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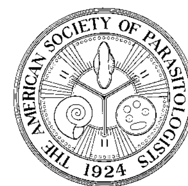

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC RELATIONSHIPS OF *PALLISENTIS* (*BREVITRITOSPINUS*) *INDICA* (ACANTHOCEPHALA: QUADRIGYRIDAE), A PARASITE OF THE SPOTTED SNAKEHEAD (*CHANNA PUNCTATUS*)

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KEY WORDS

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ABSTRACT

Pallisentis (*Brevitritospinus*) *indica* Mittal and Lal, 1976 was found infecting the spotted snakehead *Channa punctatus* Bloch and Schneider (Channidae) from Meerut, Uttar Pradesh (U.P.), India. The species was identified on the basis of proboscis hooks, trunk spines, and other structures that separate it from all described species. Molecular analysis based on *18S* rDNA placed the *P. indica* isolates within a clade of *Pallisentis* spp. but distinct from other representatives of the same genus. This study documents the first molecular characterization of *P. indica* from India.

In a parasitological survey of freshwater fishes from Meerut, Uttar Pradesh (U.P.), India, specimens of an acanthocephalan were found in the gastrointestinal tracts of the spotted snakehead *Channa punctatus* Bloch and Schneider (Channidae), which were subsequently identified as a species of *Pallisentis* Van Cleave, 1928. *Pallisentis* includes 32 species reported worldwide (Amin, 2013). There is confusion regarding the correct identity of these acanthocephalans in India. Most of the studies of acanthocephalans in India in the past few decades were based on morphological observations, some lacking informative illustrations, and type specimens were not properly deposited in some instances; thus, many similar species were described from the same species of host (Agarwal, 1958; Tadros, 1966; Mital and Lal, 1976; Gupta et al., 2015; Gautam et al., 2017). As a result, species of acanthocephalans described from Indian material have often been questioned (Pichelin and Cribb, 2001).

In this study, we clarify the taxonomy of *Pallisentis indica* Mital and Lal, 1976 using new material and using molecular data. Amin et al. (2017) redescribed *P. indica* from another locality, Kali Nadi River at Narora (28.1968°N, 78.3814°E), Bulandshahar, U.P., India in the same water system. Molecular observations of *P. indica* provided in this study make it possible to present a more complete characterization of this species.

MATERIALS AND METHODS

Spotted snakehead fish, *Channa punctatus* Bloch and Schneider (Perciformes: Channidae) (Local common name 'sauli') were originally caught in the Ganga River at Bairaj, Bijnor (29°01′N, 77°45′E) in the state of Uttar Pradesh (U.P.), India, and obtained from the local fish market of Meerut in February and May, 2017. Twenty-three specimens of *C. punctatus* were examined for parasites in the laboratory at the Department of Zoology, Chaudhary Charan Singh University, Meerut, U.P., India with a prevalence of 62% (23 of 37 fish examined) and intensity of infection 1.56 (23 male and 13 female; a total of 36 parasites from 23 infected hosts). The stomach and intestines were removed from freshly killed fish and placed in petri dishes with saline solution. Worms were collected and examined using a dissecting microscope. For microscopic identification, worms were thoroughly washed and then fixed in 70% ethanol for 24 hr. They were subsequently stained in aceto-carmine, dehydrated through an ethanol series, cleared in xylene, and mounted in Canada balsam. A light microscope (MoticSMZ-168, Motic, Xiamen, People's Republic of China) equipped with a digital image analysis system (Motic Image Plus 2.0 for Windows) was used and drawings were made using a camera lucida. Voucher specimens were deposited in the Museum of the Department of Zoology, Chaudhary Charan Singh University, U.P., India under voucher number HS/CCSU/2017/17.

For molecular analysis, total genomic DNA was extracted from 2 individual acanthocephalans using the Qiagen DNeasy™ tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Primers Worm A (5′-GCGAATGGCTCATTAAATCAG-3′) and 1270R (5′-CCGTCAATTCCTTTAAGT-3′) (Littlewood and Olson, 2001) were used to amplify the *18S* region of the ribosomal RNA gene array while primers BD1 (5′-GTCGTAACAAGGTTTCCGTA-3′); BD2 (5′-TATGCTTAAATTCAGCGGGT-3′) (Luton et al., 1992), and D1 (5′-AGGAATTCCTGGTAAGTGCAAG-3′), (5′-CGTTACTGAGGGAATCCTGGT-3′) (Galazzo et al., 2002) were used to amplify the *ITS1-5.8S-ITS2* ribosomal RNA gene array. Poly-

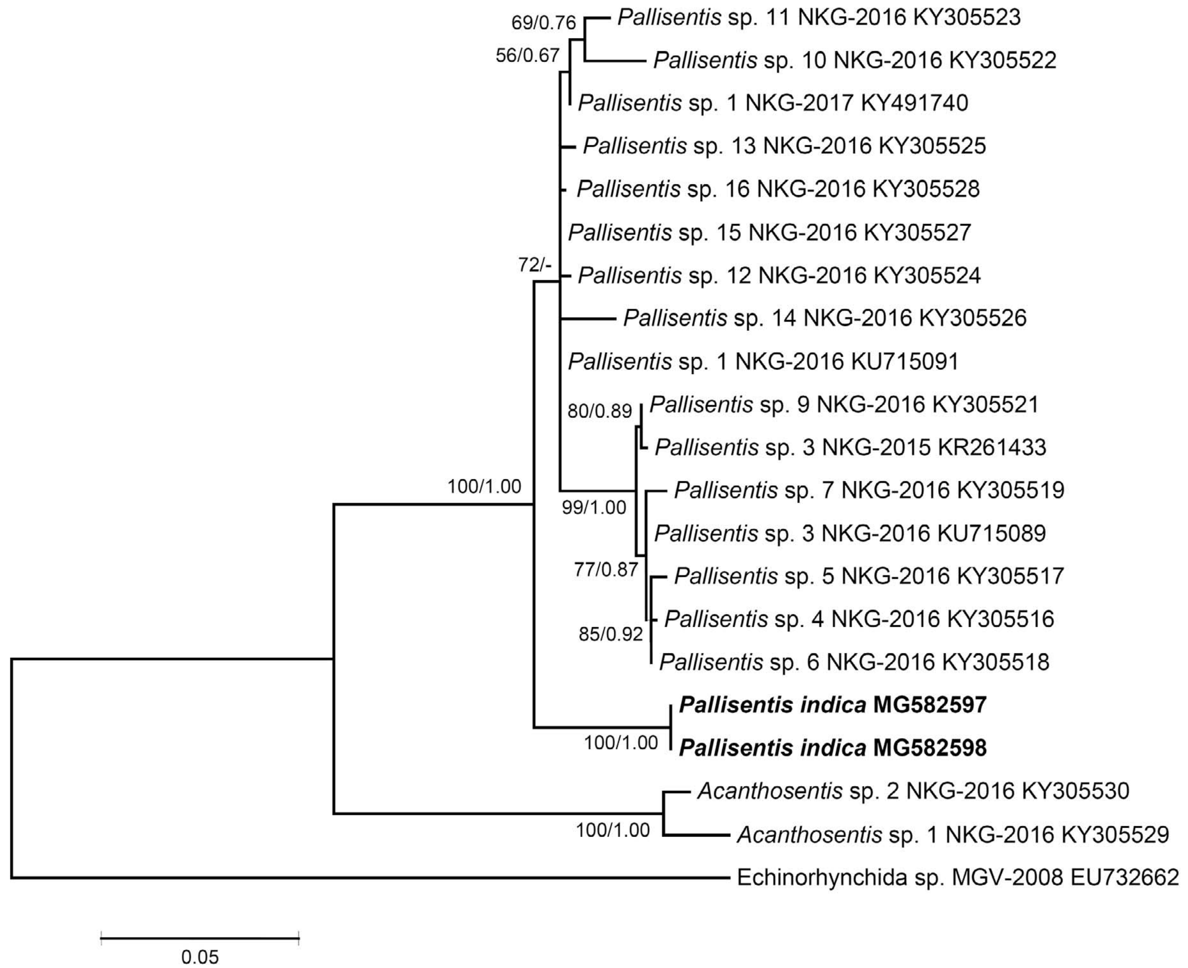

Figure 1. Phylogenetic tree generated by maximum likelihood (ML) analysis of the *18S* rDNA sequence data of *Pallisentis indica* and related species. Numbers at nodes indicate the bootstrap values (ML) and posterior probabilities (Bayesian inference; BI). Hyphen indicates node unsupported by BI. GenBank accession numbers are provided alongside the species names.

merase chain reaction (PCR) was performed in a 25-µl reaction volume containing 3 µl DNA, 2.5µl of 10X Taq buffer (Biotools, Madrid, Spain), 1 µl of Taq polymerase (1 U, Biotools), 3 µl of deoxyribonucleoside triphosphates, 1 µl of each forward and reverse primer, and 13.5 µl of water. PCR reaction cycles comprised the following steps: an initial denaturation for 3 min at 94 C followed by 40 cycles of 94 C for 40 sec, 56 C for 45 sec, and 72 C for 1 min, with a final extension of 10 min at 72 C, and then stored at 4 C. A 5-µl aliquot of PCR products were examined by 1.5% agarose-Tris-acetic acid-EDTA gel electrophoresis using ethidium bromide staining. PCR products were purified with Purelink™ Quick Gel Extraction and PCR purification Combo Kit (Invitrogen, Löhne, Germany) following the manufacturer's instructions. Purified PCR products were sent for commercial sequencing (Eurofin Genomics, Bangalore, India) with the same primers as mentioned above.

The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST) was used to compare the consensus sequences with available data in GenBank. For phylogenetic analysis, the closely related species of acanthocephalans (maximum identity more than 90%) were chosen. Multiple sequence alignment was performed using CLUSTAL W (Thompson et al., 1994). Phylogenetic trees were constructed by the maximum likelihood (ML) method using MEGA v. 6 (Tamura et al., 2013) and by Bayesian inference (BI) analysis computed by TOPALI 2.5 (Milne et al., 2009). The dataset was tested for the nucleotide substitution model of best fit and the model Akaike Information Criterion was chosen for each data set. Genetic distances were calculated by using the p-distance model in MEGA v. 6. The ML analysis was performed using the GTR + G + I model, and the bootstrap values based on 1,000 resampled datasets were generated. BI analysis was also based on the GTR + G + I model. The BI analysis was run for 1,000,000 generations, sampling every 100th tree, and discarding as 'burn in' the first 25% of the sampled trees. *Echinorhynchida* sp. MGV-2008 (EU732662) and *Acanthosentis cheni* (JX960752) were selected as out-group species for the *18S* rDNA and *ITS1-5.8S-ITS2* trees, respectively.

RESULTS

The *18S* and *ITS-5.8-ITS2* regions of rDNA sequences of *P. indica* were deposited in GenBank under the accession numbers MG582597, MG582598 (for *18S* gene), and MG737587, MG737588 (for *ITS* region). The ML and BI analyses yielded similar topologies with strong nodal support (Fig. 1). Phyloge-

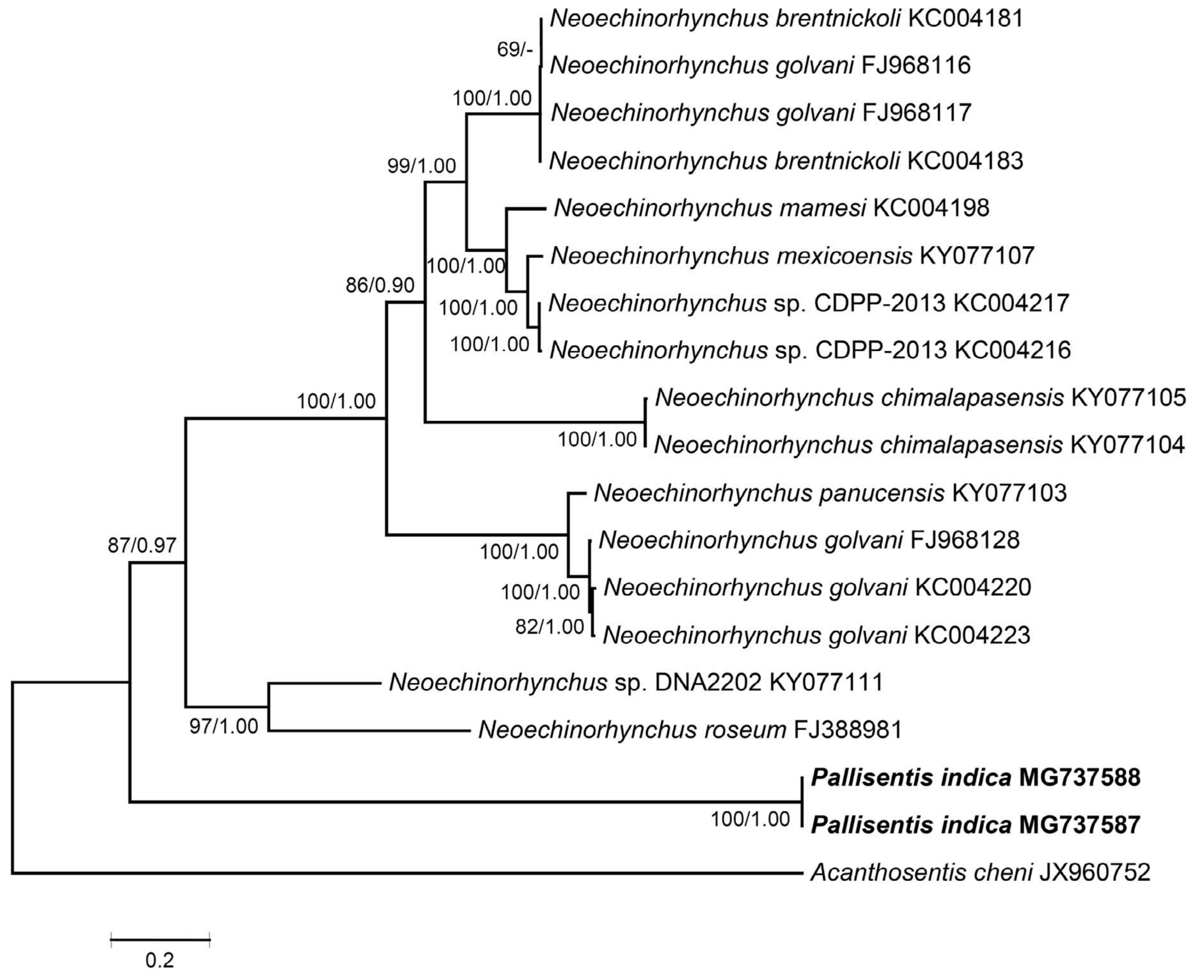

Figure 2. Phylogenetic relationships inferred by maximum likelihood (ML) analysis of *Pallisentis indica* from India based on the ribosomal *ITS1-5.8S-ITS2* gene sequences. Numbers at nodes indicate ML bootstrap values (1,000 replications) and posterior probabilities (Bayesian inference; BI), respectively, with GenBank accession numbers listed alongside the species names. Hyphen indicates node is unsupported by BI.

netic analysis inferred from the *18S* rRNA gene for *P. indica* placed the isolates within a clade of *Pallisentis* (Fig. 1). In this clade, the 2 isolates fell within a subclade supported by bootstrap values and another subclade consisting of other species of *Pallisentis*. Intraspecific variability of the *18S* rDNA sequences was not shown between the 2 isolates of *P. indica* in this study and shared 0.01–0.05% nucleotide sequence diversity with other *Pallisentis* species deposited in the GenBank database. They shared the highest sequence similarity, 96.3–98.4%, with other Indian isolates (Fig. 2). Additional data from species of *Pallisentis* are further needed for a more congruent phylogenetic analysis, as very limited sequences are available in GenBank database *18S* with which to perform a comparative analysis. Besides, both ML and BI analyses resulted in consensus trees with similar topologies for the *ITS1-5.8S-ITS2* dataset (Fig. 2). The phylogenetic analysis of the *ITS1-5.8S-ITS2* dataset provided strong support for isolates of *P. indica* forming a separate clade with high (100/1.00) bootstrap values (Fig. 2). For the *ITS1-5.8S-ITS2* region, within both isolates of *P. indica* no intraspecific variation was found. Isolates of *P. indica* were associated in the tree with species of *Neoechinorhynchus*, as no *ITS1-5.8S-ITS2* sequence is available for any *Pallisentis* species in the database for comparison (Fig. 2). Both analyses, ML and BI, confirmed the distinct status of the *P. indica* isolates and suggested that other species of this genus should be studied with the aid of molecular methods together with morphological tools to verify their identity.

DISCUSSION

The genus *Pallisentis* was established by Van Cleave (1928) with *P. umbellatus* as the type species from China. Subsequently, Amin et al. (2000) provided a detailed taxonomic treatment of 26 species of *Pallisentis* and created 3 subgenera that aimed to resolve the taxonomic challenges posed by this genus. In their classification, Amin et al. (2000) placed *P. indica* in the subgenus *Brevitritospinus* for having "proboscis hooks in circle 3 about half as long as hooks in circle 2, and cement gland usually with few giant nuclei." Later, Amin and Taraschewski (2003) updated the treatment of Amin et al. (2000) by adding 3 more species. Later, Amin et al. (2017) redescribed *P. indica* from *Channa punctatus* in Aligarh, India, and removed all uncertainties regarding its taxonomic identification by providing clear morphological details in their description. The populations described by Amin et al. (2017) and ours were comparable, especially in the size of the trunk, testes,

proboscis hooks, and trunk spines. The receptacle, lemnisci, and eggs of our specimens were relatively smaller. Specimens were collected from the same host species in 2 tributaries of the same waterway, the Ganga River, 168 km apart. The eggs in our specimens appeared to be under-developed.

More than 30 valid species have been listed in the genus *Pallisentis* (see Amin, 2013). *Pallisentis indica* was first described by Mital and Lal (1976) from *Channa gachua* Hamilton 1822 from Khurja, U.P., India. The original descriptions by Mital and Lal (1976) were incomplete and confusing as they did not clearly describe the morphological details about ducted trunk spines, the structure of spines, the female reproductive system, the proboscis, and the eggs. Amin et al. (2000) distinguished these 2 species on the basis of the shape of trunk spines being conical in *Pallisentis croftoni* and Y-shaped in *P. indica*. Furthermore, the eggs of *P. indica* are larger and the trunk spines extend posteriorly up to the level cement glands whereas *P. croftoni* has smaller eggs and the trunk spines extend only to the anterior testis.

Unfortunately, in India the identification of many species of *Pallisentis* has not been resolved and confusion persists, especially in species reported from the same host, such as *C. punctatus*. These include *Pallisentis ophiocephali* (Thapar, 1931) Baylis, 1933 (Syn. *Farzandia ophiocephali* Thapar, 1931); *Pallisentis nagpurensis* (Bhalerao, 1931) Baylis, 1933; *Pallisentis allahabadi* Agarwal, 1958 (Syn. *Pallisentis buckleyi* Tadros, 1966); *P. croftoni* Mital and Lal, 1976; *Pallisentis channai* Gupta, Maurya and Saxena, 2015; *Pallisentis vinodai* Gupta, Maurya and Saxena, 2015; *P. punctati* Gupta, Maurya and Saxena, 2015, and *Pallisentis anandai* Gautam, Upadhyay, Maurya, Verma and Saxena, 2017. Other species of *Pallisentis* reported from India include *Pallisentis nandai* Sarkar, 1953; *Pallisentis colisai* Sarkar, 1956 (Syn. *Pallisentis panadei* Rai, 1967); *Pallisentis basiri* Farooqi, 1958; *Pallisentis guntei* Sahay, Nath, and Sinha, 1967; *Pallisentis garuai* (Sahay, Sinha, and Ghosh, 1971) Jain and Gupta, 1979; *Pallisentis clupei* Gupta and Gupta, 1979; *Pallisentis cavasii* Gupta and Verma, 1980; *Pallisentis gomtii* Gupta and Verma, 1980; *Pallisentis fasciati* Gupta and Verma, 1980; *Pallisentis guptai* Gupta and Fatma, 1986; *Pallisentis mehrai* Gupta and Fatma, 1986; *Pallisentis pesteri* (Tadros, 1966) Chowhan, Gupta, Gupta, and Khera, 1987; *Pallisentis fotedari* Gupta and Sinha, 1992; *Pallisentis jagani* Koul, Raina, Bambroo, Koul, 1991, and *Pallisentis giuris* Saxena, Gupta, Ramakant, 2015. More recently, a surge of descriptions of new species of *Pallisentis* has been published including those of *P. chauhani* Saxena, Bajpai, Gupta, Gautam, Johri, and Ramakant, 2014; *P. giuris* Saxena, Gupta, Ramakant, 2015; *P. punctati* Gupta, Maurya and Saxena, 2015; *P. channai* Gupta, Maurya and Saxena, 2015; *P. vinodai* Gupta, Maurya, and Saxena, 2015; and *P. anandai* Gautam, Upadhyay, Maurya, Verma, and Saxena 2017.

Gupta, Maurya, and Saxena (2015) described *P. punctati* from *C. punctatus* and placed the species in a dendrogram of *Pallisentis* species; it is hard to verify the status of this species in the absence of expert acanthocephalan taxonomists using molecular data in India. Therefore, molecular data seem important for species differentiation in *Pallisentis* so that a more reliable and accurate accounting of valid species is possible.

In recent years, molecular methods have proved essential in acanthocephalan species identification and systematics (García-Varela and Nadler, 2006; García-Varela et al., 2009, 2011; Fontaneto and Jondelius, 2011; Tkach et al., 2013; Weber et al., 2013; Pinacho-Pinacho et al., 2014). In the present study, the phylogenetic tree based on the *18S* rRNA gene showed that *P. indica* clustered together with other species of the same genus. In our comparison within the *18S* rDNA tree, species of *Pallisentis* including *P. indica* formed a clade with high bootstrap support (Fig. 1).

Recently, a new species of *Pallisentis*, *P. anandai* (Gautam et al., 2017), was reported from India along with sequence data of the *18S* rRNA gene. However, the sequence of *P. anandai* showed a 2.25% divergence from that of *P. indica* as compared to other *Pallisentis* species included in the tree (Fig. 1), with only 0.01–0.05% genetic differences, so it could not be included in the analysis. Therefore, only *P. indica* is included in the generation of the tree due to its unambiguous position. The *18S* rDNA tree was constructed using species of *Pallisentis* that show higher genetic similarity with *P. indica*. Therefore, we can state that the genetic data from other species of *Pallisentis* is practically missing. For confirmation, we need additional evidence of more sequences of *18S* rDNA region in order to adequately test this hypothesis.

The genetic information collected in this study contributes to the understanding of the taxonomic status of *Pallisentis* spp., especially in India. Further sampling and morphological characterization are needed to supplement molecular data for other species of *Pallisentis*, which will help to establish relationships and allow for proper identification of species. Finally, we are aware that the relationships of *P. indica* need to be tested again in the future with more sequence data from other geographical isolates and other species.

CONFLICT OF INTEREST

No conflict of interest is declared by the authors.

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