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Validation of Digenetic Trematodes, Using Molecular Markers, a Case Study of Indian Species of *Mesocoelium* Odhner, 1911

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Abstract : Morphological variations in *Mesocoelium sociale* Odhner, 1911, obtained from *Duttaphrynus melanostictus* Schneider, 1799 from a water body near Barabanki have been observed. Five Indian species of *Mesocoelium* viz. *M. varunae* Baugh 1956, *M. thapari* Gupta & Jahan, 1976, *M. mithiliae* Kanth & Srivastava, 1989, *M. melanostictii* Ratnamala Rao, 1989 and *M. asymmetrovitellarius* Kumari & Verma, 1992 are considered synonyms of *M. sociale.* We also doubt inclusion of *M. burdwanense* Mukherji, 1968 under the genus *Mesocoelium*. Phylogenetic study, nucleotide composition analysis, evolutionary divergence and multiple sequence alignment, based on partial 28S rDNA, of *Mesocoelium sociale* and sequences of two species of the genus retrieved from GenBank are also done.

Keywords : Digenea. *Mesocoelium.* Taxonomy. *Duttaphrynus melanostictus.* 28SrDNA

Introduction

Mesocoelium is the type genus of the family Mesocoeliidae Dollfus, 1929. Odhner (1911) described *M. sociale,* the type species of the genus from *Bufo melanostictus* Schneider, 1799 in Burma. Agrawal and Pandey (1980) also recorded *M. sociale* from *Bufo melanostictus*. Several species were established earlier, on the basis of structural variations in India (*M.* varunae Baugh, 1956, *M.* burdwanense Mukherji, 1967, *M. thapri* Gupta & Jahan, 1976, *M.* melanostictii Ratnamala Rao, 1989, *M.* mithilae Kanth & Srivastava, 1989 and *M.* asymmetrovitellarius Kumari & Verma, 1992). The taxonomic and specific characters chosen for specific diagnosis of other Indian species of *Mesocoelium* sp. are unreliable and controversial (Calhoun & Dronen, 2012). Dronen et al., 2012 emphasized the need of additional study to validate the previously described species of *Mesocoelium.* Recently, 28S region, nucleotide sequence analysis, transition and transversion ratios have proved useful tool for differentiation of digenean species (Takach et al., 2000, Olson et al., 2003).

An extensive survey of Indian *Duttaphrynus melanostictus,* Schneider, 1799 (Syn: *Bufo melanocystis*), for an interesting digenean *Mesocoelioum* Odhner, 1911 was, therefore made, during July-August 2013-14, from local water bodies near district Barabanki. Specimens (680) collected during present work had several morphological variations. However, the molecular markers proved them to be of the same species. We also assess the extent of genetic diversity of two allopatric forms (i.e. *M.* sp1 and *M.* sp2), described in Australia and Central America, based on 28S r DNA.

Material and Methods

Duttaphrynus melanostictus, Schneider, 1799 (Syn: *Bufo melanocystis*) were collected from local water bodies near district Barabanki (26º30' and 27º19' N and 80º55' and 81º55' E). Hosts were anesthetized and dissected. Almost all visceral organs were carefully examined under binocular microscope in Petri-dishes (containing normal saline, 7.4 pH). Parasites were collected from the gut and studied alive and fixed mounted specimens. They were flattened, with the help of a cover slip in 70% alcohol (overnight), stained with Aceto-alum Carmine, dehydrated in ascending grades of alcohol, cleared in clove oil and mounted in DPX. Figures were drawn with Camera Lucida, attached to Phase Contrast Microscope (Olympus CX-41). Measurements were taken in micrometer (µm), followed by range in parenthesis. Holotype and voucher specimens were deposited in the Helminthological collection of Zoological Survey of India, Kolkata (accession no XXXX). Parasites were collected in 100% ethanol for molecular study. Single specimen was processed for DNA isolation, with slight modifications in protocol of Qiagen's DNeasy Tissue Kit (Cat. No.69504). Partial 28S rDNA region of *Mesocoelium* was amplified in an Eppendorf Master Cycler Personal (PCR), using forward (5'- ACCCGCTGAATTTAAGCAT-3') and reverse (5'-CTCTTCAGAGTACTTTTCAAC-3') primers. Each PCR amplification reaction is performed in a final volume of 12.5 µl, containing 10X buffer (100mM Tris (pH 9.0), 50 mM KCL, and 15mM MgCl2), 2.5 U Taq Polymerase enzyme, 10 mM of each dNTP's and 3µl DNA. The PCR conditions are as follows: initial denaturation at 94ºC for 5 min, 25 cycles of denaturation at 94ºC (1 min), annealing at 54ºC (1 min), extension at 72ºC (1 min), followed by a final extension at 72ºC for 5 min. PCR products were electrophoresed in 2% agarose gel in TAE buffer, stained with ethidium bromide (Etbr) and visualized under UV radiations. PCR products were sequenced with same primer by Amnion Biosciences in forward direction, using an automated sequencer (Model Name 3130x1/3130x/GA-1203-019). Sequence of our

interest was compared with *Mesocoelium* sp. *M.* sp.1 and *M.* sp.2 (retrieved from GenBank) from Australia and Central America (Table 2) using *Lechriorchis tygarti* Talbot, 1933 (family: Reniferidae Pratt, 1902) as an out group. Sequences (28S rDNA) were analyzed by Maximum Likelihood (ML) and Minimum evolution (ME) methods of MEGA 5 (Tamura *et al.*, 2011). Robustness of inferred phylogenetic trees was assessed by bootstrap value i.e., 1,000 search replicates. The sequences of 28 S rDNA regions were compared by using CLUSTAL W for each query species. Following Table 1, includes different species of *Mesocoelium*, described in India, their hosts and localities. Species wise morphometric ratios are presented in Table 3.

Table 1 List of described Indian species of *Mesocoelium*

Name of Parasites	Host	Geographical origin (locality)
M. sociale, Odhner, 1911	*Duttaphrynus melanostictus,* Schneider, 1799	South central India
M. varunae, Baugh 1956	*Bufo marinus,* Linnaeus, 1750	Northern India
M. burdwanense, Mukherji, 1968	*Calotis versicolor,* Daudin, 1803	India
M. thapari, Gupta & Jahan 1976	*Rana tigrina, Daudin….*	Northern India
M. mithilae , Kanth & Srivastava 1989	*Heteropneustes fossilis,* Bloch, 1794	Eastern India
M.melanostictii, Ratnamala Rao, 1989	*Duttaphrynus melanostictus,* Schneider, 1799	South India
M. asymmetrovitellarius, Kumari & Verma 1992	*Duttaphrynus melanostictus,* Schneider, 1799	Eastern India

Table 2 Gen Bank references used in this study, their geographical origins and Accession Number

Sl .No	Parasite species	Genbank number	Locality	Host
1	*Mesocoelium sp.1*	AY222277	Australia	*Sibon nebulata,* Liner,1994
2	*Mesocoelium sp.2*	AF433677	Guatemala(Central America)	*Bufo marinus,* Schneider, 1799
3	*Mesocoelium sp.3*	XXXXXX	India	*Duttaphrynus melanostictus,* Schneider, 1799
4	*Lechriorchis tygarti*	JF820599	USA	*Thamnophis sirtalis,* Garman, 1892

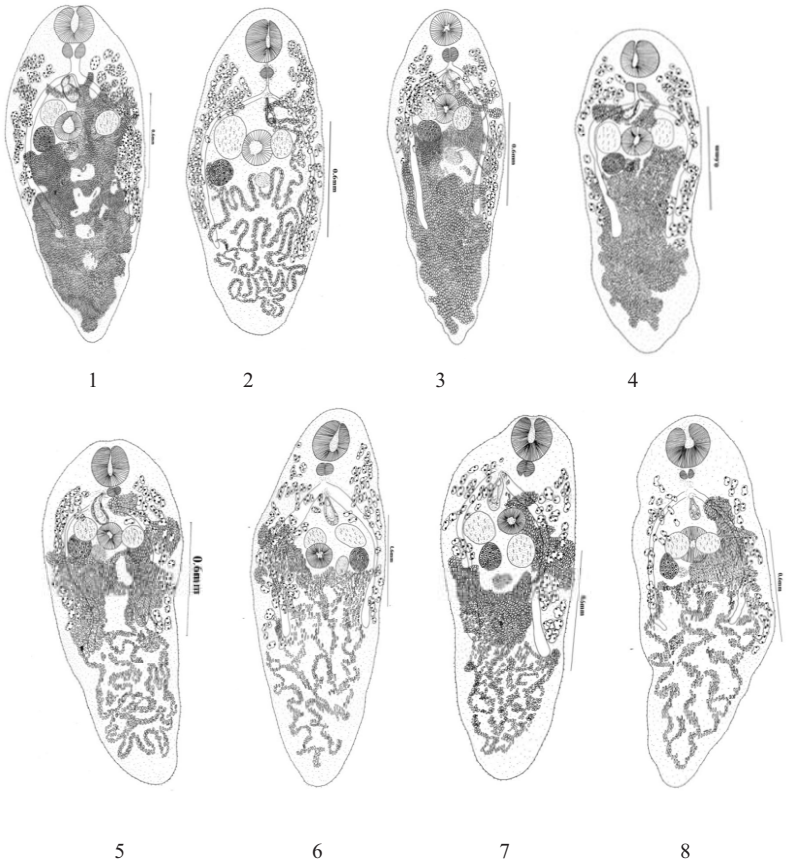
Result & Discussion

Description of *M. sociale* (Plate I. Figs 1-8):

Body elongated, anterior end oval, posterior end rounded (varying in posterior body width), narrow (Fig.1) to round (Fig. 2 & 4), beset with backwardly directed spines, 775 (760-790) long and 220 (200-240) wide. Oral sucker terminal/sub-terminal, 437 (221-432) long, 329 (213-232) wide. Pre-pharynx not observed. Pharynx round, 205 (134-142) long, 86 (81-92) wide. Esophagus very short or absent 11 (10-12) long (Fig.1 & 2). Ventral sucker smaller than oral sucker, 162 (152-173) long, 167 (164-171) wide.

Extension of intestinal caeca quite variable; equal (Fig 1, 4 & 6), unequal (Fig. 2, 3 &5), restricted up to middle region of body (Fig.6 & 8) or slightly beyond middle region. Testes two, round to oval, 213 (131-164) long, 197 (126-143) wide, symmetrical, one on either side of ventral sucker. However, in some specimens it is obliquely placed (Fig.3, 5&7) or could be abnormally developed (Fig.2). The inter-testicular distance (fig.3&8) may be quite distant. Vasa efferentia were also observed in few specimens. Cirrus sac oval, 210 (134-152) long, 105 (60-90) wide, having bipartite seminal vesicle, short pars prostatica, surrounded by a number of prostate glands and an ejaculatory duct. Genital pore is at the level with intestinal bifurcation or pre-bifurcal (5). Ovary, round to oval, post-testicular, 157 (150-165) long, 128 (120-136) wide. Receptaculum seminis, rounded. Laurer's canal is not visible. Vitellaria are glandular, laterally arranged and their distribution is also variable. They extend from pharyngeal region up to the end of intestinal caeca, mostly non confluent, sometimes confluent as well (Fig. 2, 4 & 5), could be un-equal on one side of intestinal caeca or asymmetrical (Fig. 3 & 7). Uterus is extensive, filled with eggs, convoluted, mostly confined to pre-acetabular region and extending posteriorly in the hind part of the body, in some having loose uterine coiling (Fig 2 & 5). The metraterm is highly muscular and opens into genital pore near intestinal bifurcation. Genital pore variable mostly bifurcated; in some pre bifurcal (1&5) in rests are post bifurcal. Eggs, oval and operculated 15 (10-20). Excretory bladder Y shaped. Comparative morphological variations are given from original drawings (in Plate II) and records comparative measurements of earlier described Indian species (Table 3).

Plate I. (Fig.1-8) Variation in morphological structures of *M. sociale*



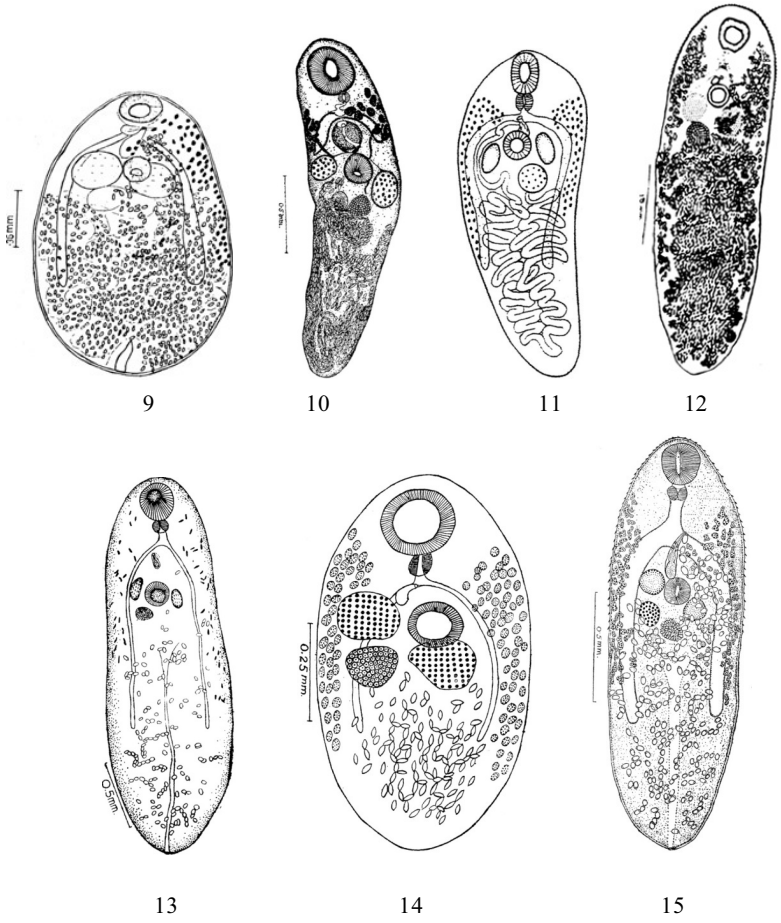
1 2 3 4

5 6 7 8

Table 3 Morphological Variations in Seven Species of *Mescoelium*

Charecteristic feature	*M.sociale*	*M.varunae*	*M. burdwanense*	*M. thapari*	*M.mithilae*	*M.melanostictii*	*M.asymmetrovitrovitellarius*
Body	spinose	*spinose*	*aspinose*	*aspinose*	aspinose	spinose	aspinose
Body length	220-775	720-1810	1845-450	2430-2800	560-960	360-1380	1840-1206
Oral sucker	329-437	180-250	270-234	250-260	165-195	330-350	180-252
Pharynx	86-205	70-80	54-72	90-100	45-45	110-150	72-108
Ventral Sucker	162-167	130-200	261	190-200	105-135	230-270	180-108
Esophagus	-	-	-	6-10	15	-	-
Cirrus sac			162-153		105x135		126-132
Position of genital pore	Bifurcal	Bifurcal	Bifurcal	Post-bifurcal	Bifurcal	Bifurcal	Bifurcal
Vitellaria	Pharynx to caecal end	Pharynx to caecal end	Ventral sucker to middle of body	Poorly developed, from Oral sucker to caecal end	From caecal bifurcation to hind end	From the level of oral sucker to caecal end	From the level of oral sucker to caecal end in one side only
Testis	311-347	105-130	T1-153-162 T2-189-135	T1-110x170-170x11 T2-130x90-160x100	T1-135-165x135-165	T1-270-360x230-380 T2-300-320x240-360	T1-370-306, T2-234-306
Ovary	150x120	140	72	90-100x60-90	135x165	240-320x230-270	180-252
Size of egg	-	62x40	264-288x14-17	45x40	45x23	38x28	36x18

Plate II. Figures (9-15) from the original literature 9. *M. assymmetrovitellarius* 10. *M. burdwanensis* 11. *M. sociale* 12. *M. melanostictii* 13. *M. thapari* 14. *M. mithlae* 15. *M. varunae*



9 10 11 12

13 14 15

Molecular study

The amplicon of 28Sr DNA gene vary in range (376; *M.* sp.3-1258; *M.* sp.1). Sequences (partial 28S rDNA) of *Mesocelium* sp. 1, *M.* sp. 2, *M.* sp. 3 are analyzed using Maximum likelihood and Minimum Evolutionary method of MEGA 5. *L. tygarti* belonging to family Telorchiidae Looss, 1899 is used as an out group. *M.* sp. 2 and *M.* sp.1 are grouped into cluster one. While *M.* sp. 3 (Indian species) formed a distinct clade. These variations are due to geographical barriers and both are dissimilar at variant nucleotide positions except C, C-2 and G-3 (Table.4). Indian isolate *M.sp3* forms basal clade by both ML & ME methods (Fig. 1a & 1b) and is different at each nucleotide positions excluding C-2 and seems to be distantly related with other two species.

The amplicon of 28Sr gene vary among genera, from 376 bp of *M. sp3* to 1258 bp of *M. sp1*. Average of 3 nucleotide sequences has total 1251 positions in the final data set. The nucleotide frequencies are 22.14% (A), 25.73% (T/U), 30.48% (C), and 21.65% (G). Nucleotide sequence analysis revealed that, fewest Cytocine (19.2%) are at first position. The degree of bias depends upon the codon composition i.e. 20.1% Cytocine in the first position, 21.8% in the second position and 21.4% at third position. All three positions are rich in Guanine at first 31.5%, the second 32.9%, and third 31.2%. Substitution pattern and rates are estimated through Tamura-Nei (1993) model (+G+1) [1].

The compositional bias differences between sequences (*M.sp2*/*M.sp3,* 0.24446, and *Msp3*/*Msp1*, 0.00240) have been found. However, this difference 0.014615 has been noticed in between *Msp2*/*Msp1*, (Table.5). The transition/transversion rate ratios are k_1=6.754 (Purines) and k_2=7.659 (Pyrimidines). The overall transition/transversion bias is R=3.564, where R= $[A*G*k_1=T*C*k_2]/[A+G]*(T+C)]$. The genetic divergence varies (*M.sp2*/*M.sp3*) is 926.00, (*Msp3*/*Msp1*) 923.00 and *Msp2*/*Msp1* 927.00 (Table.6). Estimate of average evolutionary divergence of overall sequence pairs is 595.500. There is no significant homogeneity has been observed for three species of *Mesocoelium*. The multiple sequence alignment shows both allopatric species are more similar than query species.

II. Phylogenetic Methods

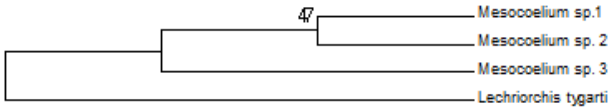


Fig1a. ML method

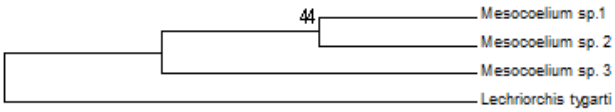


Fig 1 b. ME method

Fig.1 a & b. Phylogenetic tree of three species of *Mesocoelium*, by using MEGA5 a. Neighbor Joining (NJ) b. Minimum Evolution (ME)

Domain: Data																				
	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
M.sp. 2	25.4	21.3	21.3	32.0	1251.0	27	20.4	21.6	30.7	417.0	24	21.8	19.7	35.0	417.0	25	21.6	22.8	30.2	417.0
M.sp. 3	24.5	20.7	23.0	31.7	1129.0	22	20.7	26.3	30.8	377.0	26	21.8	21.5	31.1	376.0	26	19.7	21.3	33.2	376.0
M sp.1	25.3	21.3	21.5	31.9	1251.0	29	19.2	18.7	33.1	417.0	22	21.8	23.7	32.4	417.0	25	22.8	22.1	30.2	417.0
Avg.	25.1	21.1	21.9	31.9	1210.3	26	20.1	22.0	31.5	403.7	24	21.8	21.7	32.9	403.3	25	21.4	22.1	31.2	403.3

Table 4 Showing nucleotide sequence analysis

	1	2	3
M. sp 2			
M. sp 3	0.24446		
M. sp 1	0.00240	0.014615	

Table 5 Showing the difference in base composition bias per site

	1	2	3
M. sp 2			
M. sp 3	926.00		
M. sp 1	923.00	927.00	

Table 6 Showing the genetic divergence among three species of *Mesocoelium*

Nine body types (lanceatum, zhejiangensis, pesteri, mesembrinum, monas, brieni, sociale, leiperi, carli) have been recognized by Dronen *et al.*, 2012, on the basis of relative length of the caeca and the position of the genital pore. Genus *Mesocoelium* conforms all the morphological aspects in *M. sociale*, as is evident from the present work. *M. sociale* Odhner in 1911, the type species of the genus is characterized by spinose body, shape and size of body, ratio of suckers, moderately long intestinal caeca, symmetrical position of testis, genital pore bifurcal, condensed and profusely fused vitellaria. The shape and size of the body, size of esophagus, caecal length, arrangement of gonads, position of genital pore, extension of vitellaria greatly vary due to improper fixation of worms and coverslip pressure plays vital role in positioning of the organelles and without proper relaxation of worms, dehydration with alcohol also causes disorientation of internal structures. The placement of genital pore in reference to the caecal bifurcation and midline of body is a reliable character for distinguishing the species of *Mesocoelium* (Pojmanska, 2008, Dronen *et al.*, 2012, Calhoun and Norman, 2012). Uterine coiling has also been used to distinguish the species (Rao, 1989, Kumari & Verma, 1992). We have noticed that the younger adults have lesser number of eggs. It was also remarkable to note that fully mature worms, kept in cavity blocks containing water, start egg laying. Empty uterine coils or less number of eggs (Fig. 2, 6 &8) is thus quite common. Number of eggs, therefore, cannot be taken as a criterion of species establishment. *M. varunae* Baugh (1956) has close resemblance with *M. sociale,* except testis being smaller than ventral sucker and non- confluent vitellaria. Both the characters are variable, as is evident from the present collection (Plate I : Fig 3 & 6 and Fig. 6, 7 & 8). *M. varunae* is, therefore, regarded synonym of *M. sociale. M. burdwanense* Mukherji (1967), from the gut of *Calotis versicolor* Daudin, 1803 in West Bengal is characterized by

CLUSTAL W 2.1 multiple sequence alignment

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VI95A                           CGGGCAGAAATAACAAGGATCCCCCAGTAACGGCGAGTGAACAGGGAAAA 50
gi|18034385|gb|AF433677.1|AF43  ------------------------------CGGCGAGTGAACAGGGAAAA 20
gi|31662352|gb|AY222277.1|      --------------------------TAACGGCGAGTGAACAGGGAAAA 23
                                                          ********************

VI95A                           GCCCAGCACCGAAGCCTGTGGCCATTTGGTTACTAGGCAATGTGGTGTTT 100
gi|18034385|gb|AF433677.1|AF43  GCCCAGCACCGAAGCCTGTGGCCATTTGGTTACTAGGCAATGTGGTGTTT 70
gi|31662352|gb|AY222277.1|      GCCCAGCACCGAAGCCTGTGGCCATTTGGTT-CTAGGCAATGTGGTGTTT 72
                                ******************************* ******************

VI95A                           AGGTCGTTCCGCAGATGCTCTGCTCCACCCTAAGTCCATCAATGAGTACG 150
gi|18034385|gb|AF433677.1|AF43  AGGTCGTTCCGCAGATGCTCTGCTCCACCCTAAGTCCATCAATGAGTACG 120
gi|31662352|gb|AY222277.1|      AGGTCATTCCGCAGATACTCTGCTCCACCCCAAGTCCATCAATGAGTACG 122
                                ***** ********** ************* ******************

VI95A                           GTAGTATGGACATGGCCCATAGAGGGTGAAAGGCCCGTGGGGGTGGAGAT 200
gi|18034385|gb|AF433677.1|AF43  GTAGTATGGACATGGCCCATAGAGGGTGAAAGGCCCGTGGGGGTGGAGAT 170
gi|31662352|gb|AY222277.1|      GTAGTATGGACATGGCCCACAGAGGGTGAAAGGCCCGTGGGGGTGGAGAC 172
                                ****************** ****************************

VI95A                           TCGGCTGGTCAGAGTGTCTCTGGGTAGACCTTGGAGTCGGGTTGTTTGTG 250
gi|18034385|gb|AF433677.1|AF43  TCGGCTGGTCAGAGTGTCTCTGGGTAGACCTTGGAGTCGGGTTGTTTGTG 220
gi|31662352|gb|AY222277.1|      TCGACTGGACAGAGTGTCTCTGGGTAGACCTTGGAGTCGGGTTGTTTGTG 222
                                *** **** ****************************************

VI95A                           AATGCAGCCCAAAGTGGGTGGTAAACTCCATCCAAGGCTAAATACTTGCA 300
gi|18034385|gb|AF433677.1|AF43  AATGCAGCCCAAAGTGGGTGGTAAACTCCATCCAAGGCTAAATACTTGCA 270
gi|31662352|gb|AY222277.1|      AATGCAGCCCAAAGTGGGTGGTAAACTCCATCCAAGGCTAAATACTTGCA 272
                                **************************************************

VI95A                           CGAGTCCGATAGCGAACAAGTACCGTGAGGGAAAGTTGAAAAGTACTCTG 350
gi|18034385|gb|AF433677.1|AF43  CGAGTCCGATAGCGAACAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTG 320
gi|31662352|gb|AY222277.1|      CGAGTCCGATAGCGAACAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTG 322
                                ********************************************* **

VI95A                           AAGAGAT----------------------------------------- 357
gi|18034385|gb|AF433677.1|AF43  AAGAGAGAGTAAACAGTGCGTGAAACCGCTCAGAGGTAAACGGGTGGAGT 370
gi|31662352|gb|AY222277.1|      AAGAGAGAGTAAACAGTGCGTGAAACCGCTCAGAGGTAAACGGGTGGAGT 372
                                ******

VI95A                           ------------------------------------------------GT 359
gi|18034385|gb|AF433677.1|AF43  TGAACTGCAAGCTATGAGAATTCAGCTGATGAGTGTGATTTGAGCTTGGT 420
gi|31662352|gb|AY222277.1|      TGAACTGCAAGCTATGAGAATTCAGCTGATGAGTGTGATTTGGGCTTGGT 422
                                                                  **

VI95A                           TAAATT-------------------------------------CTCGGG 371
gi|18034385|gb|AF433677.1|AF43  CAAATTGGTGAACTCCGGGGTCTGTGTAGTAGCAGGTCTCTACCCTCGGG 470
gi|31662352|gb|AY222277.1|      CAAATTGGTGAACTCCGGGGTCTGTGTAGTAGCAGGTCTCTGCCCTCGGG 472
                                 *****                                      ******

VI95A                           TAG----------TA----------------------------------- 376
gi|18034385|gb|AF433677.1|AF43  TGGAGATGCGCGATACACTGGTCAAGTGTTGTGCGCCTCGGTTGTTT-TT 519
gi|31662352|gb|AY222277.1|      TGGGGATGCGCGATACACTGGTCAAGTGTTGTGCGCCTCGGTTGTTTGTT 522
                                * *          **

VI95A                           -------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  CGGCCTACTCGTCAGTGCACTTTCTCAGAGTGGTCACCACGACCGGCACC 569
gi|31662352|gb|AY222277.1|      CGGCCTACTCGTCAGTGCACTTTCTCAGAGTGGTCACCACGACCGGCACC 572

VI95A                           -------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  GCTGTCTGGTTGCTATGGTTAAACCGGTTTTGCATTGCACTCGTGGCTTT 619
gi|31662352|gb|AY222277.1|      GCTGTTTGGTTGCTATGGTTAAACCGGTTTTGCATTGCACTCGTGGCTTT 622

VI95A                           -------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  GCTTGATCGGGATGGCAGGTAGCTCGTTGACTTGCTGGTAGCTTGCTACT 669
gi|31662352|gb|AY222277.1|      GCTTGATCGGGATGGCAGGTAGCTCGTTGACTTGCTTGTGGCTTGCTGCA 672
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VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  GGCGTCGGTTTTCGAGTGTAATCAGCTGACCTTAGTGACTCTGTGCAGTG 719
gi|31662352|gb|AY222277.1|      GGCGTCGGTTTTCGAGTGTAATCAGCTGACCTTAGTGACTCTGTGCAGTG 722

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  TGTCGGAGACGGCGGCTTGAGGTGTGTGCGTGCTTCATGTTCTGTTGACC 769
gi|31662352|gb|AY222277.1|      TGTCGGAGACGGCGGCTTGAGGTGTGTGCGTGCTCCCTGTTCTGCTGACC 772

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  TATCCGGGTTTGGTTGTTTTGTTGCCTGTTCAAGCAGGCCTTATAATGGC 819
gi|31662352|gb|AY222277.1|      TATCCGGGTTTGGTTGTTTTGTTGCTTGTTCAAGCAGGCCTTATGATGGC 822

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  TCGGATTTGTTCGGCGGGGTGTGGGGTACGTGGCACTAATCCCAGGGCCA 869
gi|31662352|gb|AY222277.1|      TCGGATTTGTTCGGTAGGGGGTGGGGTACGTGGCACTAATCCCAGGGCCA 872

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  ATAGTCAGTGGTGTAGTGGTAGACTTTCCACCCGACCCGTCTTGAAACAC 919
gi|31662352|gb|AY222277.1|      ATAGTCAGTGGTGTAGTGGTAGACTTTCCACCTGACCCGTCTTGAAACAC 922

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  GGACCAAGGAGAGTAACATGTGCGCGAGTCATTGGGCGTTACGAAACCCA 969
gi|31662352|gb|AY222277.1|      GGACCAAGGAGAGTAACATGTACGCGAGTCATTGGGCGTTACGAAACCCA 972

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  AAGGCGCAGTGAAAGTAAAGGTTTGACTCGTTCAGACTGAGGTGAGATCC 1019
gi|31662352|gb|AY222277.1|      AAGGCGCAGTGAAAGTAAAGGTTTGACTCGTTCAGACTGAGGTGAGATCT 1022

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  TGTCGTTTCTTACGCGTGGTACCGCCAAGCATCGAGCGGCAGGCGCATCA 1069
gi|31662352|gb|AY222277.1|      TGTCGTTTCTTACGCGTGGTACCACCAAGCATCGAGCGGCAGGCGCATCA 1072

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  CCGGCCCGTCCCATGACAGTTGTTTTCGGGCAGTTTTCGGTCGGGGCGGA 1119
gi|31662352|gb|AY222277.1|      CCGGCCCGTCCCATGACAGTTGTTTTCGGGCAGTTTTCGGTCGGGGCGGA 1122

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  GCATGAGCGTACATGTTGAGACCCGAAAGATGGTGAACTATGCTTGCGCA 1169
gi|31662352|gb|AY222277.1|      GCATGAGCGTACATGTTGAGACCCGAAAGATGGTGAACTATGCTTGCGCA 1172

VI95A                           ----------------------------------------------------
gi|18034385|gb|AF433677.1|AF43  GGTTGAAGCCAGAGGAAACTCTGGTGGAGGACCGCAGCGATTCTGACGTG 1219
gi|31662352|gb|AY222277.1|      GGTTGAAGCCAGAGGAAACTCTGGTGGAGGACCGCAGCGATTCTGACGTG 1222

VI95A                           ------------------------------------
gi|18034385|gb|AF433677.1|AF43  CAAATCGATCGTCAAACGTGAGTATAGGGGCG---- 1251
gi|31662352|gb|AY222277.1|      CAAATCGATCGTCAAACGTGAGTATAGGGGCGAAAG 1258
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extension of intestinal caeca in pre-acetabular region. A review of literature shows that in all the species of *Mesocoelium,* except *M. burdwanense*, the intestinal caeca extend far beyond the ventral sucker. *M. burdwanense* cannot be placed under the genus *Mesocoelium.* In the rest of the *Mesocoelium* species, the extension of vitellaria is up to middle or hind region of body. We, therefore, doubt inclusion of this species under the genus *Mesocoelium*. The accurate placement is possible only when fresh collection is available from the type host and locality. *M. thapari* Gupta and Jahan (1976) was considered a junior synonym of *M. sociale* by Agrawal and Pandey (1980) to which we also agree. *M. mithilae* Kanth and Srivastava (1989) is also established on variable characters like ratio of suckers, unequal length of intestinal caeca, oblique testes, pre-bifurcal & post-bifurcal genital pore, distribution of vitellaria, large gonadal size and extension of uterus (observed in present colletion) Hence we consider *M. mithilae* as synonym of *M. sociale.* Description of *M. melanostictii* Rao (1989) and *M. asymmetrovitellarius* Kumari and Verma (1992) are based on a single specimen. *M. melanostictii* is also characterized by spinose body and variable characters (observed in present study, Figs 3) like ratio of suckers, extension of intestinal caeca in posterior half of body, symmetrical testes and distribution of vitellaria from oral sucker up to caecal ends. In our opinion, outer surface of improperly flattened worms give uneven appearance and when mounted under a coverslip could look like spines. Presence of spines could only be confirmed when additional specimens are available from the type locality for study. *M. melanostictii* is also considered a synonym of the type species. Regarding *M. asymmetrovitellarius,* we would like to mention that asymmetrical vitellaria is abnormal development of organ and not a specific character, and therefore the said species is none but the type species, having developed vitellaria on one side only. The appended table 3 shows variably measurements, host and locality of various parasites recorded so far in India under the type genus of the family.

Fischthal & Kuntz (1965) have stated that variation in *Mesocoelium sp*. is probably due to variation in environmental conditions in distantly related geographical areas and hosts. Morphological variability of worms may be due to wide range of host specificity, community richness on any single host species. Indian isolate *M. sociale* chiefly differs from *M.*sp1 & *M.*sp2 in terms of sequence length, composition of nucleotide bases and showed marked genetic variability. Though both species (*M.*sp1 & *M.*sp2) are forming clade but it is not significantly supported. Besides this, these two species are also different at most of the nucleotide positions as shown in Table 4. Indian species is more distantly related with other two species in terms of evolutionary divergence and dissimilarity of nucleotide sequences per site. Such pattern can be expected due to high gene flow and biogeographically barriers and ultimately leading towards diversification and speciation of parasites. We could not explore the morphology of both allopatric species because they are not identified.

Conclusions

We concluded that these species described so far in India are none but *M. sociale* except *M. burdwanensis*. We also doubt inclusion of *M. burdwanense* Mukherji, 1968 under the genus *Mesocoelium* due to extension of intestinal caeca far beyond the ventral sucker. Our molecular study also shows that *M.sociale* is more distantly related with other two allopatric forms in various molecular aspects.

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