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Rapid Communication

First molecular identification of invasive tapeworm, *Bothriocephalus acheilognathi* Yamaguti, 1934 (Cestoda: Bothriocephalidea) in India

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Abstract

During the helminthological survey of non-native fishes in Meerut region, UP, India, specimens of genus *Bothriocephalus* were collected from introduced fish green swordtail *Xiphophorus hellerii* Heckel, 1848, a native of North and Central America. The morphological and molecular study inferred with partial sequence of 18S and 28S rRNA confirmed the specimens as *B. acheilognathi*. Phylogenetic analysis further confirmed its taxonomic status, as it comes under the same clade formed by *B. acheilognathi* species reported from other geographical regions. This study first time describes the molecular identification of *B. acheilognathi* from India. The findings of the study also established its ecological impact in northern parts of India and highlights that low degree of host specificity can affect the native fish resources of India.

Key words: Cestode, Meerut, *Xiphophorus hellerii*, 18S, 28S

Introduction

Invasions by non-native parasite species on freshwater fishes have been the subject of study from many decades the world over (Bauer and Stolyarov 1961; Dogiel 1966; Kennedy 1994; Paperna 1996; Marcogliese 2008; Han et al. 2010; Choudhury et al. 2013; Muchlisin et al. 2015; Salgado-Maldonado et al. 2015). Non-native species represents one of the most serious threats to native fish host (Elvira and Almodóvar 2001; Riberio et al. 2007, 2009; Lusk et al. 2010; van Rensburg et al. 2011).

Non-native freshwater parasites have been recorded in association with their exotic host. Invasions by these parasites have attracted the attention of scientists (Daszak et al. 2000; Torchin et al. 2003; Taraschewski 2006; Kelly et al. 2009; Mastisky and Veres 2010; Paterson et al. 2011; Poulin et al. 2011; Lymbery et al. 2014) to mitigate their impact. Native taxa can be damaged by introduction of non-native species that extends parasite range by the host-switching from new native ones (Poulin et al. 2011; Lymbery et al. 2014). Despite this, non-native parasites studies are often ignored in India unless they alter the biodiversity of the freshwater ecosystem (see Zargar et al. 2012).

During a helminthological survey of non-native fishes in Meerut region, UP, India, specimens of *Bothriocephalus acheilognathi* Yamaguti, 1934 were collected from introduced ornamental fish *Xiphophorus hellerii* Heckel, 1848 also called as green swordtail. The various synonyms of *B. acheilognathi* are, *B. opsariichthydis* Yamaguti, 1934, *B. gowkongensis* Yeh, 1955, *B. kivuensis* Baer and Fain, 1958, *B. phoxini* Molnar, 1968, *B. aegyptiacus* Ryšavý and Moravec, 1975 and *Schyzocotyle fluviatilis* Akhmerov, 1960 (Kuchta and Scholz 2007). In the past, no studies have been carried out on the parasite fauna of green swordtail in India. The native range of Asian tapeworm *B. acheilognathi* comprises the Amur River that established the border between China and eastern Russia (Choudhury and Cole 2012). This worm is listed as a 'Pathogen of Regional Concern' by the US fish and Wildlife Service (2012) and can cause massive kill of fishes at high infection levels. The parasite has much potential to infect new fish species, having highly adaptable capability to naive environment (Kennedy 1994) and reported almost from every part of the world. Recent surveys showed that *B. acheilognathi* inhabits some non-cyprinid fishes also and the geographical distribution of this cestode is still goes on increasing significantly (Hoole 1994; Font and Tate 1994; Scholz 1997; Dove et al. 1997; Nie et al. 2000; Scholz et al. 2012).

Although, many morphological studies have been conducted for the presence of non-native *B. acheilognathi* in Northern India (Rukhsana et al. 2011; Sofi and Ahmad 2012; Zargar et al. 2012; Sheikh et al. 2014; Farooq et al. 2014), but none of them confirmed the presence of *B. acheilognathi* by molecular methods. Here we report the presence of non-native cestode species *B. acheilognathi* in India introduced by a non-native host, using molecular identification techniques.

Materials and methods

Parasite collection

Specimens of non-native fish *X. helleri* (n=20) were collected from growers of the aquarium trade in Meerut (29º01′N, 77º45′E), U.P., India. Identification of fish were carried out by ichthyologists, after that kill them with a sharp blow on the top of the head. Various body organs like gills, skin, body cavity and gastrointestinal tract were screened under a Motic stereomicroscope (SMZ-168 series). A total of four cestode parasites were collected from gastrointestinal tract that further processed for morphological and molecular study.

Worms were washed in saline and a small fragment from the strobila was cut and stored in 95% ethanol at -20^{0}C until DNA extraction. Rest of worm body were fixed in hot 70% ethanol after relaxation in lukewarm water for further processing of morphological study. Following staining, worms were stained with acetocarmine, dehydrated using ascending grades of alcohol series, cleared in xylene and mounted in Canada balsam. Voucher specimen i.e., hologenophores (the specimen from which the molecular sample was taken) were submitted to the Museum of the Department of Zoology, Chaudhary Charan Singh University, Meerut, U.P., India (HS/Ces/2014/01) and Natural History Museum, Geneva, Switzerland (MHNG-INVE-91838).

Molecular analysis

Genomic DNA was extracted from preserved strobila using DNAeasy Tissue Kit (Qiagen) according to manufacturer's protocol. PCR reaction were performed in a Thermal Cycler (Eppendorf Mastercycler personal) in total volume of 25 µl according to the concentrations described by Chaudhary and Singh (2012). The thermocycling profile was as follows: an initial denaturation at 94º C for 3 min, followed by 35 cycles of 94º C for 30s, 57º C for 45s, 72º C for 1 min, and was completed with a final extension at 72º C for 10 min, then stored at 4º C. Partial sequence of 28S rRNA gene (555 bp) was amplified by using the primers suggest by Mollaret et al. 2000. The ribosomal 18S gene (1916 bp) was amplified using the set of following primers, forward: 5'–GCGAATGG CTCATTAAATCAG3'; reverse: 5'–CCGTCAA TTCCTTTAAGT–3' (Littlewood and Olson 2001) and forward: 5'–TCGGTTGATCCTG CCAGTAG–3'; reverse: 5'–GTACAAAGGGCA GGGACGTA–3'. PCR products were purified by Purelink^{TM} Quick Gel Extraction Kit (Invitrogen). PCR products were sequenced directly for both strand using a Big Dye Terminator version 3.1 cycle sequencing kit in ABI 3130 Genetic Analyser, Applied Biosystems with the same primers as above.

The sequences were submitted to the GenBank with accession number KP062950 (for 28S) and KP062951 (for 18S). Sequences generated in this study were compared with sequence available in genomic database using Blast on NCBI (http://www.ncbi.nih.gov). Alignment was performed with ClustalW (Thompson et al. 1994) using default parameter settings. The alignment was corrected manually in the software MEGA 6 (Tamura et al. 2013) using alignment editor. Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were performed. The topologies resulted were compared to each other. ML was performed in MEGA 6 under GTR+G+I model. Bootstrap values based on 1,000 resampled datasets were generated.

Bayesian analysis was performed by Topali 2.5 (Milne et al. 2008) based on the GTR+G+I model. Posterior probabilities were estimated over 1,000,000 generations by two independent runs and the burn in was 25. *Parabothriocephalus segmentatus* (AB559562, AB559563 and DQ925314) were used as an out-group in the alignment for rooting the phylogenetic tree.

Figure 1. Scolex of *B. acheilognathi* from *Xiphophorus hellerii*. Scale-bar: 40 mm. Photomicrograph by Anshu Chaudhary.

Results

The tapeworm was identified as *B. acheilognathi* on the basis of characters described by Scholz (1997). Due to the lack of proper descriptions, or molecular analyses, species validity within India has been questionable in the case of many parasitic taxa. For example, the following species from India have been synonym to *B. acheilognathi* by Kuchta and Scholz, 2007 are: *Ptychobothrium phuloi* Shinde and Deshmukh, 1975; *Ptychobothrium khami* Shinde and Deshmukh, 1975; *Ptychobothrium chelai* Shinde and Deshmukh, 1975; *Ptychobothrium nayarensis* Malhotra, 1983; *Bothriocephalus teleostei* Malhotra, 1984 and *Capooria barilii* Malhotra, 1985. Figure 1 show the typical scolex of specimens collected from *X. hellerii*. Morphological examination showed that cestode having an arrow or heart shaped head, antero-laterally directed scolex, narrow slit like grooves, without distinct neck and proglottids begin directly behind the scolex. Besides these, other characteristics of the strobila of parasite are the same as described by Scholz (1997).

Molecular analyses inferred from partial sequences of the 28S rDNA have shown close relationships with all the sequences available of same species (Figure 2). This gene 28S shows a more conserved region in *B. acheilognathi* sequences. The maximum likelihood and Bayesian analysis performed from 28S sequences represents the same topology, so the tree generated by ML is shown here (Figure 2) with high bootstrap values. Figure 3 is based on the 18S rRNA gene and it shows deep phylogenetic relationships as compare to 28S rRNA gene in *B. acheilognathi* sequences. All the *B. acheilognathi* isolates were divided into three clades. One clade for the isolates of China, France and Panama, another for the isolates of Czech Republic, while the remaining were for the isolates of India, South Korea, Czech Republic and USA (Figure 3). The Indian isolate showed close relationship with same species represented in the GenBank from South Korean (GU979860), European (AY340106) and USA (DQ866988 to DQ866992) isolates (Figure 3). Both analysis, ML and BI recovered the same clade and topologies indicating the affiliation by the bootstrap support (Figure 3). Pairwise comparisons among the 18S rDNA sequences for *B. acheilognathi* species showed that Indian isolate exhibited a higher percentage of identity to above isolates (100%).

Discussion

Our study enabled the identification of an invasive cestode from Indian water. It also provides the first genetic evidence for *B. acheilognathi* from India. However, previous observations showed *B. acheilognathi* establishment in the northern part of India (Rukhsana et al. 2011; Sofi and Ahmad 2012; Zargar et al. 2012; Sheikh et al. 2014; Farooq et al. 2014) based on morphological characters. Parasite species identification based only on morphological tools is complicated. In general, additional molecular analysis is necessary to confirm species identification. Although morphological characters, in the case of *B. acheilognathi*, are sufficient for correct identification, precision can be achieved by utilising supporting molecular analysis.

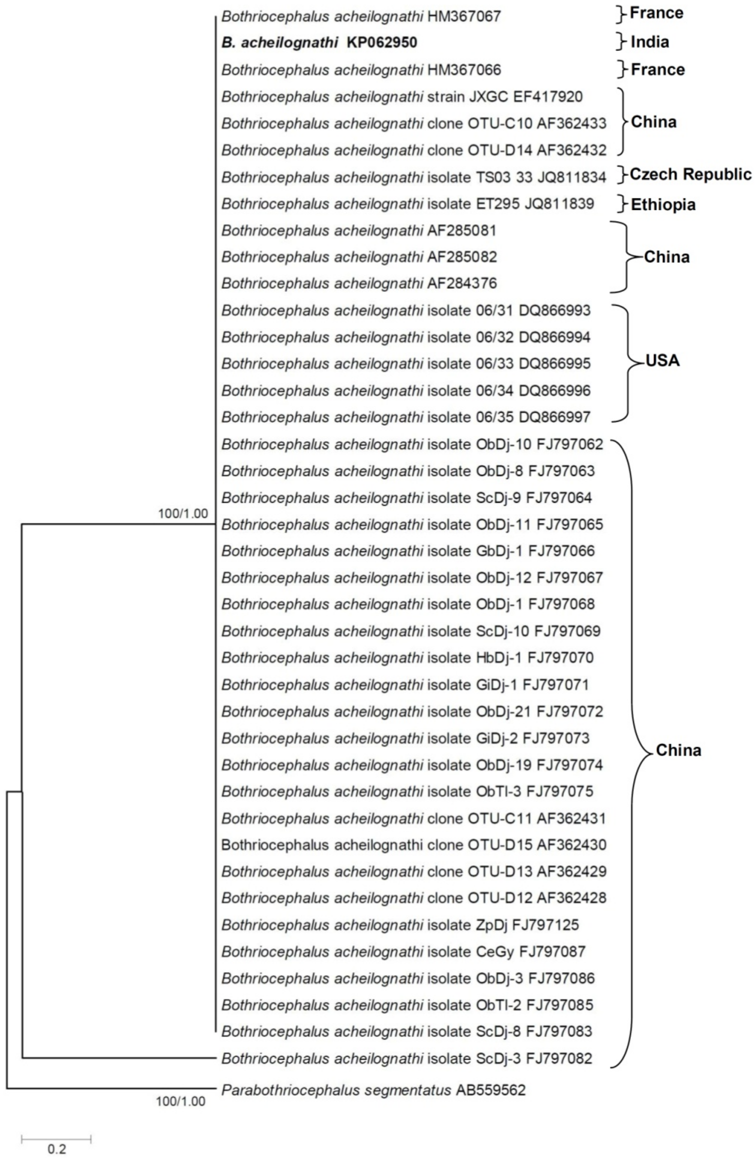

Figure 2. Phylogenetic tree generated for Indian isolate *B. acheilognathi* from swordtail. The tree was constructed on the basis of the 28S rDNA sequences using maximum likelihood analysis along with Bayesian inference probabilities. *P. segmentatus* was used as out-group.

It has been shown in many studies, that geographical distribution of this cestode is still increasing significantly (Hoole 1994, Font and Tate 1994; Scholz 1997; Dove et al. 1997; Nie et al. 2000; Scholz et al. 2012; Choudhary et al. 2013; Muchlisin et al. 2015; Salgado-Maldonado et al. 2015). The species has been documented in almost every part of the world (Scholz 1989; Scholz and Dicave 1993; Paperna 1996; Dove et al. 1997; Font 1998; Rejo et al. 1999; Dove and Fletcher 2000; Luo et al. 2002; Choudhary et al. 2006; Marcogliese 2008; Perez-Pence de Leon et al. 2010; Scholz et al. 2012; Zargar et al. 2012; Kuchta et al. 2012; Choudhary et al. 2013). An example of previously cited observations, was the recorded presence of *B. acheilognathi* in the Kashmir valley, where it quickly infested the indigenous host *Schizothorax niger* Heckel, 1838, a fish species with subsequently reduced populations in the Kashmir valley (Rukhsana et al. 2011; Sofi and Ahmad 2012; Zargar et al. 2012; Sheikh et al. 2014; Farooq et al. 2014).

Most of the research about the presence of *B. acheilognathi* in India was from northern part, around lakes of Srinagar, Jammu and Kashmir (Figure 4). The presence of the Asian fish tapeworm

Figure 3. Phylogenetic tree constructed on the basis of maximum likelohood (ML) analysis of the 18S rDNA sequences of *B. acheilognathi*. Number at nodes showed the bootstrap values (ML) and posterior probabilities (BI). *P. segmentatus* was used as out-group. The scale-bar represents the expected number of substitutions per site.

from various lakes in Srinagar testifies that this worm has the potential to cause infection in the indigenous freshwater fishes of India with potential to impact freshwater ecosystems. The low specificity regarding the host has easily allowed this worm to spread in a new host and new regions.

However, information is very scarce about the possible impacts of this tapeworm on the endemic fishes in other parts of India. Although, the initial introduction of *X. helleri* into India was not well documented, it was possibly introduced to revitalize the aquarium market. However, no consideration was made with regard to its parasitic fauna. Besides this, we predict that the infection of *B. acheilognathi* probably comes by an overlooked factor, i.e. an establishment of parasites in the intermediate hosts. Tapeworms with low host specificity can enter aquariums harboured in live planktonic copepods (intermediate host) caught from the wild used for feeding purposes. Of

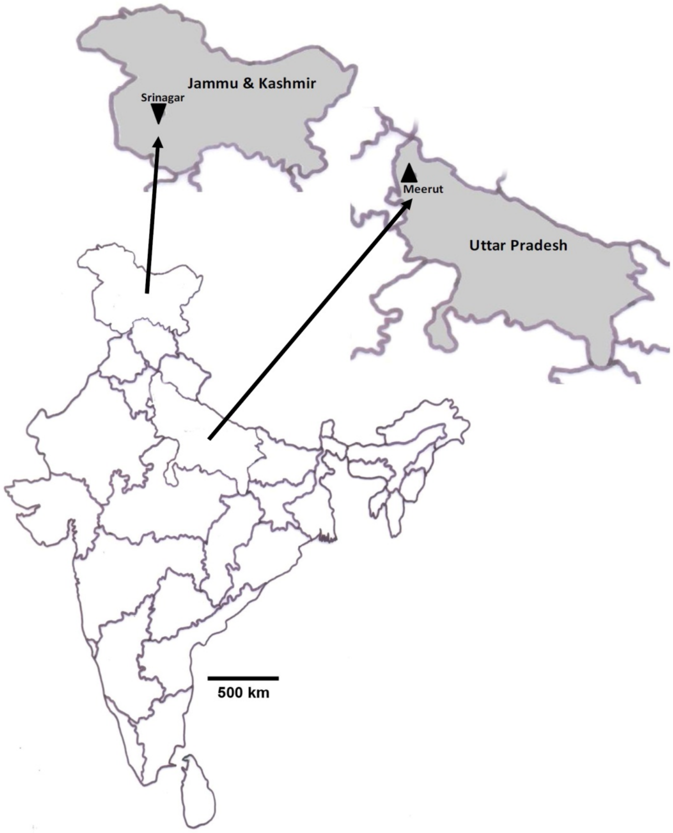

Figure 4. Map showing the distribution and presence of *B. acheilognathi* in two states of India i.e., Jammu and Kashmir and Uttar Pradesh comprises two districts, Srinagar and Meerut.

particular interest is the fact that in India risk of spreading of invasive species via imported fish is an actual threat for ornamental breeding fish. Further release of infected ornamental fish into freshwater ecosystems may represent a serious risk for the spreading of this worm.

The 28S rRNA gene used for *B. acheilognathi* studies (Kuchta et al. 2012) shows a high degree of conservation in the sequences of isolates (Figure 2), that is why we also chose the 18S rRNA gene for discrimination among different isolates (Figure 3) as several molecular studies have been focused on 18S for this group of parasites (Mariaux 1998; Kodedová et al. 2000; Škeříková 2004; Kuchta et al. 2008). The 18S gene in the present investigation seems to be more useful for resolving phylogenetic relationship in the tree. The three closely related clades of isolates reported from different geographical regions exhibit unequivocal host-specificity, as display by the clades. The results also clearly indicate that *B. acheilognathi* isolates are genetically polymorphic species. Supplementary investigations of *B. acheilognathi* is required from various parts of India, based on molecular markers, to know the distribution and potential impacts of this parasite on the indigenous fish fauna of India.

Moreover, the study also emphasizes the need for more intensive fish sampling from different regions to provide better understanding and documentation. Monitoring programmes including fish veterinary inspections should also be undertaken as control measures for parasites.

Conclusions

Non-native parasite species in India are mostly identified based on morphological traits that may lead to erroneous conclusions. In contrast, the molecular data can provide an unequivocal tool for identification. This study strengthens the importance of genetic analysis for the study of *B. acheilognathi* present in Indian freshwater. In future, the molecular data from Indian region can help in the identification of the species for further comparison. In addition, we confirm the presence of *B. acheilognathi* in India, based on genetic data analyzed for the first time in this country.

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