Volume-4, Issue-8, August-2015 • ISSN No 2277 - 8160

Research Paper Zoology

Morphological and Molecular Characterization of *Raillietina* Sp.(Cestoda: Davaineidea) Infecting Chickens (*Gallus Gallus Domesticus*) from Aurangabad District (M.S.) India.

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ABSTRACT *Fuhrmann, 1920 established the genus Raillietina mostly harbours the class aves and well studied gastrointestinal parasite of family davaineidae (Cestoda:Cyclophyllidea).It is reported to be large in size, scolex globular in shape, mature proglottids broader than long rectangular in shape, testes smaller in size 33 to 40 in numbers, cirrus pouch flask shaped, ovary irregular. In the present study morphological methods coupled with molecular analysis of genomic DNA were employed for precise identification of cestode parasite.*

KEYWORDS : Aves, Cyclophyllidea,Genomic, Raillietina.

INTRODUCTION

The red jungle fowl, *Gallus gallus domesticus* economically important as a food and also succeptible to some gastrointestinal parasites. The *Raillietina* group of Cyclophyllidean eucestodes conventionally treated as one genus viz *Raillietina* furmann, 1920(Davainidea), comprises a large assemblage of over 200 known species and is considered monophyletic group. Systemic relationship between cestodes of the order cyclophyllidea are so far based mainly on morphological criteria, which are mostly of little significant for phylogeny(Mariaux1996). The phylogeny of eucestoda has been a matter of controversy for a century (Mariaux 1996;Hoberg et al 1997). Initial cladistic studies based on morphological characters were attempted to identify and specify the relationship among the major lineages of eucestoda.

Molecular techniques have become widely accepted all over the world .They provide a more specific method than methods conventionally employed in epidemiological studies(Coote 1990; Erlich et al 1991 ; Barker 1994;Rognlie et al 1994; Kramer and Schnieder 1998 and Ademir, 2006). Molecular techniques such as PCR and its variants are used for the diagnosis of parasitic disease and identification of parasite(Ademir, 2006). Molecular approches are most effective and accurate means for the detection of many organisms and for screening of genetic variation among population (Wongsawad and wongsawad 2010)

In the present study the cestode parasite morphologically studied and shows close resemblance to *Raillietina tetragona* and with the help of molecular data the phylogenetic position of cestode parasite *Raillietina* sp found in *Gallus gallus domesticus* has been recorded.

MATERIAL AND METHODS

1] Morphological identification:

Live tapeworms(*Raillietina* Sp) were collected from the small intestine of *Gallus gallus domesticus* at Aurangabad district (M.S) India. These parasites washed in saline solution and after killing preserved in hot 4% formalin and stained with Acetocaramine or Harris haematoxylin passed through various alcoholic grades cleared in xylin mounted in DPX and drawings are made with the aid of camera lucida. All measurements are given in millimeters. The parasite identified with the help of systema helminthum.

2] Molecular identification:

Cestodes envisioned for molecular exploration were fixed with 95% ethyl alcohol. DNA Extraction was carried out using Genelute Mammalian Genomic DNA extraction kit (Sigma, G1N70-1KT). 25mg of tissue was minced and transferred to 1.5ml microcentrifuge tube. 180µl of Lysis solution T and 20 µl of proteinase K were added. The samples were mixed and incubated at 550C to digest the tissue completely. 20 µl of RNase A solution was added and incubated at room temperature for 2min. Then 200µl of lysis solution C was added and incubated at 700C for 10 min. The column was prepared for binding by adding 500µl of Column preparation solution to each pre-assembled GenEluteMiniprep Binding Column and centrifuge at 12,000 rpm for 1 min. 200µl of ethanol was added to the lysate and mixed by vortexing. The entire lysate was transferred into the treated binding column and centrifuge at 10,000rpm for 1 min. The binding column was then placed in fresh 2ml collection tube. 500µl of Wash solution was added to the binding column and centrifuge at 10,000 rpm for 3min. This step was repeated twice. The column was again transferred to a new tube. 200µl of elution buffer was added directly into the centre of the binding column and centrifuge at 10,000rpm for 1min. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation A11454806498). The DNA was stored at -200C for further use.

The DNA isolated was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). The PCR reaction mix contained 2.5µl of 10X buffer, 1µl of each primer , 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA and 8.5µl nuclease free water. The PCR amplification cycle consist of, a cycle of 5 min at 94°C; 35 cycles of 1min at 94°C, 1 min at 56°C, 2 min at 72°C; and additionally 1 cycle of 7 min at 72°C. The reagents used are procured from GeNei .Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The band size obtained for amplification of partial 18S rRNA region is ~1095bp.

The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). 100µl of PCR-A buffer was added to the 25µl of reaction. The sample was mixed and transferred to column placed in 2ml collection tube and centrifuge at 10,000 rpm for 1min. the filtrate was discarded. 700µl of W2 buffer was added to the column and centrifuge at 10,000rpm for 2min. This step was repeated twice. The column was transferred to a new tube. 25µl of Eluent was added into the column and incubated at room temperature for 2min. Then centrifuge at 10,000rpm for 5min. It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. For sequencing of 18S rRNA PCR product 18s 5F- 5′ (CTGGTTGATYCTGCCAGT 3′) sequencing primer was used and for sequencing 28S rRNA PCR product LSU5F 5′ (TAGGTCGACCCGCTGAAYTTAAGCA) sequencing primer was used.

The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Centre for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of Worms. Altogether twenty sequences, including sample were used to generate phylogenetic tree (Figure 1-4). The tree was constructed by using MEGA 5 software (Saitou N. and Nei M.,1987; Felsenstein J.1985 and Tamura K. et al 2011).

RESULT

Morphological Exploration:

Fifteen specimens , of the cestode parasites , were collected , from the intestine of country hen, *Gallus gallus domesticus* at Aurangabad city M.S india, in the month of january 2015. The worms were medium in length consists of scolex , numerous immature and mature proglottids.

The worms were flattened , preserved in 4% formalin, stained with Harris haematoxylin or acetocaramine stain, passed through different alcoholic grades cleared in xylene mounted in DPX and whole mount slides were prepared for further taxonomic observation.

The scolex is small in size, globular in shape, slightly longer than broad , broad in the middle , not distinct off from the strobila and measures 1.373mm(1.182-1.564) in length and 1.239mm(1.068-1.411) in width.

The rostellum is medium, oval and measures 0.266 (0.228-0.305) in length and 0.457 (0.419-0.495) in width , it is armed with single rows of minute hooks. Four suckers are medium in size, oval in shape arranged in pairs in each half of the scolex and occupy major portion of the scolex with wide border and measures 0.648(0.534-0.763) in length and 0.533(0.495-0.572) in width.

The neck is long, wide longer than broad and measures 5.662 in length and 0.858(0.495-0.572) in widht.

The mature proglottids are medium in size, rectangular in shape, broader than long with irregular concave or convex lateral margins,with short blunt round projections at the corner of the segments and measures 05.662 in length and 3.586 (3.510-3.662) in width.

The testes are medium in size, oval in shape 30 -40(33)in number unevenly distributed in two lateral fields in between to longitudinal excretory canals and measures 0.495(0.457-0.534) in length and 0.095(0.076-0.114) in width.

The cirrus pouch is large in size , flask shaped situated posterior to the middle of the segments opens marginally and measures 0.228 in length and 0.038 in width.

The cirrus is thin, straight, contained within the cirrus pouch and measures 0.247(0.152-0.343) in length 0.209(0.152-0.267) in width.

The vas deferens is thin ,coiled obliquely placed directed anteriorly then runs transversely and measures 0.114mm in length and 0.038 in width The ovary is large in size , irregular with separate, finger shaped blunt acini located in the central region of the segments and measures 0.572mm(0.534-0.61) in length and 0.533(0.381-0.686) in width.

The vagina is thin , placed posterior to the cirrus pouch, starts from the genital pore, extends obliquely for a short distance, turns posteriorly towards the centre of segments reaches and opens into the ootype and measures 0.953 in length and 0.038 in width.

The ootype is small in size , round in shape, situated ventral to the ovary and measures 0.171(0.152-0.19)in length and 0.152 (0.114-0.19) in width.

The genital pores large in size , oval in shape, marginal, regular and measures 0.534 in length and 0.076 in width.

The excretory canal are medium in width and measures 0.076 in width.

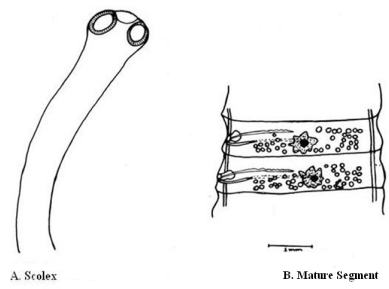


Fig. Raillietina teragona, Fuhrmann, 1920

Molecular data:

After going through partial sequence of the 18s rRNA gene of the existing cestode with those of other cestodes, in a phylogenetic context, provided further support for redescribing this species as *Raillietina tetragona* accordingly confirming taxonomic conclusion based on morphological data.

In the phylogenetic trees(fig 2) obtained by maximum parsimony analysis of the 18s rRNA sequence data seat, a close to the species *Raillietina micracantha* isclear with maximum identity (97%) , *Raillietina tunetensis(* 95%) and *Raillietina mitchelli* (94%) respectively(Table no. 1). After partial 18s rRNA gene sequence of *Raillietina* sample DNA length is 1041bp(fig 3).

Table 1: Phylogenetic neighbors of *Raillietina* sp based on partial 18s rRNAgene sequence

Description	Max score	Query cover	E value	Ident	Accession
Raillietinamitchelli 18S ribosomal RNA gene, complete sequence	1921	95%	0.0	94%	AY382315.1
Raillietina sp. 1 Costa Rica 18S small subunit ribosomal RNA gene, partial sequence	1917	92%	0.0	95%	EU665464.1
Raillietina sp. 3 Nebraska 18S small subunit ribosomal RNA gene, partial sequence	1912	92%	0.0	95%	EU665467.1
Raillietinatunetensis 18S small subunit ribosomal RNA gene, partial sequence	1901	92%	0.0	95%	EU665465.1
Fuhrmannettamalakartis 18S small subunit ribosomal RNA gene, partial sequence	1892	92%	0.0	95%	EU665463.1
Raillietina sp. 2 Nebraska 18S small subunit ribosomal RNA gene, partial sequence	1890	92%	0.0	95%	EU665466.1
Raillietinaaustralis 18S ribosomal RNA gene, complete sequence	1873	95%	0.0	94%	AY382311.1
Raillietinasonini 18S small subunit ribosomal RNA gene, partial sequence	1868	92%	0.0	95%	EU665468.1
*Raillietinachiltoni*18S ribosomal RNA gene, complete sequence	1862	95%	0.0	93%	AY382313.1
Raillietinadromaius 18S ribosomal RNA gene, complete sequence	1851	95%	0.0	94%	AY382314.1

Raillietinaaustralis 18S ribosomal RNA gene, complete sequence	1799	95%	0.0	93%	AF286980.1
*Raillietinabever-idgei*18S ribosomal RNA gene, complete sequence	1794	95%	0.0	93%	AY382312.1
Lyruterinanigropunc-tata partial 18S rRNA gene, fragment 1	1157	72%	0.0	95%	AJ555173.1
Raillietinamicracan-tha partial 18S rRNA gene, fragment 1	1107	50%	0.0	97%	AJ555178.1
*Avitellinacentripunc-tata*isolate Minxian 18S ribosomal RNA gene, complete sequence	1018	74%	0.0	93%	JQ609343.1
Dilepisundula 18S ribosomal RNA gene, complete sequence	972	82%	0.0	93%	AF286981.1
*Echinococcusgran-ulosus*18S ribosomal RNA gene, complete sequence	944	86%	0.0	91%	GQ260092.1
Lanfrediellaamphi-cirrus 18S ribosomal RNA gene, partial sequence	924	74%	0.0	92%	HM185494.1
Monieziabenedeni isolate 2 clone 2 18S ribosomal RNA gene, partial sequence	918	82%	0.0	91%	GU817404.1
Monieziabenedeni isolate 2 clone 1 18S ribosomal RNA gene, partial sequence	918	82%	0.0	91%	GU817403.1

Figure 2: Phylogenetic tree for *Raillietina* sp using partial 18s rRNAgene sequence

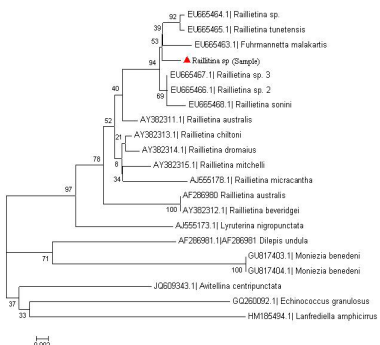
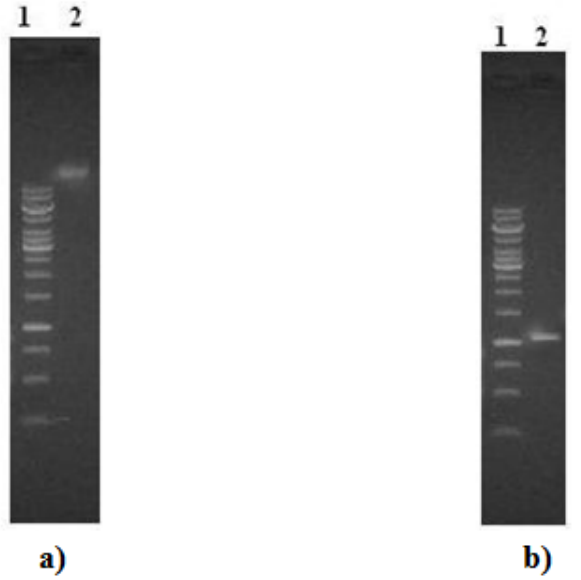


Fig.3. a) Genomic DNA extracted from *Raillietina* sp
b) Amplification of partial 18S and 28S rRNA gene for *Raillietina* sp



a) Lane 1: 1kb DNA Ladder of Fermentas

Lane 2: Genomic DNA of Sample of *Raillietina* sample

b) Lane 1: 1Kb DNA Ladder of Fermantas

Lane 2: Amplified PCR product

DISCUSSION

The genus *Raillietina* was established by Fuhrmann in 1920. After going through the literature, the worm under discussion have characteristic features such as scolex globular, rostellum oval with minute hooks, mature proglottids broader than long, rectangular in shape, testes small round to oval, 30 to 40(33) in numbers, cirrus pouch flask shaped, ovary irregular with several acini.

The present worm resembles *Raillietina teragona* in many characters but differs from it in few characters given below,

1) The present cestodes differ from *Raillietina tetragona* Molin,1858 in the position of suckers, arranged in pairs in each half of the scolex and occupy major portion of the scolex with wide borders.
2) The present tapeworm differs from it , with irregular concave or convex lateral margins with short blunt round projections at the corner of the segments .
3) The present worm differs from *Raillietina tetragona* Molin 1858, in the size of the ovary , distinctly irregular as against compact ovary.

As the above characters are minor , it is redescribed as ***Raillietina tetragona,*** Molin 1858.

After morphological and molecular observation we conclude that, this species should be considered to be a member of genus *Raillietina* but differs at species level. The present worm morphologically come closer to *Raillietina tetragona* and the molecular exploration reveals, on the basis of, position of sequence of the given Raillietina sample in the phylogenetic tree, the sample showed nearby resemblance with *Raillietina micracantha (*97%), *Raillietina tunetensis(* 95%), and *Raillietina mitchelli* (94%) repectively(Table no.1)

ACKNOWLEGMENTS

Author is thankful to the Head, Department of Zoology, Dr.BabasahebAmbedkarMarathwada University, Aurangabad for providing the laboratory facilities during this research work and also thanks to Codon Biosciences Pvt. Ltd. Panaji, Goa laboratory for providing necessary facilities

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