Genetic characterisation of *Tanqua* (von Linstow, 1879) (*Nematoda: Gnathostomatidae*) larval forms including new host and locality records

Michelle Williams\textsuperscript{a,}\textsuperscript{*}, Marta Hernandez-Jover\textsuperscript{a}, Md Shafaet Hossen\textsuperscript{a,b}, Shokoofeh Shamsi\textsuperscript{a}

\textsuperscript{a} School of Agriculture, Environmental and Veterinary Sciences & Graham Centre for Agricultural Innovation, Charles Sturt University, Wagga Wagga, NSW, 2678, Australia
\textsuperscript{b} Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh, 2202, Bangladesh

\textbf{A R T I C L E   I N F O}

\textbf{Keywords:}

*Channa punctata*

*Tanqua* species

Bangladesh

\textbf{A B S T R A C T}

In an unrelated study of spotted snakehead fish *Channa punctata* (Bloch) of family Channidae (N = 103) from Bangladesh, ten fish had taupe and clear coloured cysts attached to the intestinal mesentery. Investigation of the cysts revealed larval nematodes. The larvae were damaged and not suitable for detailed morphological study, however, key features such as tooth like projections of the pseudolabia and lateral pseudolabium were observed in specimens with undamaged cephalic regions. Molecular characterisation was undertaken and although the parasite genetic material was poor, five of the twelve nematode larvae through sequencing of the 18S ribosomal RNA gene, showed 98.17\% match with sequences assigned for *Tanqua tiara* (accession number JF934728) deposited in GenBank. The prevalence of infection was 9.7\% and the mean intensity 2.70. *Tanqua* has not previously been identified in fish, or from the definitive host, the Asian water monitor *Varanus salvator* (Laurenti, 1768) of family Varanidae (class Reptilia), in Bangladesh. Therefore, this study represents a new host and locality record for this nematode species. In many previous reports from this region, nematode larvae have been identified morphologically and assigned to a diverse range of nematode genera. Some confusion therefore exists regarding their accuracy and further investigations are required using molecular methodology to clarify the species of larval nematodes which infect edible fish in Bangladesh.

1. Introduction

Spotted snakehead fish *Channa punctata*, is a hardy, air breathing, benthopelagic and potamodromous freshwater species (Prasad et al., 2011; Karnatak et al., 2020) which is distributed throughout the Indian sub-continent (Pakistan, India, Sri Lanka, Nepal, Bangladesh) (Qadir and Malik, 2011; Karnatak et al., 2020), Yunnan in China and Myanmar (Chaudhry et al., 2019; Islam et al., 2020). Due to the plethora of freshwater environments in Bangladesh, species of small indigenous fish are widely available and provide a dietary source of high-quality protein, which is also cheap to purchase (Islam et al., 2020). In a study of *C. punctata* from Bangladesh, commercially obtained from a fish market in Dhaka, fish were found to have a proximate protein composition of 15.91 ± 0.34\% (Hossain et al., 1980). *Channa* are also claimed to be medically important and consumption of *C. striata*, for example, has been anecdotally linked to rapid wound healing and reduced pain after surgery (Gam et al., 2006).

*Channa punctata* is distributed in swamps, ponds and ditches and as adults in muddy streams and stagnant water bodies (Alam et al., 2019). *Channa* spp. are known as carnivorous and voracious predators of small fish/fries, frogs, young turtles and may even prey on ducklings (Mustafa et al., 2012; Deshmukh et al., 2020). Due to these feeding habits, *C. punctata* may become highly infected with intestinal parasites (Chowdhury and Hossain, 2015). Many nematode species have been described in *C. punctata* however, there is a great deal of taxonomic confusion regarding larval nematodes identified from *C. punctata* in Bangladesh (Table 1). It is recognised that identification of larval nematodes to a species level morphologically is not reliable (Borges et al., 2012; Shamsi and Suthar, 2016a; Moravec and Nagasawa, 2018; Mazzone et al., 2019; Abro et al., 2020) because most features are absent in larval specimens. Therefore, the aim of this study was to identify larval nematodes recovered from the intestinal mesentery of *C. punctata* (Bangladesh) using molecular methodology.

\textsuperscript{*} Corresponding author.

\textit{E-mail address:} miwilliams@csu.edu.au (M. Williams).

https://doi.org/10.1016/j.ijppaw.2022.01.001

Received 17 October 2021; Received in revised form 17 December 2021; Accepted 3 January 2022

Available online 6 January 2022

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2. Materials and methods

2.1. Parasite collection

Fish were thawed before dissection. It is unknown how long the fish had been frozen preceding examination. The methods used for fish examination, parasite collection and isolation followed the description in Shamsi and Suthar (2016b). Fish were then lightly blended and artificially digested in a pepsin physiological saline solution (concentration of 15 mg/L) following the method described in Bier et al. (2001). The pepsin/fish solution was processed at 37 °C, 100–120 rpm for 16–20 h. The digesta was passed through a 1000-μm mesh strainer and the clarity adjusted with physiological saline before examining under a dissecting microscope (Leica EZ4 Stereo Microscope). Collected parasites were stored in 1.5 mL sterile Eppendorf® tubes containing 70% ethanol pending molecular identification.

2.2. Parasite processing

The prevalence (P), mean intensity (MI), and mean abundance (MA) of larval nematodes were calculated following Bush et al. (1997). For molecular study, a small piece was excised from the mid-body of twelve larval nematodes using a sterile scalpel blade and the excised piece transferred to 1.5 mL sterile Eppendorf® tubes as described in Shamsi et al. (2008). The anterior and posterior portion of each nematode were then slide mounted and cleared with lactophenol for image capture using an Upright Motorized Microscope ECLIPSE Ni-E, Nikon, Japan.

2.3. Genetic characterisation

Genomic DNA from 12 larval nematodes were extracted according to the method described in Shamsi et al. (2019) using DNeasy Blood & Tissue Kits (QIAGEN, Germany) and eluted by 40 μl of elution buffer. Since the 18S gene has been successfully used as a genetic marker for studying Tanqua and other Spirurina parasites in freshwater aquatic species (Laetsch et al., 2012; Choudhury and Nadler, 2018; Schoeman et al., 2020), samples from 12 larval nematodes were amplified using the primer sets Forward: 35 fm (5’-TATAATGTTGAAACCGCAACGGC-3’) and Reverse: 18gMm (5’-GGAAACCTTGTTACGACTTTTGCC-3’). The cycling conditions for PCR were as follows, 95 °C for 2 min followed by 95 °C for 30 s, 48 °C for 45 s, 72 °C for 1 min for 40 cycles and a final extension of 72 °C for 10 min.

For each amplicon, a 3 μl aliquot was examined on a 1.5% w/v agarose gel, was stained with GelRed™ after which a photograph was taken using a gel documentation system. Five of the 12 amplified samples were sent for sequencing to the Australian Genome Research Facility (AGRF). The chromatogram and sequence data were observed using the Sequence Scanner software (Applied Biosystems® Genetic Analysers). BLAST search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to conduct homology searches. The sequences generated from AGRF were cleaned first and then aligned using MUSCLE (in MEGA v. 10) (Kumar et al., 2016, 2018) to verify any variation between sequences.

The 18S sequences of Gnathostomatidae available in GenBank were included in phylogenetic analyses. Five new sequences generated in the present study for Tanqua sp. from the type-host Channa punctata in Bangladesh were also added (Table 2). Cucullanus robustus (accession number JF934726) was used as the outgroup based on earlier Gnathostomatidae-related phylogenetic analysis (Laetsch et al., 2012). The phylogenetic tree was inferred using the Maximum Likelihood method (MEGA v. 10) and is presented as Fig. 1.

3. Results

Laval nematodes were encysted in or on the intestinal mesentery, some in round taupe-coloured resilient sacks, others in fragile transparent cysts and three were encysted in musculature. A total of ten C. punctata were infected with 27 larval nematodes, prevalence was 9.7%; mean intensity 2.70 and mean abundance 0.30 (Table 3).

3.1. Larval morphology

No morphological description was provided for adult Tanqua tiara from Varanus indicus, Australia in Laetsch et al. (2012 Supp. data 18.S. haplotypes) however Schoeman et al. (2020) provided morphological description for larval stage (L3) Tanqua sp. from Xenopus laevis, South Africa. Twelve of the specimens in the present study used for DNA extraction were entire, and although internal structures were damaged (Fig. 2A) the cephalic region of the 12 entire specimens followed the description of the cephalic region of adult Tanqua tiara in Sou (2020) in which large lateral pseudolabia and tooth like projections were described. Fig. 2A and B shows the anterior of two of the 12 specimens used for DNA extraction and identified at Tanqua sp. Fig. 2 B at 40X magnification shows the tooth like projections of the pseudolabia (II) and the large lateral pseudolabia (lp). Schoeman et al. (2020) describes the pseudolabia as minute and although tooth like projections are not described in text these can be observed in Fig. 6B of the publication. The entire body, of all of the 12 specimens used for DNA extraction in the

| Hosts | Parasite taxa | Family | Site | Localities | Reference |
|-------|---------------|--------|------|-----------|-----------|
| C. punctata | Ascaridia sp. | Compositae | Digestive tract, viscera, body cavity | Dhaka, Bangladesh | Huq et al. (1985) |
| Ascaridia sp. | Compositae | Intestine | Mymensingh, Bangladesh | Farzana et al. (2019) |
| Ascaris sp. | Compositae | Stomach | Dhaka, Bangladesh | Huq et al. (1985) |
| Camallanus sp. | Camallanidae | Unknown | Bangladesh | Chandra (2006) |
| Camallanus intestinalis | Camallanidae | Intestine | Dhaka and Sylhet, Bangladesh | Bashirullah (1974) and Khalil et al. (2014) |
| Camallanus pearsi | Camallanidae | Unknown | Bangladesh | Chandra (2006) |
| Contracecum sp. larva | Anisiakidae | Intestine | Mymensingh, Bangladesh | Ali (1968) and Farzana et al. (2019) |
| Echinococclus sp. | Gnathostomatidae | Unknown | Bangladesh | Chandra (2006) |
| Neocamallanus sp. | Camallanidae | Pyloric caeca | Chittagong, Bangladesh | Arthur and Ahmed (2002) |
| Neocamallanus ophiocephali | Camallanidae | Pyloric caeca, intestine | Dhaka or Sylhet, Bangladesh | Ahmed (1981); Bashirullah (1973) |
| Paracamallanus spiculogubernaculus | Camallanidae | Unknown | Bangladesh | Chandra (2006) |
| Paracamallanus sweeti | Camallanidae | Unknown | Bangladesh | Chandra (2006) |
| Porcupacum sp. | Camallanidae | Intestine | Mymensingh, Bangladesh | Farzana et al. (2019) |
| Procamallanus sp. | Camallanidae | Intestine | Dhaka, Bangladesh | Huq et al. (1985) |
| Procamallanus (Spirococulum) | Camallanidae | Stomach, intestine | Chittagong, Dhaka, Sylhet, Bangladesh | Bashirullah (1973) and Ahmed (1981) |
present study, were heavily annulated and although characteristic of a number of other genera of nematodes (Moravec et al., 2007; Pereira et al., 2014; Velarde-Aguilar et al., 2015; Ocampo-Salinas et al., 2021), are also described in adult Tanqua tiara (Sou, 2020). Specimens identified as Tanqua sp. (Fig. 2C and D) in the present study show the trunk is heavily annulated and these annulations cover the entire parasite. Schoeman et al. (2020) did not describe annulations in the morphological description of L3 Tanqua sp.

3.2. Molecular identification

The 18S sequences of three specimens (voucher numbers 674-1; 678-9), were explored at 1581 bp long. Voucher numbers 678-12 and 678-10 showed bp of 1405 and 1001 long, respectively. However, all of the 12 specimens used for DNA extraction, despite molecular characterisation, were identified as Tanqua sp. in Schoeman et al. (2020). Intraspecific morphological variations of adult T. tiara has been reported (Sou, 2020). The specimens in the present study showed low genetic variability with adult T. tiara (1.8%: JF934728) in Laetsch et al. (2012) and by L3 Tanqua sp. (1.2%: MN526252) in Schoeman et al. (2020). The differences in body annulations and size of the lateral pseudolabia between L3 larvae in Schoeman et al. (2020) and the present study may be a consequence of a the difference in geographical locality, host or larval developmental stage. Further morphological and genetic study is required to explore these relationships.

The phylogenetic tree clustered the sequences obtained in the present study with the sequences registered for Tanqua species in GenBank with a strong bootstrap value (Fig. 1). Therefore, the present study confirms the C. punctata as a host of Tanqua species in Bangladesh. As the specimens in the present study were larval stage and not suitable for detailed morphological study authors have assigned our specimens to Tanqua sp.

A search of the literature for published records of Tanqua sp. in the definitive host, the Asian water monitor, Varanus salvator, or any species of fish from Bangladesh was unsuccessful. Previous records of nematodes identified in Channa punctata from Bangladesh are included in Table 1. The Asian water monitor V. salvator, commonly found in Asia and the Indian sub-continent, is considered the definitive host of Tanqua sp., although other members of Varanus genus have also been identified as hosts (Schoeman et al., 2020). In Bangladesh, V. salvator is distributed mainly in mangroves where they scavenge a huge array of prey, particularly during the wet season when most foraging activity occurs (Rahman et al., 2017b). Crabs, toads, small fishes, frogs, shrimp- prawns, birds’ eggs, water birds and kitchen scraps are all foraged by V. salvator in Bangladesh (Rahman et al., 2017a). Although distribution of V. salvator in Bangladesh is in mangroves this species also frequent, ponds, swamps, sewers and drains which reflects the habitat where C. punctata is commonly distributed (Froese and Pauly, 2018). Eggs shed in the faeces of V. salvator infect multiple aquatic species (Agustin et al., 2017) many of which are also foods predated by C. punctata (Deshmukh et al., 2020).

In the present study, 27 larval nematode specimens were recovered from cysts attached to the mesentery or the musculature of ten C. punctata from Bangladesh. Five of the twelve nematodes used for molecular characterisation, were identified as Tanqua sp. representing a new host and locality record for this genus. Molecular identification of seven of the 12 larval nematodes used for DNA extraction was not possible in the present study due to the poor quality of genetic material. However, all of the 12 specimens used for DNA extraction, despite having damage to the trunk and internal structures, had the head and tail intact. All of the 12 specimens were annulated over the entire body and had similar toothlike projections and large lateral pseudolabia described in adult T. tiara by Sou (2020). Of the remaining 15 larval nematodes, not subjected to DNA extraction, and with undamaged cephalic regions the same features were identified. This may suggest that many of the remaining 15 specimens were also be Tanqua sp. Specimens in the present study were heavily annulated which differed from the description of larval Tanqua sp. in Schoeman et al. (2020). Intraspecific morphological variations of adult T. tiara has been reported (Sou, 2020). The specimens in the present study showed low genetic variability with adult T. tiara (1.8%: JF934728) in Laetsch et al. (2012) and by L3 Tanqua sp. (1.2%: MN526252) in Schoeman et al. (2020). The differences in body annulations and size of the lateral pseudolabia between L3 larvae in Schoeman et al. (2020) and the present study may be a consequence of a the difference in geographical locality, host or larval developmental stage. Further morphological and genetic study is required to explore these relationships.

Table 2

| Phylum | Species name | Length (bp) | Reference |
|--------|--------------|------------|-----------|
| Nematoda | *Cucullanus robustus* (Outgroup) | JF934726 | - |
| Echinoclophus carpiae | KC493258 | Cyprinus carpio | - |
| Gnathostoma lineatum | JF934729 | Heptodiscus portulacensis | - |
| Gnathostoma turgidum | Z96496 | Pool of eight larvae | - |
| Gnathostoma turgidum | Z96497 | - | - |
| Gnathostoma turgidum | KT894809 | - | #Unpublished |
| Spiricysea contortas | MN629917 | Eryx orbiculatus | Polenis National Park (South-Eastern Poland) |
| Spiricysea harsaki | AB183383 | Andrias japonicus | Hyogo, Japan |
| Spiricysea japonica | AB183382 | Rana nigromaculata | Niigata, Tokamachi, Japan |
| Spiricysea japonica | KM846295 | Pelophylax nigromaculatus | Yingtian, Jiangxi Province, China |
| Tanqua tiara | JF934728 | Varanus indicus (Isolate N691) | Malingrida, Northern Territory, Australia |
| Tanqua sp. | MN526252 | Xenopus laevis | Limpopo Province, South Africa |
| Tanqua sp. | OLI800839 | Channa punctata | Bangladesh |
| Tanqua sp. | OLI800840 | Channa punctata | Bangladesh |
| Tanqua sp. | OLI800841 | Channa punctata | Bangladesh |
| Tanqua sp. | OLI800842 | Channa punctata | Bangladesh |
| Tanqua sp. | OLI800843 | Channa punctata | Bangladesh |

Information at GenBank only from unpublished PhD in Spanish

Host species, geographical origin of the sample and research reference for each specimen.

*Unpublished
Agustin et al. (2017) speculated that *T. tiara* has zoonotic potential and mammals may become infected from drinking worm eggs in contaminated water (Agustin et al., 2017). The authors of the present publication found no evidence to support this, however *T. tiara* is a species of one of four genera within the family Gnathostomatidae. One of the genera, *Gnathostoma*, has members species which have been identified in multiple cases of human infection globally (Chandenier et al., 2001; Chappuis et al., 2001; Chai et al., 2003; Görgolas et al., 2003; Basak et al., 2004; Barua et al., 2007). In addition, cases of gnathostomiasis in countries where *Gnathostoma* species have not been identified in local fish may have been misidentified according to Shamsi et al. (2021) and could be *Echinocephalus* sp., also of family Gnathostomatidae. *Channa punctata* in this study was infected with third stage larvae, which is the developmental stage infective to humans, and three specimens were embedded in the fish musculature. Perhaps the zoonotic potential of *Tanqua* sp. requires further investigation.

The habitat of *V. salvator* has declined in Bangladesh due to many anthropogenically driven factors (Khandakar et al., 2020). The consequence of this continued habitat degradation will be a blurring of boundaries between man and *V. salvator*. Fish, as the most favoured

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**Fig. 1.** Phylogenetic tree (of 18S sequences of nematodes) inferred using the Maximum Likelihood Method. The bootstrap values higher than 80 are indicated next to the branches. The new sequences generated from this study are indicated with asterisks.
scavenged food of Bengali Varanus spp. (Rahman et al., 2015), places this genus of monitors at the interface of fish polyculture in Bangladesh, essential to establish food security for the growing population (Bogard et al., 2015). It is important to identify parasites accurately using molecular method to better understand this developing dynamic between Bengali people, fish production, and wildlife.

Declaraton of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Michelle Williams is the grateful recipient of the PhD scholarship from Australian Research Training Program Scholarship through Charles Sturt University.

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Table 3

Infection data for larval nematodes and Tanqua sp. Genomic DNA was extracted from 12 of the 27 unidentified nematodes (row 1). Of these 12 nematodes used for extraction of DNA five were sent for sequencing and identified as Tanqua sp. (row 2).

| Fish and number (N =) | Parasite species | No. of fish infected | Range in infected fish | Prevalence (%) | Total no. of parasites found or identified | Mean intensity | Mean abundance | GenBank accession number this study | GenBank accession number match |
|-----------------------|------------------|---------------------|----------------------|----------------|------------------------------------------|---------------|---------------|-----------------------------------|--------------------------------|
| 1. Channa punctata (N = 103) | Larval nematodes | 10 | 0–13 | 09.7 | 27.0 | 2.70 | 0.30 | OLA830839 | JF934728 |
| 2. Tanqua sp. | | 5 | | | | | | OLA830840 | OLA830841 | OLA830842 | OLA830843 |

Fig. 2. Larval nematodes identified as Tanqua sp. 2A specimen 674-1 anterior tip (20x); 2B specimen 678-1 showing tooth like projections of pseudolabia (fl) and lateral pseudolabium (lp) (40x); 2C specimen 674-1 posterior trunk (4x) showing annulations (an). and 2D specimen 678-9 tail (20x) respectively showing annulations (an) and anus (as). The circled area in Fig. 2A is indicative of the damage to internal structures which precluded detailed morphological examination.
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