosomal subunit (Table 4, 5), were obtained. All the sequences are submitted to the GenBank, National centre for biotechnology information (NCBI) and their accession numbers are given in Table 3. Blastn searches performed for 28S region depicted that most of the members showed the highest similarity with C. agarwali (Table 4) while Blastn search for 18S region, showed similarity with C. susanai (Table 5). The distribution of ATGC among different Cornudiscoides species deviated from species to species for both ribo-
Phylogenetic analysis was conducted among the members of the family Dactylogyridae for 28S and 18S ribosomal subunits. Newly obtained sequences along with twenty sequences belonging to different subfamilies members of the family Dactylogyridae, retrieved from GenBank, were used to evaluate their phylogenetic relationship. Evolutionary analyses were conducted in MEGA7 version 7.0 software (Kumar et al., 2016), using neighbour-Joining (NJ) and Minimum Evolution (ME) methods. Substitution, including transitions, transversions, gaps and missing data, were decimated. In the analyses, the codon positions (first, second and third) and non-coding sites were also included. The nodal values were estimated by bootstrapping (n=1000). Phylogenetic analysis based on these two methods produced identical tree topologies with the branch length 0.10 and 0.05 for 28S and 18S. The evolutionary distances were estimated with the help of the p-distance method.

However, the phylogenetic tree generated from both 28S and 18S rDNA region depicted that the members of the family Dactylogyridae are separated on the basis of subfamilies i.e. members of subfamily Ancylodiscoidinae, Ancyrocephalinae, Pseudodactylogyridinae and Dactylogyridinae. They clustered separately, forming sister clades, with each other and separate clade with a member of Sundanonchidae for 28S rDNA region (Fig. 1, 2). In the clade of Ancylodiscoidinae, all the species of *Cornudiscoides* clustered together and formed sister clade with *Thaparocleidus* spp. It is worth to note that, in the phylogenetic analysis of partial sequences of 28S and 18S ribosomal units, similar results were found, showing genetic relatedness among *Cornudiscoides* spp. while the separation of others reflects inter-specific/ intergeneric dissimilarities.

The species of the genus *Cornudiscoides* are chiefly differentiated by structure of their hard parts (haptor and genital armature) (Agrawal et al., 2020). The molecular study is conducted to complement morphometric analysis. Genetic distance portrays a degree of heterogeneity in the genetic constitution of taxa, therefore, becomes an ideal systematic tool (Fergusson, 2002). The ribosomal DNA of monogenoideans was used to evaluate phylogenetic relationship at the level of families and subfamilies (Plaisance et al., 2005; Šimková et al., 2003, 2006). In the identification of closely related parasites, PCR-based DNA sequencing technology provides an alternative approach (Nolan and Cribb, 2005). Nucleotide sequences of monogenoideans have sufficient phylogenetic information to decode the relationship among them (Cunningham et al., 2000).

For the first time, the attempt has been taken for comprehensive analysis of sixteen species of *Cornudiscoides*, using 28S and 18S rDNA partial sequences to show the genetic relatedness among them. Different sets of primers amplified different range of 327 to 1041 base pairs for 28S region and 483 to 1109 base
pairs for 18S region. The Blastn search disclosed the obscure relationship of these parasites with other species and showed the highest similarity with Cornudiscoides spp. for large as well as for small ribosomal subunit (Table 4 and 5).

The dispersion of ATGC bases (Table 4 and 5) for the two regions among Cornudiscoides spp. found deviated from species to species and exhibit clear-cut interspecific differences.

Among several sequences used in phylogenetic/cladistic analysis, it was found that rDNA is extremely useful in evidencing intra and inter-specific variations among parasitic species since they evolve with varying rates from extremely conserved (18S, 5.8S and 28S) to extremely variable (transcribed and non-transcribed or IGS) regions (Hillis and Dixon 1991). Comparative study of 28S and 18S regions of ribosomal DNA reveals considerable sequence similarity as well as differences to define relativeness and phylogenetic relationship. These subunits have a wide range of phylogenetic utili-
ty (high sequence similarity with the highly variable region) providing an easy alignment between taxa (Littlewood et al., 1998) and are useful in genetic characterization of species.

In the computed phylogenetic tree for 28S and 18S regions, the species of Cornudiscoides, formed a different clade with other subfamily members of the family Dactylogyridae. All Cornudiscoides species clustered together in a single clade, forming sister clade with Thaparoleidus spp. showing a close relationship

**Table 4.** Distribution of A, T, G, C contents % of GC and amplicon size along with blastn similarity of 28S rDNA of Cornudiscoides species.

| Cornudiscoides spp. | Adenine (A) | Thymine (T) | Guanine (G) | Cytosine (C) | GC (%) | Amplicon size (bp) | Blast similarity |
|---------------------|-------------|-------------|-------------|--------------|--------|--------------------|------------------|
| C. heterotylus      | 154         | 212         | 169         | 114          | 43.6   | 649                | 96.00% with C. susanai |
| C. mystusi          | 238         | 295         | 269         | 198          | 46.7   | 1000               | 92.24% with C. aori |
| C. proximus         | 220         | 274         | 236         | 156          | 44.2   | 886                | 95.30% with C. agarwali |
| C. geminus          | 216         | 266         | 238         | 153          | 44.8   | 873                | 97.41% with C. susanai |
| C. agarwali         | 255         | 295         | 257         | 182          | 44.4   | 989                | 97.78% with C. agarwali |
| C. tukarami         | 269         | 297         | 283         | 192          | 46.6   | 1041               | 95.34% with C. agarwali |
| C. bleekerai        | 89          | 76          | 93          | 69           | 49.5   | 327                | 96.59% with C. agarwali |
| C. susanai          | 190         | 239         | 185         | 126          | 42     | 740                | 96.00% with C. agarwali |
| C. sclerovaginalis  | 170         | 226         | 187         | 125          | 44.1   | 708                | 98.72% with C. aori |
| C. longicirrus      | 285         | 262         | 279         | 209          | 46.9   | 1030               | 92.23% with C. agarwali |
| C. aori             | 246         | 294         | 265         | 183          | 45.3   | 988                | 99.72% with C. sclerovaginalis |
| C. tripathii        | 178         | 228         | 206         | 147          | 46.5   | 759                | 98.05% with C. geminus |
| C. speratai         | 161         | 219         | 177         | 122          | 44     | 679                | 99.47% with C. species LW |
| C. indicus          | 252         | 283         | 260         | 182          | 45.2   | 977                | 92.25% with C. Proximus |
| C. falcatum         | 209         | 279         | 240         | 175          | 46     | 903                | 98.92% with C. longicirrus |
with each other and confirming the distinction from other Dactylogyrids. Thus, the tree topologies further confirm our preliminary results.

Conclusion

As in previous molecular studies conducted on monogenean parasites, results showed that the 18S gene is a better potential marker than the 28S gene (Blair and Barker, 1993; Cunningham et al., 1995; Zhu et al., 1998; Matejusová et al., 2001) but in the present study, we have found both the markers useful for the characterization of parasites. It is, therefore concluded that molecular markers strongly demonstrate the genetic delineation and confirms the validation of C. heterotylus, C. mystusi, C. proximus, C. geminus, C. agarwali, C. tukarami, C. bleekerai, C. susanai, C. gussevi, C. sclerovaginalis, C. longicirrus, C. aori, C. tripthii n. sp., C. indicus n. sp., C. speratai n. sp. and C. falcatum n. sp. Thus, molecular study substantiated the morphological identity of different Cornudiscoides species and proves them genetically distinct species.

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Conflict of interest

The authors declare that they have no conflict of interest.

Table 5. Distribution of A, T, G, C contents % of GC and amplicon size of 18S rDNA of Cornudiscoides species.

| Cornudiscoides spp. | Adenine (A) | Thymine (T) | Guanine (G) | Cytosine (C) | GC (%) | Amplicon size (bp) | Blastn similarity |
|---------------------|-------------|-------------|-------------|--------------|--------|--------------------|------------------|
| C. heterotylus       | 163         | 159         | 175         | 149          | 50.2   | 646                | 96.68% with C. susanai |
| C. mystusi          | 294         | 288         | 300         | 227          | 47.5   | 1109               | 98.24% with C. longicirrus |
| C. proximus         | 161         | 152         | 161         | 126          | 47.8   | 600                | 97.31% with C. susanai |
| C. geminus          | 132         | 122         | 125         | 104          | 47.4   | 483                | 98.17% with C. proximus |
| C. agarwali         | 148         | 140         | 159         | 148          | 51.6   | 595                | 99.44% with C. susanai |
| C. tukarami         | 152         | 172         | 180         | 134          | 49.2   | 638                | 98.65% with C. susanai |
| C. gussevi          | 142         | 129         | 144         | 144          | 48-8   | 529                | 99.81% with C. susanai |
| C. bleekerai        | 181         | 204         | 211         | 164          | 49.3   | 760                | 98.79% with C. susanai |
| C. susanai          | 171         | 178         | 188         | 143          | 48     | 680                | 97.31% with C. proximus |
| C. sclerovaginalis  | 156         | 159         | 174         | 158          | 51.3   | 647                | 98.24% with C. mystusi |
| C. longicirrus      | 285         | 262         | 274         | 209          | 46.9   | 1030               | 98.24% with C. mystusi |
| C. aori             | 246         | 233         | 253         | 196          | 48.4   | 928                | 97.14% with C. mystusi |
| C. tripthii         | 128         | 124         | 122         | 109          | 47.8   | 483                | 98.51% with C. geminus |
| C. speratai         | 197         | 217         | 211         | 164          | 47.5   | 789                | 94.86% with C. susanai |
| C. falcatum         | 252         | 246         | 230         | 215          | 47.2   | 943                | 78.62% with C. susanai |

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