Molecular characterization of the Indian poultry nodular tapeworm, *Raillietina echinobothrida* (Cestoda: Cyclophyllidea: Davaineidae) based on rDNA internal transcribed spacer 2 region

Ramnath · D. B. Jyrwa · A. K. Dutta ·
B. Das · V. Tandon

Received: 29 May 2012 / Accepted: 25 September 2012 / Published online: 7 October 2012
© Indian Society for Parasitology 2012

Abstract The nodular tapeworm, *Raillietina echinobothrida* is a well studied avian gastrointestinal parasite of family Davaineidae (Cestoda: Cyclophyllidea). It is reported to be the largest in size and second most prevalent species infecting chicken in north-east India. In the present study, morphometrical methods coupled with the molecular analysis of the second internal transcribed spacer (ITS2) region of ribosomal DNA were employed for precise identification of the parasite. The annotated ITS2 region was found to be 446 bp long and further utilized to elucidate the phylogenetic relationships and its species-inter-relationships at the molecular level. In phylogenetic analysis similar topology was observed among the trees obtained by distance-based neighbor-joining as well as character-based maximum parsimony tree building methods. The query sequence *R. echinobothrida* is well aligned and placed within the Davaineidae group, with all *Raillietina* species well separated from the other cyclophyllidean (taeniid and hymenolepid) cestodes, while *Diphyllbothrium latum* (Pseudophyllidea: Diphyllbothriidae) was rooted as an out-group. Sequence similarities indeed confirmed our hypothesis that *Raillietina* spp. are neighboring the position with other studied species of order Cyclophyllidea against the out-group order Pseudophyllidea. The present study strengthens the potential of ITS2 as a reliable marker for phylogenetic reconstructions.

Keywords Cestode · ITS2 · Phylogeny ·
Raillietina echinobothrida · rDNA

Introduction

The *Raillietina* group of the cyclophyllidean eucestodes conventionally treated as one genus viz. *Raillietina* Fuhrmann, 1920 (Davaineidae), comprises a large assemblage of over 200 known species and is considered a monophyletic group. Members of the genus *Raillietina* Megnin, 1881 are obligate, widely-dispersed cestode parasites of avian and mammalian hosts and display a wide range of body forms, life histories, and host associations. Of these, *R. echinobothrida* is amongst the most prevalent pathogenic cestode parasites of birds throughout the world (Schmidt 1986; Khalil et al. 1994; Mariaux 1996, 1998; Hoberg et al. 1997, 1999) and earns the distinction of being the second most prevalent helminth recorded from the domestic fowl in north-east India (Yadav and Tandon 1991). The adult tapeworm is found in the intestinal lumen of its definitive host, *Gallus domesticus* and causes characteristic hyperplastic enteritis associated with the formation of granuloma on the intestinal wall of the infected birds (Soulsby 1982), whereas the larval stage resides in various tissues of the insect intermediate host, *Tetramorium* spp. (Jones and Horsfall 1935; Horsfall 1938; Gogoi and Hazarika 1975). The taxonomy of Cyclophyllidea traditionally depends upon the accurate descriptions of small differences in the size and shape of the scolex, rostellum (unarmed or armed with rows of hooks), suckers and gravid proglottids (Sawada 1965). However, with a rich resource of burgeoning data the molecular approach is proving to be more rapid, sensible and reliable than that based on morphological data alone for phylogenetic analysis (Brooks and Hoberg 2001).

The rDNA internal transcribed spacer (ITS) sequence data have revolutionized the phylogenetic analysis as a powerful tool in resolving remarkable taxonomic issues and discriminating genera and species across a large variety of

Ramnath · D. B. Jyrwa · A. K. Dutta · B. Das · V. Tandon (✉)
Department of Zoology, North-Eastern Hill University,
Shillong 793022, Meghalaya, India
e-mail: tandonveena@hotmail.com

organisms, because these regions ITS spacers are more variable and more informative than other parts of the rDNA locus (Hershkovitz and Lewis 1996; Coleman 2003). Furthermore, the ITS2 markers have also been proposed for use in species barcoding and DNA microarrays, thus increasing the detection power of closely related species (Park et al. 2007; Engelmann et al. 2009). In recent years, several molecular tools including sequence data related to conserved domains have unveiled their utility in molecular identification, taxonomy, systematics and phylogeny of floral, faunal and microbial species, too (Huang et al. 2006; Prasad et al. 2009a, b; Wiemers et al. 2009; Yao et al. 2010; Sharma et al. 2012). Since Woese and Fox (1977), the nuclear ribosomal repeat cistrons have also been widely implied for parasites (Littlewood and Olson 2001; Waeschenbach et al. 2007; Jyrwa et al. 2009; Goswami et al. 2009; Tandon et al. 2010; Prasad et al. 2011; Ghatani et al. 2012). Molecular phylogeny has advanced our understanding of comparative studies in basic and applied biology and more generally of their position within the phylum Platyhelminthes (Brooks and Hoberg 2001; Tandon et al. 2010).

The objective of the present study was to broaden the molecular and genetic information on the invasive tapeworm, *R. echinobothrida*, supplementing the morphological observation. In this study, we assess the validity of the selected ITS2 fragment of *R. echinobothrida* in discriminating closely related cestode species. Further validation needs to be well established, so as to exploit the ITS2 as well as other markers for robust molecular characterization of this avian parasite.

Materials and methods

The parasite

Live tapeworms (*R. echinobothrida*) were collected from the small intestine of naturally infected and freshly sacrificed domestic chicken in Shillong and its suburbs, Meghalaya. This species is characterized by the presence of a heavily armed rostellum with two rows of hooklets and four circular suckers on the scolex (Soulsby 1982).

Isolation of genomic DNA and amplification of ITS2 region

Genomic DNA (gDNA) from a single adult worm was isolated using standard phenol/chloroform/iso-amyl alcohol method (Sambrook and Russel 2001). DNA integrity was analyzed on 0.8 % (w/v) agarose gel containing 0.05 % ethidium bromide. ITS2 sequences were obtained by PCR amplification of gDNA using universal flatworm (*Schistosoma*) primers (3S: 5'-GGTACCGGTGGATCACTCGGC TCGTG-3' as the forward primer and A28: 5'-GGGATC

CTGGTTAGTTTCTTTTCCTCCGC-3' as the reverse primer) following Bowles et al. (1995). In brief, PCR amplification was performed in a 25 µl reaction mixture containing 1× PCR buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 10 pmol of each primer, 1 U of Taq DNA polymerase and 30–50 ng of gDNA using an automated thermal cycler (AB Veriti™-4375786). The cycling conditions were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 45 s, 72 °C for 60 s and final extension at 72 °C for 10 min. The amplicons were resolved on 1.5 % (w/v) agarose gel containing 0.05 % ethidium bromide. PCR products were gel eluted and purified using Sigma gel elution kit (GenElute™-NA1111). The amplicons were resuspended in nuclease-free water and sequenced on an automated sequencer at The Centre for Genomic Applications (TCGA), New Delhi.

Sequence analysis and construction of phylogenetic trees

Nucleotide sequences were aligned using ClustalW and BioEdit softwares (Thompson et al. 1994). After alignment, ambiguous regions (i.e., those containing gaps) were removed. Analysis was carried out on all substitutions. Sequences were deposited in GenBank and their accession numbers obtained.

Phylogenetic trees were constructed by comparing the ITS2 sequences of *R. echinobothrida* with ITS2 sequences of other cyclophyllidean cestodes available in GenBank using BLAST (Basic Local Alignment Search Tool; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were entered, edited, and aligned using ClustalX, in MEGA 5.05 (Tamura et al. 2011). For tree construction distance-based neighbor-joining (NJ) as well as the character-based maximum parsimony (MP) methods were used with branch support was given using 1000 bootstrap replicates.

The evolutionary history was inferred using the NJ method (Saitou and Nei 1987) and a consensus tree inferred from 100 bootstrap replicates taken to represent the evolutionary history of taxa analyzed. The evolutionary distances were computed using the maximum composite likelihood (MCL) method and are in the units of the number of base substitutions per site. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar 2000) with search level 3 in which the initial trees were obtained with the random addition of sequences (100 replicates). All positions containing gaps and missing data were eliminated.

Results and discussion

The PCR-amplified product was successfully obtained using the trematode universal primers and is shown in Fig. 1. Sequence analysis of the ITS2 PCR product

Fig. 1 PCR-amplified product of ITS2-gel picture (Lane 1 DNA Ladder (100 bp), Lane 2 purified product)

revealed the size of 600 bp with flanking regions of 5.8S and 28S region; the annotated product ITS2 region was found to be 446 bp in length spanning the ITS2 region. The query sequence was compared with ITS2 sequences of other cyclophyllidean cestodes available in public domain. The ITS2 region of the latter revealed a wide range in size, i.e., 367–938 bp in length. All these species used in the study along with their GenBank accession numbers are listed in Table 1.

Phylogenetic trees were obtained by comparing the ITS2 query sequences of *R. echinobothrida* with those of other cestode species available in GenBank; *Diphylobothrium latum* (a representative of the order Pseudophyllidea and a parasite of piscivorous mammals) was taken as the out-group. Similar topology was observed among the trees obtained by

distance-based (NJ) as well as character-based (MP) tree building methods in phylogenetic analysis (Fig. 2). The species included in the analysis mainly clustered into three major clades: the Davaineidae, Hymenolepididae, Taeniidae, with *D. latum* (Pseudophyllidea: Diphylobothriidae) at the base of the branch rooted as an out-group, which may indicate its early evolutionary history. The query sequence *R. echinobothrida* is well aligned and placed within the Davaineidae group, with all species of *Raillietina* well separated from the taeniid and hymenolepid cestodes (Fig. 2).

It is now widely accepted that rDNA ITS2 region is a useful tool for differentiation of taxa at the level of phylogeny due to the fact that ITS sequences are variable (Wolf et al. 2005). Because of its fast rate of evolution the ITS2 region continues to be applied for phylogenetic reconstructions at the species and genus levels (Alvarez and Wendel 2003; Wolf et al. 2005; Ashokan and Pillai 2008). In context of the cestode group of parasites the orders Cyclophyllidea, Pseudophyllidea, Caryophyllidea and Trypanorhyncha have been assessed on the basis of morphological, ultrastructural and ontogenetic characters (Hoberg et al. 1997, 2001; Beveridge et al. 1999; Bray et al. 1999) and several controversial phylogenetic disputes of cestode parasites have been published (Haukisalmi et al. 2001; Literak et al. 2006). Since in contrast to the bordering regions of ribosomal subunits; sequence is not conserved evolutionarily, thus genetic differentiation is detectable even in closely related groups of organisms. Sequence similarities indeed confirmed our hypothesis that *Raillietina* spp. are neighboring the position with other studied species of Order Cyclophyllidea against the out-group Order Pseudophyllidea. Morphological variation is either limited or the homology of morphological features remains unclear in tapeworms of the genus *Raillietina*. Hence, molecular characterization supplements and validates unambiguous identification at species level.

Table 1 Cestode species used in this study and GenBank accession numbers for their corresponding ITS2 sequences

Species	Country	GenBank accession no.	Order: family	Annotated ITS2 region bp
<i>Raillietina echinobothrida</i> ^a	India	JN797628	Cyclophyllidea: Davaineidae	446
<i>Raillietina dromaius</i>	Australia	AY382320.1	Cyclophyllidea: Davaineidae	830
<i>Raillietina chiltoni</i>	Australia	AY382319.1	Cyclophyllidea: Davaineidae	781
<i>Raillietina beveridgei</i>	Australia	AY382318.1	Cyclophyllidea: Davaineidae	938
<i>Raillietina australis</i>	Australia	AY382317.1	Cyclophyllidea: Davaineidae	841
<i>Hymenolepis diminuta</i>	China	JF766714.1	Cyclophyllidea: Hymenolepididae	791
<i>Hymenolepis nana</i>	Mexico	HM536187.1	Cyclophyllidea: Hymenolepididae	531
<i>Hymenolepis microstoma</i>	Japan	AB494478.1	Cyclophyllidea: Hymenolepididae	501
<i>Taenia hydatigena</i>	China	FJ886761.1	Cyclophyllidea: Taeniidae	367
<i>Taenia saginata</i>	China	AY825542.1	Cyclophyllidea: Taeniidae	733
<i>Taenia multiceps</i>	China	FJ886762.1	Cyclophyllidea: Taeniidae	507
<i>Diphylobothrium latum</i> ^b	France	DQ768176.1	Pseudophyllidea: Diphylobothriidae	Incomplete

^a Query sequence generated as per this study

^b Used as out-group

Fig. 2 NJ and MP phylogenetic trees of *Raillietina* spp. and other cyclophyllidean cestode spp. based on ITS sequences (* Query sequence generated as per this study; ** Used as out-group)

Acknowledgments Authors thank the Department of Biotechnology (Government of India), New Delhi, for their financial support for the study. Thanks are also due to the Head, Department of Zoology, NEHU, for providing infrastructural facilities.

References

- Alvarez I, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. *Mol Phylogenet Evol* 29:417–434
- Ashokan KV, Pillai MM (2008) In silico characterization of silk fibroin protein using computational tools and servers. *Asian J Exp Sci* 22:265–274
- Beveridge I, Campbell RA, Palm HW (1999) Preliminary cladistic analysis of genera of the cestode order Trypanorhyncha Diesing, 1863. *Syst Parasitol* 42:29–49
- Bowles J, Blair D, McManus DP (1995) A molecular phylogeny of the genus *Echinococcus*. *Parasitology* 110:317–328
- Bray RA, Jones A, Hoberg EP (1999) Observations on the phylogeny of the cestode order Pseudophyllidea Carus, 1863. *Syst Parasitol* 42:13–20
- Brooks DR, Hoberg EP (2001) Parasite systematics in the 21st century: opportunities and obstacles. *Trends Parasitol* 17: 273–275
- Coleman A (2003) ITS2 is a double-edged tool for eukaryotic evolutionary comparisons. *Trends Genet* 19:370–375
- Engelmann JC, Rahmann S, Wolf M, Schultz J, Fritzilas E, Kneitz S, Dandekar T, Müller T (2009) Modeling cross-hybridization on phylogenetic DNA microarrays increases the detection power of closely related species. *Mol Ecol Resour* 9:83–93
- Ghatani S, Shylla JA, Tandon V, Chatterjee A, Roy B (2012) Molecular characterization of pouched amphistome parasites (Trematoda: Gastrothylacidae) using ribosomal ITS2 sequence and secondary structures. *J Helminthol* 86:117–124
- Gogoi AR, Hazarika RN (1975) *Tetramorium tortosum* (Rog), an intermediate host of poultry tapeworms *Raillietina echinobothrida* and *Raillietina tetragona*. *Indian Vet J* 52:939–941
- Goswami LM, Prasad PK, Tandon V, Chatterjee A (2009) Molecular characterization of *Gastrodiscoides hominis* (Platyhelminthes: Trematoda: Digenea) inferred from ITS rDNA sequence analysis. *Parasitol Res* 104:1485–1490
- Haukisalimi V, Wickstrom LM, Hantula J, Henttonen H (2001) Taxonomy, genetic differentiation and Holarctic biogeography of *Paranoplocephala* spp. (Cestoda: Anoplocephallidae) in collared lemmings (*Dicrostonyx*; Arvicolinae). *Biol J Linn Soc* 74:171–196
- Hershkovitz M, Lewis L (1996) Deep-level diagnostic value of the rDNA-ITS region. *Mol Biol Evol* 13:1276–1295
- Hoberg EP, Mariaux J, Justine JL, Brooks DR, Weekes PJ (1997) Phylogeny of the orders of the Eucestoda (Cercaromorphae) based on comparative morphology: historical perspectives and a new working hypothesis. *J Parasitol* 83:1128–1147
- Hoberg EP, Jones A, Bray RA (1999) Phylogenetic analysis among the families of the Cyclophyllidea (Eucestoda) based on comparative morphology, with new hypotheses for co-evolution in vertebrates. *Syst Parasitol* 42:51–73
- Hoberg EP, Mariaux J, Brooks DR (2001) Phylogeny among orders of the Eucestoda (Cercaromorphae): integrating morphology, molecules and total evidence. In: Littlewood DJT, Bray RA (eds) *Interrelationships of the Platyhelminthes*. Taylor & Francis, London, pp 112–126
- Horsfall MW (1938) Observations on the Life History of *Raillietina echinobothrida* and of *R. tetragona* (Cestoda). *J Parasitol* 24:409–421
- Huang A, Li JW, Shen ZQ, Wang XW, Jin M (2006) High-throughput identification of clinical pathogenic fungi by hybridization to oligonucleotide microarray. *J Clin Microbiol* 44:3299–3305
- Jones MF, Horsfall MW (1935) Ants as intermediate hosts for two species of *Raillietina* parasitic in chickens. *J Parasitol* 21:442–443
- Jyrwa DB, Tandon V, Lyngdoh RD (2009) Molecular characterization of *Lytocestus* spp. (Eucestoda: Caryophyllidea) from *Clarias batrachus* (L.) in Meghalaya. In: Tandon V, Yadav AK, Roy B (eds) *Current trends in parasitology. Proceedings of the 20th national congress of parasitology, India, November 3–5, 2008*. Panima Publishing Corporation, New Delhi, p 137–146
- Khalil LF, Jones A, Bray RA (eds) (1994) *Keys to the cestode parasites of vertebrates*. Commonwealth Agriculture Bureaux International, Wallingford, pp 407–441

- Literak F, Tenora V, Letkova M, Goldova N, Torres J, Olson PD (2006) *Mesocostoides litteratus* (Batsch, 1786) (Cestoda: Cyclophyllidae: Mesocostoidae) from the red fox: morphological and 18S rDNA characterization of European isolates. *Helminthologia* 43:191–195
- Littlewood DTJ, Olson PD (2001) Small subunit rDNA and the phylum Platyhelminthes: signal, noise, conflict and compromise. In: Littlewood DTJ, Bray RA (eds) *Interrelationships of the Platyhelminthes*. Taylor & Francis, London, pp 262–278
- Mariaux J (1996) Cestode systematics: any progress? *Int J Parasitol* 26:231–243
- Mariaux J (1998) A molecular phylogeny of the Eucestoda. *J Parasitol* 84:114–124
- Nei M, Kumar S (2000) *Molecular evolution and phylogenetics*. Oxford University Press, New York, p 333
- Park MH, Sim CJ, Baek J, Min GS (2007) Identification of genes suitable for DNA barcoding of morphologically indistinguishable Korean Halichondriidae sponges. *Mol Cells* 23:220–227
- Prasad PK, Tandon V, Biswal DK, Goswami LM, Chatterjee A (2009a) Phylogenetic reconstruction using secondary structures and sequence motifs of ITS2 rDNA of *Paragonimus westermani* (Kerbert, 1878) Braun, 1899 (Digenea: Paragonimidae) and related species. *BMC Genomics* 10(Suppl 3):S25
- Prasad PK, Tandon V, Biswal DK, Goswami LM, Chatterjee A (2009b) Use of sequence motifs as barcodes and secondary structures of internal transcribed spacer 2 (ITS2, rDNA) for identification of the Indian liver fluke, *Fasciola* (Trematoda: Fasciolidae). *Bioinformation* 3:314–320
- Prasad PK, Goswami LM, Tandon V, Chatterjee A (2011) PCR-based molecular characterization and in silico analysis of food-borne trematode parasites *Paragonimus westermani*, *Fasciolopsis buski* and *Fasciola gigantica* from northeast India using ITS2 rDNA. *Bioinformation* 6:64–68
- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Sambrook J, Russel D (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Springs Harbour Press, New York
- Sawada I (1965) On the genus *Raillietina* fuhrmann 1920 (II). *J Nara Gakugei Univ (Nat)* 13:5–38
- Schmidt GD (1986) *CRC Handbook of tapeworm identification*. CRC Press, Boca Raton 675 pp
- Sharma SK, Dkhar J, Kumaria S, Tandon P, Rao SR (2012) Assessment of phylogenetic inter-relationships in the genus *Cymbidium* (Orchidaceae) based on internal transcribed spacer region of rDNA. *Gene* 495:10–15
- Soulsby EJJ (1982) *Helminths, Arthropods and Protozoa of domesticated animals*, 7th edn. ELBS and Bailliere Tindall, London
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony method. *Mol Biol Evol* 28:2731–2739
- Tandon V, Biswal DK, Prasad PK, Malsawmtluangi C (2010) Reconstructing the phylogenetic relationships of the cyclophyllidean cestodes: a case study using ITS2 rDNA and sequence structure alignment. In: Fred A, Filipe J, Gamboa H (eds) *Biomedical engineering systems and technologies*. BIOSTEC 2010, CCIS 127. Springer, Berlin, pp 309–321
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Waeschbach A, Webster BL, Bray RA, Littlewood DTJ (2007) Added resolution among ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with complete small and large subunit nuclear ribosomal RNA genes. *Mol Phylogenet Evol* 45:311–325
- Wiemers M, Keller A, Wolf M (2009) ITS2 secondary structure improves phylogeny estimation in a radiation of blue butterflies of the subgenus *Agrodiaetus* (Lepidoptera: Lycaenidae: *Polyommatus*). *BMC Evol Biol* 9:300
- Woese CR, Fox GE (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc Natl Acad Sci USA* 74: 5088–5090
- Wolf M, Achtziger M, Schultz J, Dandekar T, Müller T (2005) Homology modeling revealed more than 20,000 rRNA internal transcribed spacer 2 (ITS2) secondary structures. *RNA* 11: 1616–1623
- Yadav AK, Tandon V (1991) Helminth parasitism of domestic fowl (*Gallus domesticus* L.) in a subtropical high-rainfall area of India. *Beitr Trop Landwirtschaft Veterinarmed* 29:97–104
- Yao H, Song J, Liu C, Luo K, Han J et al (2010) Use of ITS2 Region as the universal DNA barcode for plants and animals. *Plos One* 5:e13102