

Validation of *Bifurcohaptor* spp. (Monogeneoidea: Dactylogyridae) reported from India using molecular methods with inclusion of insilico study: A brief report on its host-specificity

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Abstract. A total of 17 species of the genus *Bifurcohaptor* Jain, 1958 have been reported from two fish families namely Bagridae Bleeker, 1858 (*Mystus vittatus* (Bloch, 1794), *M. tengara* (Hamilton, 1822), *M. keletius* (Valenciennes, 1840), *Hemibagrus nemurus* (Valenciennes, 1840), *Rita rita* (Hamilton, 1822) and *Sperata seenghala* (Sykes, 1839)) and Sisoridae Bleeker, 1858 (*Bagarius bagarius* (Hamilton, 1822)). Out of these, only two species viz. *B. indicus* and *B. giganticus* are found valid in India, parasitizing gills of *Mystus* spp. and *Bagarius* sp. Taxonomic studies suggest, present specimen of *B. indicus* and *B. giganticus*, both are morphologically close to species described by Jain (1958), except morphometric variations and possess 7 pairs of marginal hooks instead of 6 pairs. Present manuscript delves with the characterization of *B. indicus* and *B. giganticus* reported from India, using molecular techniques. Partial mt COI nucleotide sequence based insilico protein analysis and partial 28S and ITS-1 rDNA based phylogenetic analysis, estimated by Neighbour-joining (NJ) and Minimum Evolution (ME) methods revealed that the species of the genus *Bifurcohaptor* are genetically distinct and valid. The grouping of *Bifurcohaptor* spp. with other representatives of family Dactylogyridae supports morphology based placement into family Dactylogyridae. Present and previous host-parasite information suggests both *Bifurcohaptor* spp. are species specialist however, the genus *Bifurcohaptor* is generalist at generic level.

INTRODUCTION

The genus *Bifurcohaptor* was established by Jain (1958) with *B. indicus* (Figure 6a) as type species from the gills of *Mystus vittatus* of family Bagridae Bleeker, 1858 at Lucknow along with *B. giganticus* Jain, 1958 (Figure 6b) from *Mystus seenghala* (now known as *Sperata seenghala*). *Bifurcohaptor* is a large sized parasite, have unique mode of attachment. The species of *Bifurcohaptor* possess long dorsal anchors, about one fourth to one half of body length, used as forceps or claws that almost completely embrace the gill filaments of

host with preference for marginal edges (Gusev, 1976; Kearns & Kumar, 1997). The genus *Bifurcohaptor* and its historical background is very complex and status of its species is still under question (Table 1). We have made a wide survey to collect specimens from Arunachal Pradesh (Itanagar), Bihar (Patna), Meghalaya (Shillong), Telangana (Hyderabad) and Uttar Pradesh (Gorakhpur, Lucknow, Kanpur, Mathura, Sitapur and Varanasi) and recorded only two species i.e. *B. indicus* and *B. giganticus* for the genus *Bifurcohaptor*. The mitochondrial (COI) and nuclear (28S and ITS-1 rDNA) DNA based studies also affirm

Table 1. Reports of *Bifurcohaptor* spp., their hosts and localities

<i>Bifurcohaptor</i> sp. (valid)	Hosts	Locality
<i>Bifurcohaptor indicus</i> Jain, 1958 (type species) Syn: <i>Bifurcohaptor minutum</i> Kulkarni, 1969 <i>Bifurcohaptor sohani</i> Agarwal & Singh, 1982	<i>Mystus vittatus</i> <i>M. tengara</i> <i>M. vittatus</i>	Lucknow and Meghalaya Hyderabad Lucknow
<i>Bifurcohaptor giganticus</i> Jain, 1958 Syn: <i>Bifurcohaptor hemlatae</i> Gupta, 1983 <i>Bifurcohaptor son</i> (Tripathi, 1959) Yamguti, 1963	<i>M. seenghala</i> <i>Rita rita</i> <i>Bagarius bagarius</i>	Lucknow Kanpur Son River at Dehri-on-Son in Bihar
<i>Bifurcohaptor chauhani</i> Agarwal & Sharma, 1986 <i>Bifurcohaptor chauhani</i> Swarup & Jain, 1984 <i>Bifurcohaptor kulkarni</i> Swarup & Jain, 1984 <i>Bifurcohaptor ramlingami</i> Swarup & Jain, 1984 <i>Bifurcohaptor lucknowensis</i> Agarwal & Sharma, 1990 <i>Bifurcohaptor mulleri</i> Gupta & Sharma, 1981 <i>Bifurcohaptor tripathii</i> Gupta & Sharma, 1981	<i>Bagarius bagarius</i> <i>Bagarius bagarius</i> <i>Bagarius bagarius</i> <i>Bagarius bagarius</i> <i>Bagarius bagarius</i> <i>Bagarius bagarius</i> <i>Channa striata</i> (misidentified)	Lucknow Yamuna at Mathura Yamuna at Mathura Yamuna at Mathura Yamuna at Mathura Lucknow River Gomati, Lucknow
<i>Bifurcohaptor gorakhnathai</i> Kumar & Agarwal, 1982 <i>Bifurcohaptor vishwanathai</i> Agarwal & Kumar, 1977	<i>Bagarius bagarius</i> <i>Bagarius bagarius</i>	Ramgarh Tal, Gorakhpur River Ganga, Dashashwamedha Ghat, Varanasi
<i>Bifurcohaptor lanki</i> Gussev, 1976	<i>M. keletius</i>	Water bodies of Nugegoda near Colombo, Sri Lanka
<i>Bifurcohaptor baungi</i> Lim & Furtado, 1983	<i>Hemibagrus nemurus</i>	Malaysian Peninsula and Thailand
<i>Bifurcohaptor</i> sp.	<i>Bagarius bagarius</i>	Thailand

genetic validity of *B. indicus* and *B. giganticus* in India. Mitochondrial (COI) region based 3D structure of proteins and 28S and ITS-1 rDNA based molecular phylogenetics seems a novel insilico approach for species discrimination and validation as well. The molecular portrayal thus approves, morpho-taxonomy based species differentiation and systematic placement of *Bifurcohaptor* in family Dactylogyridae. The present records evince that species of *Bifurcohaptor* parasitize only to fish of the families Bagridae (*M. vittatus*, *M. tengara*, *M. keletius* and *Hemibagrus nemurus*) and Sisoridae (*Bagarius bagarius*), distributed across some Asian countries (Figure 1). *B. indicus* and *B. giganticus*, both species have very narrow host specificity range and are therefore specialists. However, the genus *Bifurcohaptor* is a generalist (Table 2).

MATERIALS AND METHODS

Live fish were procured from local fish markets and various water bodies of Arunachal Pradesh (27°08'35.1"N, 93°45'48.7"E, Doimukh, Itanagar), Bihar (25°36'38.3"N, 85°12'31.3"E, Sadikpur, Patna), Meghalaya (25°36'48.4"N, 91°54'38.0"E, Shillong), Telangana (17°29'33.7"N, 78°23'36.8"E, fish markets nearby Jawaharlal Nehru Technological University, Hyderabad) and Uttar Pradesh (Gorakhpur (26°44'36.7"N, 83°25'09.3"E, Girdharganj), Lucknow (26°50'54.9"N, 80°55'52.0"E, Qaiserbaugh fish market), Kanpur (26°22'36.5"N, 80°29'31.2"E, Chaudanpur), Mathura (27°29'46.1"N, 77°41'45.5"E, Sadar Bazar), Sitapur (27°34'23.1"N, 80°40'03.9"E, Sarayan river, Kenchi Bridge) and Varanasi (25°19'58.5"N, 83°03'02.1"E, Panchkosi road, Kotwa)). Fish



Figure 1. Map depicting distribution of *Bifurcohaptor* spp. and their hosts.

Table 2. Details of host and parasite along with host-specificity of *Bifurcohaptor* spp.

S. No.	Fish-family	Fish	Distribution of fish	No. of host examined/ found infected with <i>Bifurcohaptor</i> sp.	Parasite collected (Previous records)	Parasite collected in present work (No. of parasite collected)	Specificity of <i>Bifurcohaptor</i> at specific level	Specificity of <i>Bifurcohaptor</i> at generic level
1	Bagridae	<i>M. vittatus</i> (Bloch, 1794)	Pakistan, India, Sri Lanka, Nepal, Bangladesh, Malaysia, Laos, Bhutan, Vietnam and Cambodia	50/15	<i>B. indicus</i>	<i>B. indicus</i> (30)	Mesostenoxenous of Mesoxenous category (Specialist parasite)	Generalist (does not parasitize on <i>S. seenghala</i> , <i>R. rita</i> and <i>C. striata</i>)
					<i>Cornudiscoides proximus</i> Gusev, 1976	<i>Cornudiscoides proximus</i> Gusev, 1976 (26)	Mesostenoxenous of Mesoxenous category (Specialist parasite), also reported from <i>M. tengra</i>	
					<i>C. geminus</i> Gusev, 1976	<i>C. geminus</i> Gusev, 1976 (21)	Mesostenoxenous of Mesoxenous category (Specialist parasite), also reported from <i>M. tengra</i>	
					<i>C. agrawali</i> Agrawal and Vishwakarma, 1996	<i>C. agrawali</i> Agrawal and Vishwakarma, 1996 (15)	Mesostenoxenous of Mesoxenous category (Specialist parasite), also reported from <i>M. bleekeri</i>	
2	Bagridae	<i>M. tengara</i> (Hamilton, 1822)	Pakistan, India, Nepal, Bangladesh and Afghanistan	50/20	<i>B. indicus</i>	<i>B. indicus</i> (26)	Mesostenoxenous of Mesoxenous category (Specialist parasite)	
					<i>C. heterotylus</i> Kulkarni, 1969	<i>C. heterotylus</i> Kulkarni, 1969 (22)	Mesostenoxenous of Mesoxenous category (Specialist parasite), also reported from <i>M. cavasius</i>	
3	Bagridae	<i>M. bleekeri</i>	Pakistan, India, Bangladesh, Nepal, Myanmar and Indonesia. Also Bhutan		<i>C. bleekeri</i> Agrawal and Vishwakarma, 1996	<i>C. bleekeri</i> Agrawal and Vishwakarma, 1996 (26)	Mesostenoxenous of Mesoxenous category (Specialist parasite), also reported from <i>M. vittatus</i>	
					<i>C. gussevi</i> Agrawal and Vishwakarma, 1996	<i>C. gussevi</i> Agrawal and Vishwakarma, 1996 (40)	Mesostenoxenous of Mesoxenous category (Specialist parasite), also reported from <i>M. cavasius</i>	
					<i>C. susanae</i> Agrawal and Vishwakarma, 1996	<i>C. susanae</i> Agrawal and Vishwakarma, 1996 (32)	Mesostenoxenous of Mesoxenous category (Specialist parasite), also reported from <i>M. cavasius</i>	
					<i>C. tukarami</i> Agrawal and Vishwakarma, 1996	<i>C. tukarami</i> Agrawal and Vishwakarma, 1996 (36)	Mesostenoxenous of Mesoxenous category (Specialist parasite), also reported from <i>M. cavasius</i>	

4	Bagridae	<i>M. keletius</i> (Valenciennes, 1840)	India and Sri Lanka	Not collected	<i>B. lanki</i>	N/A	Oioxenous
5	Bagridae	<i>Sperata seenghala</i> (Sykes, 1839)	Bangladesh, India and Nepal	50/0	<i>B. giganticus</i>	Not found	Oioxenous, parasitize only <i>B. bagarius</i>
6	Sisoridae	<i>Bagarius bagarius</i> (Hamilton, 1822)	Bangladesh; Bhutan; India; Nepal	25/20	<i>B. giganticus</i>	<i>B. giganticus</i> (50)	
7	Bagridae	<i>Rita rita</i> (Hamilton, 1822)	Afghanistan, Pakistan, India, Nepal, Bangladesh and Myanmar	25/0	<i>B. giganticus</i>	Not found	
8	Channidae	<i>Channa striata</i> (Bloch, 1793)	Bangladesh; Cambodia; China; India; Indonesia; Lao People's Democratic Republic; Malaysia; Myanmar; Nepal; Pakistan; Sri Lanka; Thailand; Vietnam	50/0	<i>B. giganticus</i>	Not found	
9	Bagridae	<i>Hemibagrus nemurus</i> (Valenciennes, 1840)	Java in Indonesia, Malay Peninsula, Sumatra and Borneo	Not collected	<i>B. baungi</i>	Not examined	Oioxenous
10	Siluridae	<i>Wallago attu</i> (Bloch & Schneider, 1801)	Pakistan to Vietnam, Indonesia, Afghanistan, Western Ghats, India	50/50	<i>T. wallagonius</i> Jain, 1952 <i>T. gontius</i> (Jain, 1952) Lim, 1996 <i>T. indicus</i> (Kulkarni, 1969) Lim, 1996 <i>T. sudhakari</i> (Gusev, 1976) Lim, 1996 <i>T. yogendrait</i> Agrawal, 1981 <i>Mizelleus indicus</i> Jain, 1957 <i>Mizelleus longicirrus</i> (Tripathi, 1959) Venkatanraiah & Kulkarni, 1981	<i>T. wallagonius</i> Jain, 1952 (20) <i>T. gontius</i> (Jain, 1952) Lim, 1996 (50) <i>T. indicus</i> (Kulkarni, 1969) Lim, 1996 (60) <i>T. sudhakari</i> (Gusev, 1976) Lim, 1996 (62) <i>T. yogendrait</i> Agrawal, 1981 (53) <i>Mizelleus indicus</i> Jain, 1957 (30) <i>Mizelleus longicirrus</i> (Tripathi, 1959) Venkatanraiah & Kulkarni, 1981 (20)	Oioxenous
11	Pangasiidae	<i>Pangasianodon hypophthalmus</i> (Sauvage, 1878)	Mekong, Chao Phraya, and MaeKlong basins. Introduced into additional river basins for aquaculture.	50/25	<i>T. siamensis</i> (Lim, 1990) Lim, 1996 <i>T. caecus</i> (Mizelle and Kritsky, 1969) Lim, 1996	<i>T. siamensis</i> (Lim, 1990) Lim, 1996 (51) <i>T. caecus</i> (Mizelle and Kritsky, 1969) Lim, 1996 (40)	Oioxenous
12	Notopteridae	<i>Notopterus notopterus</i> (Pallas, 1769)	India, peninsular Thailand and Malaysia, Indonesia (Sumatra and Java)	20/15	<i>Malayanodiscoides indicus</i> Agrawal, Vishwakarma and Gaur, 1998	<i>Malayanodiscoides indicus</i> Agrawal, Vishwakarma and Gaur, 1998 (20)	Oioxenous
13	Notopteridae	<i>Chitala chitala</i> (Hamilton, 1822)	India, Thailand and Indo-China.	25/15	<i>Notopterodiscoides indicus</i> Jain, 1955	<i>Notopterodiscoides indicus</i> Jain, 1955 (25)	Oioxenous
14	Bagridae	<i>Sperata aor</i> (Hamilton, 1822)	Pakistan, India, Nepal, Bangladesh and upper Myanmar.	30/20	<i>T. aori</i> (Rizvi, 1971) Lim, 1996 <i>T. speratai</i> Agrawal et al. (2004) <i>T. Susanae</i> Rajvanshi and Agrawal, 2013 <i>C. mystusi</i> (Rizvi, 1971) Dubey et al., 1992	<i>T. aori</i> (Rizvi, 1971) Lim, 1996 (25) <i>T. speratai</i> Agrawal et al. (2004) (15) <i>T. Susanae</i> Rajvanshi and Agrawal, 2013 (32) <i>C. mystusi</i> (Rizvi, 1971) Dubey et al., 1992 (23)	Oioxenous

15	Osphronemidae	<i>Colisa fasciatus</i> Bloch & Schneider, 1801	Pakistan, India, Nepal, Bangladesh and upper Myanmar. Widely transported around the world	50/20	<i>Heteronchocheilus lucknowensis</i> Agarwal and Bhatnagar, 1997	<i>Heteronchocheilus lucknowensis</i> Agarwal and Bhatnagar, 1997 (30)	Oioxenous
16	Schilbeidae	<i>Eutropiichthys vacha</i> (Hamilton, 1822)	Pakistan, India, Bangladesh, Nepal, Myanmar and Thailand.	50/20	<i>T. kritskyi</i> Rajvanshi and Agrawal, 2014a <i>T. singularis</i> Rajvanshi and Agrawal, 2014	<i>T. kritskyi</i> Rajvanshi and Agrawal, 2014a (30) <i>T. singularis</i> Rajvanshi and Agrawal, 2014 (25)	Oioxenous
17	Mastacembelidae	<i>Macrogynathus pancalus</i> (Hamilton, 1822)	Pakistan, India, and Bangladesh and Nepal	50/25	<i>Mastacembelocleidus bam</i> (Tripathi, 1959) Kritsky, Pandey, Agrawal and Abdullah, 2004	<i>Mastacembelocleidus bam</i> (Tripathi, 1959) Kritsky, Pandey, Agrawal and Abdullah, 2004 (36)	Oioxenous
18	Mastacembelidae	<i>Macrogynathus aculeatus</i> (Bloch, 1786)	India, Bangladesh, Nepal, Vietnam, Thailand, Malay-Peninsula, Indonesia (Borneo)	50/23	<i>Mastacembelocleidus bam</i> (Tripathi, 1959) Kritsky, Pandey, Agrawal and Abdullah, 2004	<i>Mastacembelocleidus bam</i> (Tripathi, 1959) Kritsky, Pandey, Agrawal and Abdullah, 2004 (40)	Oioxenous
19	Mastacembelidae	<i>Mastacembalus armatus</i> (Lacepede, 1800)	Pakistan to Vietnam and Indonesia.	50/32	<i>M. heteranchorus</i> (Kulkarni, 1969) Kritsky, Pandey, Agrawal and Abdullah, 2004 <i>Mastacembelocleidus bam</i> (Tripathi, 1959) Kritsky, Pandey, Agrawal and Abdullah, 2004	<i>M. heteranchorus</i> (Kulkarni, 1969) Kritsky, Pandey, Agrawal and Abdullah, 2004 (36) <i>Mastacembelocleidus bam</i> (Tripathi, 1959) Kritsky, Pandey, Agrawal and Abdullah, 2004 (41)	Oioxenous
20	Mastacembelidae	<i>Mastacembalus mastacembalus</i> (Banks and Solander, 1794)	Tigris and Euphrates basin.	50/22	<i>M. heteranchorus</i> (Kulkarni, 1969) Kritsky, Pandey, Agrawal and Abdullah, 2004	<i>M. heteranchorus</i> (Kulkarni, 1969) Kritsky, Pandey, Agrawal and Abdullah, 2004 (17)	Oioxenous
21	Ambassidae	<i>Chanda nama</i> (Hamilton, 1822)	Pakistan, India, Nepal, Bangladesh, and Myanmar.	50/22	<i>Spicocleidus nama</i> Agrawal, Tripathi and Shukla, 2005 <i>Chandacleidus recurvatus</i> (Jain, 1961) Agrawal et al., 2006 <i>Chandacleidus saiensis</i> Agrawal et al., 2006 <i>Chandacleidus lucknowensis</i> Agrawal et al., 2006	<i>Spicocleidus nama</i> Agrawal, Tripathi and Shukla, 2005 (36) <i>Chandacleidus recurvatus</i> (Jain, 1961) Agrawal et al., 2006 (28) <i>Chandacleidus saiensis</i> Agrawal et al., 2006 (39) <i>Chandacleidus lucknowensis</i> Agrawal et al., 2006 (19)	Oioxenous
22	Belonidae	<i>Xenentodon cancila</i> (Hamilton, 1822)	Sri Lanka and India eastward to the Mekong.	50/26	<i>Xenentocleidus xenentodoni</i> (Jain, 1961) Tripathi et al., 2007	<i>Xenentocleidus xenentodoni</i> (Jain, 1961) Tripathi et al., 2007 (41)	Oioxenous

were identified with the help of FishBase (Froese and Pauly, 2016). Parasites were identified with Pandey & Agrawal (2008) (Table 2). Parasites were dislodged using micro needles in glass Petri-dishes. Live parasites (under cover slip containing water) and 3% formalin preserved (diluted with lukewarm water) were examined under Phase Contrast microscope (Olympus BX-51, Tokyo, Japan). The method of staining,

mounting and illustration of parasites was that of Kristsky *et al.* (1986) and numbering of hooks is according to Kulwiec (1927). Unstained glycerine mounts, sealed with sealant (nail enamel) were used for measurements, taken in μm , using a calibrated micrometer (Table 3). Image-Pro Express 6.0 software (for Image analysis) was used for measurements. Drawings were made with the aid of Camera Lucida

Table 3. Comparative measurements of *Bifurcohaptor* spp. reported from India

Characters		<i>B. indicus</i> Present records (µm)	<i>B. indicus</i> Jain, 1958 Previous records (µm)	<i>B. giganticus</i> Present records (µm)	<i>B. giganticus</i> Jain, 1958 Previous records (µm)
Body length/ breadth		450-620x65-80	550-820x85	27130-2155x390-452	3250-3760x600
Pharynx		36-46x	36x35	130-126x127-120	160x150
Opithohaptor		170-180	200-270x150-290	438-445-x330-350	800-1200x600-850
Dorsal anchors	Inner length	145-155 (Total length)	160-200	293-275	800-1125x140 (Total length)
	Outer length	160-165	–	289-310	–
	Recurve Point	25-32	–	43-59	–
Ventral anchors	Inner length	16-24	16-25 (total length)	32-39	45-51 (total length)
	Outer length	12-18	–	29-35	–
	Recurve Point	16-20	–	27-35	–
Dorsal bar		35-46	41-45	118-125x 130-140	140-150x160-170
Ventral bar (each piece)		32-40	35-42	120-130	165-190
Hooks		7 pair11-12	6 pair10-12	7 pair12-14	6 pair11-13
Ovary length/ breadth		74-82x30-36	74-82x30-36	196-220x123-146	200-230x100-130
Testis length/ breadth		86-110x40-52	90-120x170-210	235-275x145-170	250-310x150-190
Accessory piece		26-34	21-25	Three pieced 64-72 (I st piece) 83-90 (II nd piece) and 60-64 (III rd piece)	Two pieced 110-121 (I st piece) and 90-120 (II nd piece)
Cop. tube		70-87	70-75	240-251	230-260x25
Egg		Oval, unipolar, 80x50.1	Triangular, each side, 35, 42 and 20 long	–	–
Host		<i>Mystus vittatus</i>	<i>Mystus vittatus</i>	<i>Bagarius bagarius</i>	<i>Mystus seenghala</i>
Locality		Lucknow	Lucknow	Lucnow	Lucknow

attached to the microscope. Ethanol-preserved (100%) specimens were used to extract genomic DNA using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per manufacturer's protocol. Partial 28S, ITS-1 rDNA and mt COI region were amplified by polymerase chain reaction (PCR) with the aid of respective set of primers (Table 4). Each PCR amplification reaction was performed in a final volume of 12.5 µl, containing 10×buffer (100 mM Tris, pH 9.0), 50 mM KCl and 15 mM MgCl₂, 2.5 U Taq polymerase enzyme, 10 mM of each deoxynucleotide triphosphate (dNTP's) and 3 µl DNA. The PCR conditions were as follows: initial denaturation at 94°C for 5 min; annealing for 28S at 58°C (1.10 min), ITS-1 and COI at 56°C (1.10 min) and final extension at 72°C for 10 min. Ethidium Bromide (EtBr) stained PCR products were visualized on 2% Agarose gel in TAE (Tris–Acetic Acid–EDTA) buffer, 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 ITS-1 rDNA) of *Bifurcohaptor* spp. were used for phylogenetic analysis along with published sequences, retrieved from GenBank (Table 5).

Translation of obtained mitochondrial nucleotide sequences was done by Emboss

transeq tool version 6.6.0 of Expasy (Goujon *et al.*, 2010; Rice *et al.*, 2000). All asterisks were removed from 1D dataset (amino acid(s)) of *B. indicus* and *B. giganticus*. Statistical values of translated amino acid sequences were predicted by ExPASy ProtParam (Gasteiger *et al.*, 2005). Threading was used to predict 3D conformation of *B. indicus* and *B. giganticus* (sequences that have query coverage below 80%). Phyre2 generated three dimensional conformations of *B. indicus* and *B. giganticus* was based on HMM-HMM (Hidden Markov Model), which is adept to search homologous template even for distant sequence (Kelley *et al.*, 2015). The target-template complex was achieved using Chimera 1.10.1 (Pettersen *et al.*, 2004; Meng *et al.*, 2006). Target and template PDB files were uploaded in Chimera software to get the target-template complex protein conformation. Target and template protein conformation was annotated with different colours (*B. giganticus* is coded with red and *B. indicus* with purple colour and template protein with cyan blue colour). Root mean square values (RMSD) were used to estimate sequence divergence between target and template protein sequence (Pettersen *et al.*, 2004; Meng *et al.*, 2006).

Query sequences were subjected to Clustal Omega for multiple sequence alignment (<http://www.ebi.ac.uk/Tools/>

Table 4. Details of genes, primers and their sources

Genes	Primer Name	Sequence 5' to 3'	Source
28S (Used for <i>B. giganticus</i>)	(Forward) (Reverse)	ACCCGCTGAATTTAAGCAT CTCTTCAGAGTACTTTTCAA	Mollaret <i>et al.</i> , 2000
28S (Used for <i>B. indicus</i>)	Ancy55 (Forward) LSU1200R (Reverse)	GAGATTAGCCCATCACCGAAGG GCATAGTTCACCATCTTTTCGG	Plaisance <i>et al.</i> , 2005
ITS-1 (Used for <i>B. giganticus</i>)	BD1 (Forward) BD2 (Reverse)	GTCGTAACAAGGTTTCCGTATCTA GATGCGTTTCCA(G/A)TGTCGATG	Bowles <i>et al.</i> , 1995
ITS-1 (Used for <i>B. indicus</i>)	P3b (Forward) F3 (Forward)	TAGGTGACCCTGCAGAAGGATCA TTGCTGCACTCTTCATC	Harris <i>et al.</i> , 1999
mt COI (Used for <i>B. giganticus</i>)	JB3 (Forward) JB45 (Reverse)	TTTTTTGGGCATCCTGAGGTTTAT TAAAGAACATAATGAAAATG	Bowles <i>et al.</i> , 1993
mt COI (Used for <i>B. indicus</i>)	Asmit1 (Forward) Asmit2 (Reverse)	TTTTTTGGGCATCCTGAGGTTTAT TAAAGAAAGAACATAATGAAAATG	Bowles <i>et al.</i> , 1992

Table 5. GenBank accession number of Monogenoideans, their host and family used in the present study

Species name	Hosts	Family	GenBank accession number		
			28S	ITS-1	COI
<i>Bifurcochapter indicus</i> Jain, 1958	<i>Mystus vittatus</i>	Dactylogyridae	KX863730	KX863729	Submitted
<i>B. giganticus</i> Jain, 1958	<i>Mystus seenghala</i>	Dactylogyridae	KY5547887	KY554788	Submitted
<i>Thaparocleidus gontius</i>	<i>Wallago attu</i>	Dactylogyridae	KC92229	–	–
<i>Thaparocleidus indicus</i>	<i>Wallago attu</i>	Dactylogyridae	JX960419	–	–
<i>Thaparocleidus sudhakari</i>	<i>Wallago attu</i>	Dactylogyridae	JX984666	–	–
<i>Malayanodiscoides indicus</i>	<i>Notopterus chitala</i>	Dactylogyridae	GU830882	–	–
<i>Mastacembelocleidus bam</i>	<i>Mastacembelus armatus</i>	Dactylogyridae	KC437381	–	–
<i>Mastacembelocleidus heteranchorus</i>	<i>Mastacembalus armatus</i>	Dactylogyridae	KC763980	–	–
<i>Dactylogyrus achmerowi</i>	–	Dactylogyridae	–	KX369224	–
<i>Dactylogyrus vastator</i>	–	Dactylogyridae	–	KX369223	–
<i>Dactylogyrus dulkeiti</i>	–	Dactylogyridae	–	KX369217	–
<i>Pseudodactylogyrus anguillae</i>	–	Dactylogyridae	–	AJ490162	–
<i>Pseudodactylogyrus bini</i>	–	Dactylogyridae	–	AJ490163	–
<i>Thaparocleidus rukyanii</i>	–	Dactylogyridae	–	FJ493163	–
<i>Cichlidogyrus</i> sp. KalLobLab3	–	Dactylogyridae	–	KT037205	–
<i>Cichlidogyrus</i> sp. Kal_LolLa2	–	Dactylogyridae	–	KT037200	–

msa/clustalo/). Phylogenetic trees were constructed by the Neighbor-joining (NJ) and Minimum Evolution (ME) methods of MEGA software, version 6.06 using two molecular markers viz. partial 28S and ITS-1 (Tamura *et al.*, 2013). The host specificity of parasites is according to Pojmańska & Niewiadomska (2012).

RESULTS

The description of Jain (1958) for the species *B. indicus* and *B. giganticus* was based on ordinary microscopes. Thus, restudy of the genus *Bifurcochapter* is required to check the morphological and morphometric variations for the species described herein (Table 3). The morphometric variations among all examined specimens of *B. indicus* and *B. giganticus* (50 specimens each) are closer to each

other while both species are widely different in morphology and measurements. The consistency in morphometry of both species follows concept of host-specificity as both are host specific species and demonstrate good relationship between host size and parasite size. Sasal *et al.* (1999) have also suggested direct relationship between host and parasite. Jain (1958) described 6 pairs marginal hook for *B. indicus* and *B. giganticus* respectively which are fixed here to 7 pairs for each species.

3D conformation of protein

The amino acid composition (primary data set) of *B. giganticus* and *B. indicus* is completely different (Table 6). Main characteristic features of 2D conformation (disordered, α -helix, Beta-strand and TM helix) are also peculiar for both *Bifurcochapter* spp. *Bifurcochapter giganticus* has 6%, 98%, 0% and 73%,

Table 6. Characteristic features of protein data set with their best hit templates

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	<i>B. giganticus</i>	123	14810.45	7/7	d1eysh2	11%/50.1%/62%/0.000Å
2	<i>B. indicus</i>	134	16516.16	4/4	d1nxia	8%/29.3%/64%/0.000Å

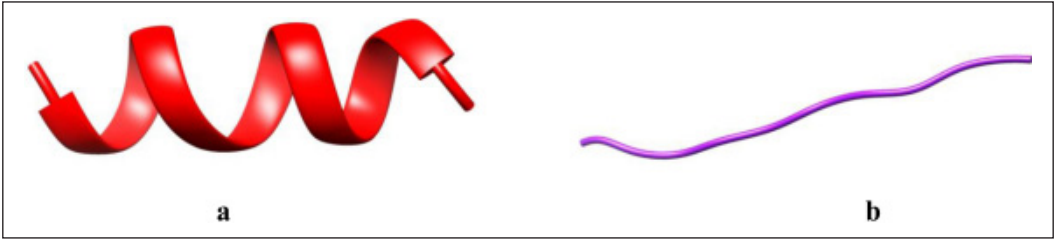


Figure 2. Mt.COI region based three dimensional (3D) protein models of *B. giganticus* (a) and *B. indicus* (b) predicted by Phyre2.

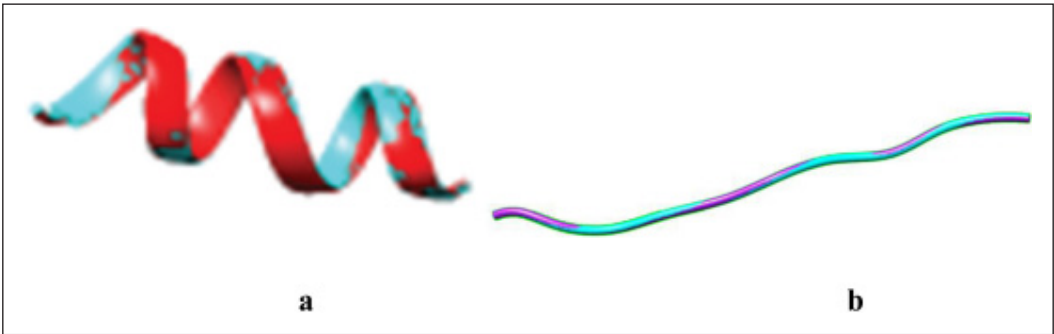


Figure 3. Target-template complex (a) *B. giganticus* (b) *B. indicus* predicted by Chimera.

disordered, α -helix, Beta-strand and TM helix value while *B. indicus* has 5%, 54%, 28% and 59% values, respectively. Phyre2 generated three dimensional conformation of *B. indicus* (coded with purple) and *B. giganticus* (coded with red) are completely distinct that are resultant of inter/intra (physico-chemical properties of amino acid residues arranged in string) polypeptide interactions (Figure 2 a-b). Structural identities between 3D conformations of target and template proteins facilitate superimposition of both on each other. In target-template complex, the identical amino acid sequences of target (*B.*

giganticus is coded with red and *B. indicus* with purple colour) protein and template protein (for both species) get the same colour (Cyan blue colour), while similar amino acid sequences remain in their original colour code (Figure 3 a-b).

However, 3D conformation of both query species (*B. indicus* and *B. giganticus*) are not superimposing on each other (as there is less than three amino acids are in alignment) due to genetic variations, evincing genetically distinct and valid species. Howbeit, RMSD (Root mean square value) values of both target-template complex sequences is 0.000 Å (increases

with sequence divergence), demonstrating sequence identity between target-template complexes (Table 6).

Phylogenetic analysis

The alignment of both species of *Bifurcohaptor* showed 65.5% identity, 5.2% gap and 29.0% transition/transversion for 28S region (alignment length, 361bp) and 53.11% identity, 5.48% gap and 41.39% transitions/transversion for ITS-1 region (alignment length, 401 bp) expressing genetic alliance and variation, respectively. Different taxa of the family Dactylogyridae were used to confirm their validity and earlier taxonomic placement of the genus *Bifurcohaptor*. 28S rDNA based tree depicted clustering of the genus *Bifurcohaptor* with other freshwater members of the family Dactylogyridae and forming a sister clade with members of the *Thaparocleidus*, supporting grouping with four anchored monogenoids (Figure 4). However, ITS-1 region based phylogenetic tree also showed grouping of *Bifurcohaptor* with other members of family Dactylogyridae. Present study, thus, supports earlier morpho-

taxonomy based systematic placement under the family Dactylogyridae. Sequences (encompassing ITS-1 region) of four as well as three anchored parasites were used for the construction of phylogenetic tree for ITS-1 region because very limited number of sequences of freshwater fish-parasite (four anchored) belonging to family Dactylogyridae are present on NCBI.

Host-specificity

On the basis of previous and present records, we are at the opinion, the genus *Bifurcohaptor* is a generalist parasite as it parasitizes to gills of *Mystus* spp., *Bagarius* sp. and *Hemibagrus* sp. (Table 2) while its species namely *Bifurcohaptor indicus* and *B. giganticus*, both are specialist monogenoid, where *B. indicus* belongs to sub-category mesostenoxenous (parasitize on fish species of same genus instead of two different genera of a family) of category mesoxenous while *B. giganticus* is a oioxenous parasite (parasitize only to *B. bagarius*). *B. indicus* and *B. giganticus*, collected from *Mystus* spp. and *Bagarius*

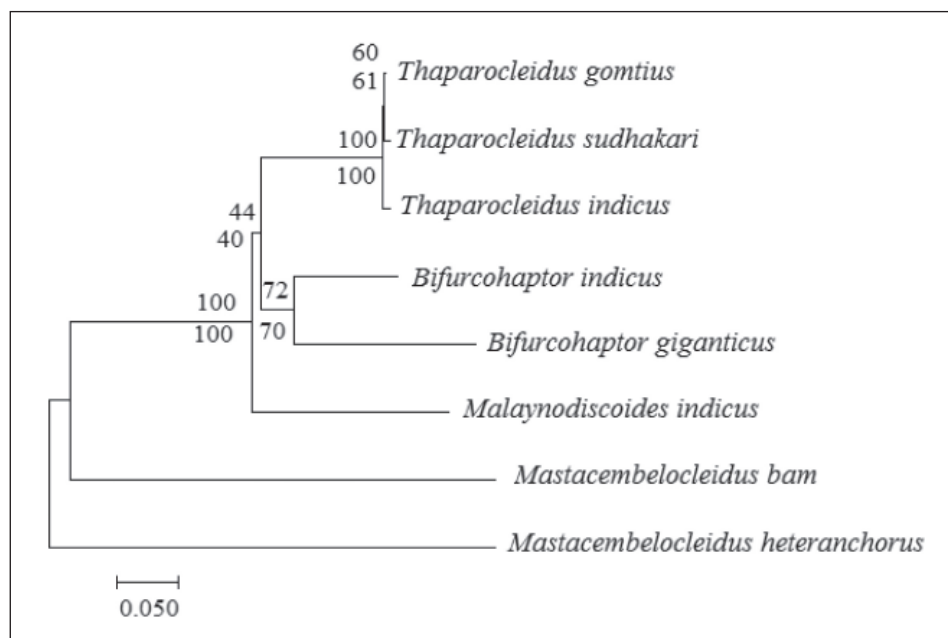


Figure 4. Phylogenetic tree (based on NJ and ME method) inferred from 28S ribosomal DNA. Bootstrap values ($n=1000$ replicate) are shown above and below the branches for ME and NJ respectively.

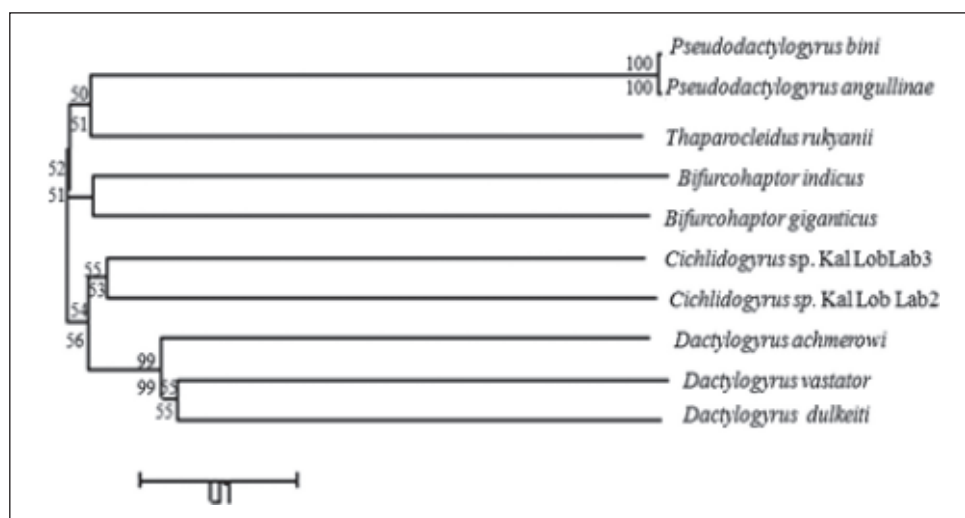


Figure 5. Phylogenetic tree (based on NJ and ME method) inferred from ITS-1 ribosomal DNA. Bootstrap values ($n=1000$ replicate) are shown above and below the branch for NJ and ME respectively.

bagarius respectively, are demonstrating host-specificity rather than host switching. Parasitological records also corroborate that *B. baungi* and *B. lanki* are host-specialist/oioxenous parasites (categorization of host specificity is according to Pojmańska & Niewiadomska, 2012) as these parasites do not parasitize to any other fish host. Since, March, 2011, a number of fish (Table 2) were collected from various localities of India and examined for *Bifurcohaptor* spp. Not a single species of *Bifurcohaptor* was collected from *Channa striata*, *Rita rita* and *Sperata seenghala* (earlier considered as host for *Bifurcohaptor*). Lim *et al.* (2001) have also suggested *Channa striata* is not a host for *Bifurcohaptor*.

DISCUSSION

Taxonomic history of the genus *Bifurcohaptor* is quite complex and validity of its species (Table 1) has been queried several times (Pandey & Singh, 1989; Lim *et al.*, 2001; Pandey *et al.*, 2002; Pandey & Agrawal, 2008). *B. indicus* and *B. giganticus* (Figure 6 a-b) are distinguishable by shape and size of haptor (attachment

parts), vagina and copulatory complex (reproductive parts) where haptor parts of parasite are of generic importance (Bychowsky, 1937; Gusev, 1976; Yamaguti 1963), while male copulatory complex and vaginal armature are species specific. It seems that occurrence of only two species in India shows genetic material of the genus *Bifurcohaptor* has not evolved over a long time period (due to slow rate of genomic DNA modification).

Protein data sets have been proved indispensable and staple source of information other than DNA (Telford *et al.*, 2000). The 1D data set based information also assists the genetic heterogeneity between both *Bifurcohaptor* spp. that turns into unique type of 3D conformation. The protein conformation (secondary and tertiary), based on translated mt COI sequence comparison suggests genetic distinction and validation of both species of *Bifurcohaptor*. The structural variations in 3D conformation of protein-coding mt COI region are excellent and explaining well genetic modifications, incorporated under influence of new ecosystem. The structural divergence between 3D conformation of *Bifurcohaptor* spp. is crucial for intra/inter species distinction in addition to nucleotide

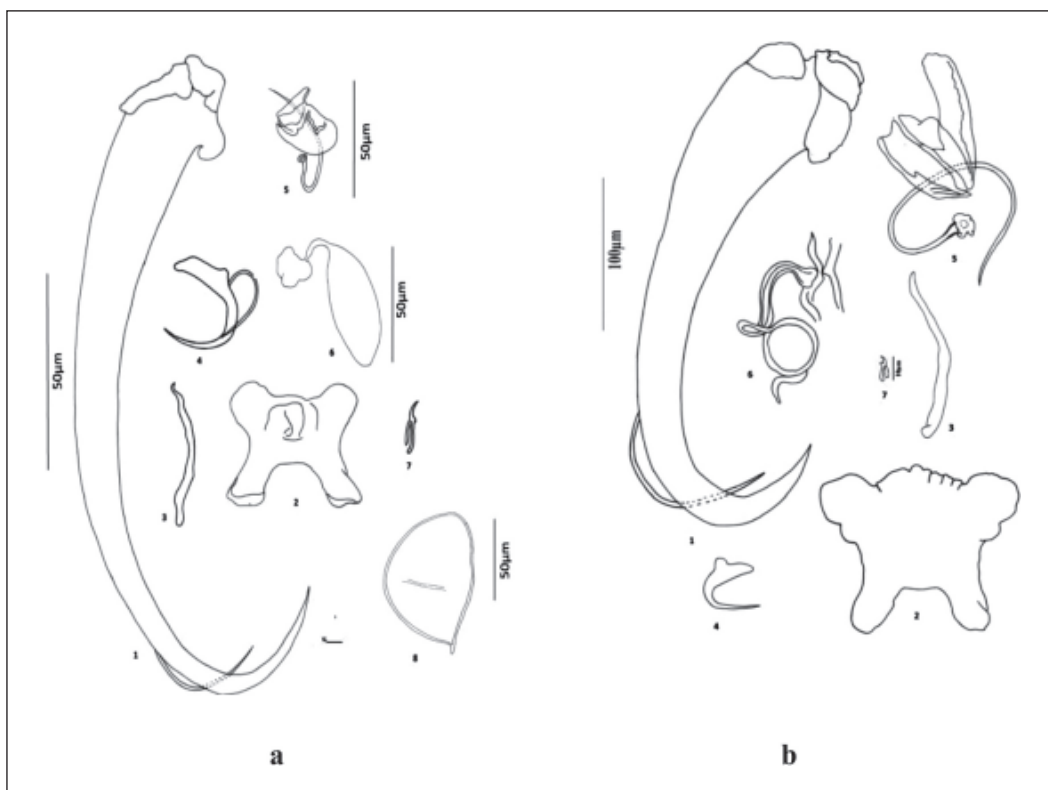


Figure 6. Sclerotized parts of (a) *B. indicus* and (b) *B. giganticus* (1. Dorsal anchor, 2. Dorsal bar, 3. Ventral bar, 4. Ventral anchor, 5. Copulatory complex, 6. Vagina, 7. Hook and 8. Egg).

depended identification. Involvement of protein conformation is a step ahead that further strengthens and explores morphological and molecular indagation as these structures offer quick view for genetic differentiation of species in comparison to X-ray crystallography and NMR structures (Sanchez & Sali, 1997). The target-template alignment improves as the sequence identity between target-template increases (Sutcliffe *et al.*, 1987; Bajorath *et al.*, 1994; Johnson *et al.*, 1994; Rost & Sander, 1996) which decreases with increased RMSD values in Angstrom (Gan *et al.*, 2002). This feature assists to evaluate the geographical impact on genetic material of *Bifurcohaptor* spp., parasitizing fish-fauna, as it is direct under climate control and is foremost for survival of progeny. Often these characters accumulate and pave the way for establishment of new species with completely different set of morphological and genetic characters (Orengo & Thronton, 2005).

Alteration in core region of protein is the main ground for establishment of new species. Thus, 3D protein conformation based insilico study self-explains species variation and validation of *Bifurcohaptor* spp.

The intraspecific genetic divergence between *B. indicus* and *B. giganticus* is 34.3% for large subunits and 46.87% for ITS-1. 28S and ITS-1 region based phylogenetic results provide novel insights on genetic validity of *Bifurcohaptor* spp. and relationship with other representatives of the family Dactylogyridae. The phylogenetic analysis showed nesting of *Bifurcohaptor* spp. with other members of family Dactylogyridae, which supports morphology based placement of the genus *Bifurcohaptor* under Dactylogyridae. Molecular approach has proved here, an effective tool for unerring species identification and differentiation. It also authenticates species identification of

immature parasites, being incompetent for morphological distinction of genus/species that can mislead the results. Characterization of two *Bifurcohaptor* spp. through molecular techniques are providing here first time genetic information.

Host specificity

Our studies suggest, the genus *Bifurcohaptor* parasitizes only to the family Bagridae (*Mystus vittatus*, *M. tengara*, *M. keletius* and *Hemibagrus nemurus*) and Sisoridae (*Bagarius bagarius*). The host-switching case reports for the genus *Bifurcohaptor* are not yet recorded. We have observed that *B. indicus* and *B. giganticus* are specialist (host-specific) parasite, where, *B. giganticus* is a strictly host specific/oioxenous parasite while *B. indicus* is a specialist and belongs to mesostenoxenous of mesoxenous category. However, the genus *Bifurcohaptor* displays characteristic for euryxenous/polixenous/generalist (Pojmańska & Niewiadomska, 2012). The genus *Bifurcohaptor* follows general concept of host specificity where haptor parts of parasite adaptively radiate according to host specificity. Parasites with variable haptor armature are basically generalist while rest are specialist (do not have much variation). *Bifurcohaptor* reports from Meghalaya (Thapa *et al.*, 2011), Sri Lanka (Gusev, 1976), Malaysian Peninsula (Lim & Furtado, 1983) and Thailand (Lerssutthichawal & Lim, 2005) also suggest occurrence of *Bifurcohaptor* on *Mystus*, *Hemibagrus* and *Bagarius*, confirming the specificity of the genus *Bifurcohaptor* and its species. The world wide survey of Bagrids and Sisorids is urgently needed to access the actual position of *Bifurcohaptor* as the host-specificity often treated as bio-marker/biological tag for error-free host-identification (Hayward, 2005; Rajvanshi *et al.*, 2015; Agrawal *et al.*, 2016). Host-specificity may also play an important role in population reduction or extinction of parasites along with host under unfavourable environmental conditions. Bio-invasion of host along with inevitable parasite having similar niche to that of native species that can be a cause for

reduction/extinction of host with its parasites, if it is dominant over native species (Rajvanshi *et al.*, 2015). Enhancement in niche-size can favour survival of parasites in modified environment (Agrawal *et al.*, 2016).

Thus, the morphological and molecular study portrays the genus *Bifurcohaptor* encompasses only two species in India viz. *B. indicus* and *B. giganticus* within the family Dactylogyridae. *B. indicus* and *B. giganticus*, both are genetically distinct and valid species. *B. indicus* and *B. giganticus*, both being specialist belongs to mesostenoxenous of mesoxenous and oioxenous category respectively where as the genus *Bifurcohaptor* is generalist.

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