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Research Note

Molecular and ultrastructure characterization of two nematodes (*Thelandros scleratus* and *Physalopteroides dactyluris*) based on ribosomal and mitochondrial DNA sequences

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Summary

The phylogenetic relationships of the nematode species *Thelandros scleratus* (Oxyurida: Pharyngodonidae) and *Physalopteroides dactyluris* (Spirurida: Physalopteridae) were analyzed using the ribosomal 18S rRNA and the mitochondrial cytochrome C oxidase subunit genes. The nematodes were recovered from Brook's house gecko, *Hemidactylus brooki* (Reptilia: Gekkonidae) from Hastinapur, Meerut (U.P.), India. The results demonstrated that *T. scleratus* shows 100% similarity with another sequence available from the same species and a close relationship (98-99%) with species of *Parapharyngodon* in both 18S rRNA and cox 1 regions. Regarding the nematode *Physalopteroides*, analysis showed a close phylogenetic relationship between *P. dactyluris* and several species of *Physaloptera*. This is the first sequence of 18S available for any species of the genus *Physalopteroides*.
Keywords: *Hemidactylus brooki,* Nematodes, Phylogenetics, ribosomal DNA, mitochondrial DNA

Introduction

Various parasitological studies on nematode infecting *Hemidactylus brooki* (Brooke's house gecko), have been performed in India for many years. Regarding the intestinal nematode parasites of *H. brooki*, several species of different genera have been described (Sood, 1999). This includes *Thelandros* Wedl, 1861 and *Physalopteroides* Wu & Liu, 1940 living in the intestine of *H. brooki*. During the past decades, at least 11 *Thelandros* species have been described in this Indian region (Sood, 1999).

Parasitic nematode *Parapharyngodon maplestoni* found in the intestine of *Calotes versicolor* lizard was described in 1933 by Chatterji. Later, Baylis 1936 considered *P. maplestoni*, a synonym of *Thelandros* Wedl, 1861. *Thelandros scleratus*, from the same host, was described by Travassos, 1923. The differentiation between *Parapharyngodon* Chatterji, 1933 and *Thelandros* Wedl, 1861 has been controversial by reason of the morphological similarities. Several authors considered them as synonym (Baylis, 1936; Karve, 1938; Vicente *et al.,* 1993), while others considered them as different genera (Ramallo *et al.,* 2002; Bursey & Goldberg, 2005). Both genera can be differentiated from each other on the basis of posterior end morphology in both sexes (Bursey & Goldberg, 2005). Status of *Thelandros* species in India has also been questionable due to lack of molecular tools. Some phylogenetic studies of *T. scleratus* have been carried out with nuclear genes like 18S and 28S (Kumari *et al.,* 2011; Chaudhary *et al.,* 2014).

The genus *Physalopteroides* was recognized in China by Wu & Liu, 1940 with the type species of *P. dryophisi* collected from *Dryophis prasinus*. Excluding *P. dactyluris* (Karve, 1938; Chabaud & Brygoo, 1960) the, other species of this genus described in India include *P. asymmetrica* (Baylis, 1930; Chabaud & Brygoo, 1960), *P. quadridentata* (Khera, 1951; Chabaud & Brygoo, 1960) and *P. versicoloris* (Deshmukh, 1969). The status of genus *Physalopteroides* in India has also been controversial for long time for the reason that different species show similar morphologies and there is no molecular data available to compare them precisely. Nema-

tode taxonomy is mostly based on morphological features such as oesophagus structure, pharynx and structures of males and females reproductive organs. Although, morphological characteristics contribute to the species identification further authentication DNA sequence data is needed for a complete description and identification of a species. Morphological diagnosis in nematodes is very difficult because the characters that serve to distinguish between each other overlap. For molecular analysis of nematodes ribosomal RNA gene has been widely applied in phylogenetic and systematic studies to discriminate among species (Bae *et al.,* 2008; van Megen *et al.,* 2009; Bhadury & Austen, 2010; Perera *et al.,* 2013). However, less attention has been paid to the mitochondrial DNA (mt DNA). Mitochondrial gene analysis has been used successfully in studies regarding identification and phylogenetic relationships in some nematode groups (Blouin *et al.,* 1997; Keddie *et al.,* 1998; Hoberg *et al.,* 1999; Denver *et al.,* 2000; Blouin, 2002; Lavrov & Brown, 2001; Liu *et al.,* 2013). The cytochrome c-oxidase subunit1 (Cox 1) gene has been successfully used to resolve the phylogenies of closely allied species in most animal phyla including nematodes (Kumazawa & Nishida, 1993; Hebert *et al.,* 2003; Prosser *et al*., 2013).

Previous to this study, no molecular information from *Physalopteroides* was available in GenBank. The present study analyzes for the first time the phylogenetic relationships the nematode species of *Thelandros* and *Physalopteroides* belonging to the same geographical area using the partial 18S and Cox 1 genes.

Materials and Methods

Brook's house gecko (N=15), *Hemidactylus brooki* infected with nematodes were collected from Hastinapur (29°01'N and 77°45'E), Meerut (U.P.), India and were sacrificed using chloroform or ether anesthetic. Their digestive tracts were removed for further examination. Nematodes were collected into 0.6 % saline solution, then fixed with 70 % hot alcohol, mounted in glycerin for morphological analysis, and then subjected to light microscopy and photography. The surface morphology analysis by scanning electron microscopy (SEM) was also performed. For the SEM, parasites were fixed in 70 % ethanol, dried using critical point-drier, mounted on SEM stubs, coated with a thin layer of gold and finally examined with a JOEL Neoscope JCM5000 SEM at an accelerating voltage of 10 kV.

For molecular analysis, nematodes were fixed in 95 % ethanol and genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR were used to amplify the 18S and Cox 1 regions using the primers, Nem 18S F (5'-CGCGAATRGCTCATTACAACAGC-3') and Nem 18S R (5'-GGGCGGTATCTGATCGCC-3') (Floyd *et al.,* 2005); LCO1490 F (5'-GGTCAACAAATCATAAAGATATTGG-3') and HC02198 R (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.,* 1994), respectively. The PCR were performed in volumes of 25 µl under standard conditions according to Singh *et al.,* 2013. The PCR products were electrophoresed in 1 % agarose gel in TAE buffer, stained with ethidium bromide. Subsequently, PCR products were purified using Purelink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Germany) following the manufacturer's instructions. Amplicons were sequenced (in both directions) with Big Dye Terminator version 3.1 cycle sequencing kit in an ABI 3130 Genetic Analyzer (Applied Biosystems).

To identify related sequences, a BLAST search was carried out on NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/). The 18S and COX 1 sequences were aligned using the Clustal W algorithm (Thompson *et al.,* 1994) implemented in MEGA 6.0 (Tamura *et al.,* 2013) with defaulted parameters. Maximum likelihood (ML) and Bayesian interference (BI) phylogenetic analyses were performed for congruence among topologies. The ML analysis was performed in MEGA 6.0 with GTR + G + I model and the bootstrap values generated were based on 1,000 resampled datasets. The dataset was tested on MEGA 6.0 for the nucleotide substitution model of best fit and the best-fitting model according to the Akaike Information Criterion (AIC). The substitution models were tested by the Bayesian Information Criterion as determined by the model test algorithm of the software and GTR + G + I model was selected to be the best fit for analysis. BI analysis was computed using TOPALi 2.5 interface (Milne *et al.,* 2009). Posterior probabilities were estimated over 1,000,000 generations conducted by two independent runs of four simultaneous MCMCMC chains. The burn in was 25.

Results and Discussion

The following two nematode species were found to parasitize *H. brooki*.

Thelandros scleratus Travassos, 1923 (Fig. 1 A – F)

Description: T. scleratus was identified on the basis of their mouth bounded by three bilobed lips and oesophagus type. Buccal cavity short, without chitinous walls. Oesophagus display very short pharynx and posterior bulb. In females, tail is constricted behind anus to form a terminal spike, vulva positioned little behind the middle of body. Uterine branches parallel, oviparous, eggs oval, not embryonated when laid. In males, posterior extremity is truncated with tail process mid-dorsally, caudal alae absent, spicule short, sharp, pointed and absent gubernaculum.

Host: Hemidactylus brooki (Brook's house gecko); *Site:* Intestine; *Locality:* Hastinapur (29°01'N and 77°45'E), Meerut, Uttar Pradesh, India; *Reference material: Thelandros scleratus*; Family: Pharyngodonidae. Prepared slides were deposited in the Museum of the Department of Zoology, Chaudhary Charan Singh University, Meerut, Uttar Pradesh, India, under the voucher number Nem/2014/01.

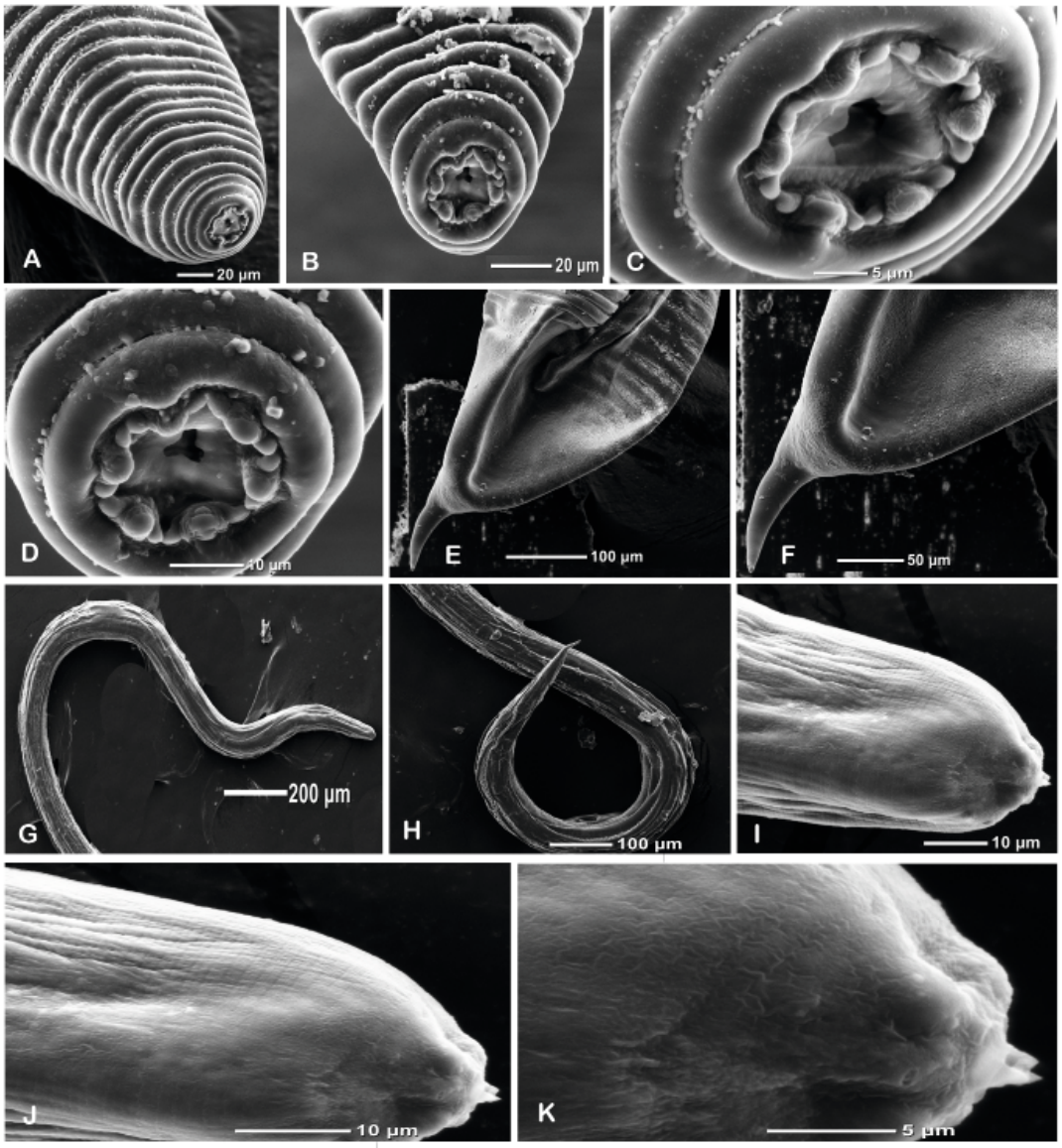

Fig. 1. The SEM photographs of females of *T. scleratus* (A – F) and *P. dactyluris* (G – K). A and B, SEM photograph of anterior portion of *T. scleratus*. C and D, Enlarged view of mouth and lips. E and F, Tail at greater magnification. G, Anterior part of body. H, Posterior part of body. I – K, Lower to higher magnification of head region

Molecular characterization: The 18S (800 bp) and Cox 1 (612 bp) sequences obtained from *T. scleratus* were deposited in GenBank under the accession numbers KP338604 and KP338607, respectively. Sequences of the 18S rDNA and mt Cox 1 genes showed 100 % similarity with the sequences isolated from the same species available in GenBank. Sequences were also closely related (98 – 99 %) to the genus *Parapharyngodon* as they have shown only 0.01 to 0.02 % divergence for the 18S region. The results from the phylogenetic analysis based on ML and BI are shown in figure 2 A and B. In Figure 2 A, the 18S analysis depicts that *T. scleratus* clusters with another species of the same genus, *T. tinerfensis* which was reported from Spain through high bootstrap support (98/0.99). Moreover, *T. scleratus* always grouped together with *Parapharyngodon* species (Figs 2 A and B) in phylogenetic analysis based on 18S and Cox 1 gene sequences. The findings of the study also indicate that except *T. scleratus*, no molecular data is available from any other *Thelandros* species for comparison of mt Cox 1 region (Fig. 2 B).

Physalopteroides dactyluris Karve, 1938 (Fig. 1 G – K)

Description: Identification of this parasite is based on its oesophagus, caudal extremity and pedunculated papillae. Body is delicate, slender, mouth comprises two simple lateral lips. Collar cuticular are present at the base of muscular lips. In females, vulva in pre-equatorial position, uterine branches parallel. Thick shelled eggs contain larvae at the deposition and with conical tail.

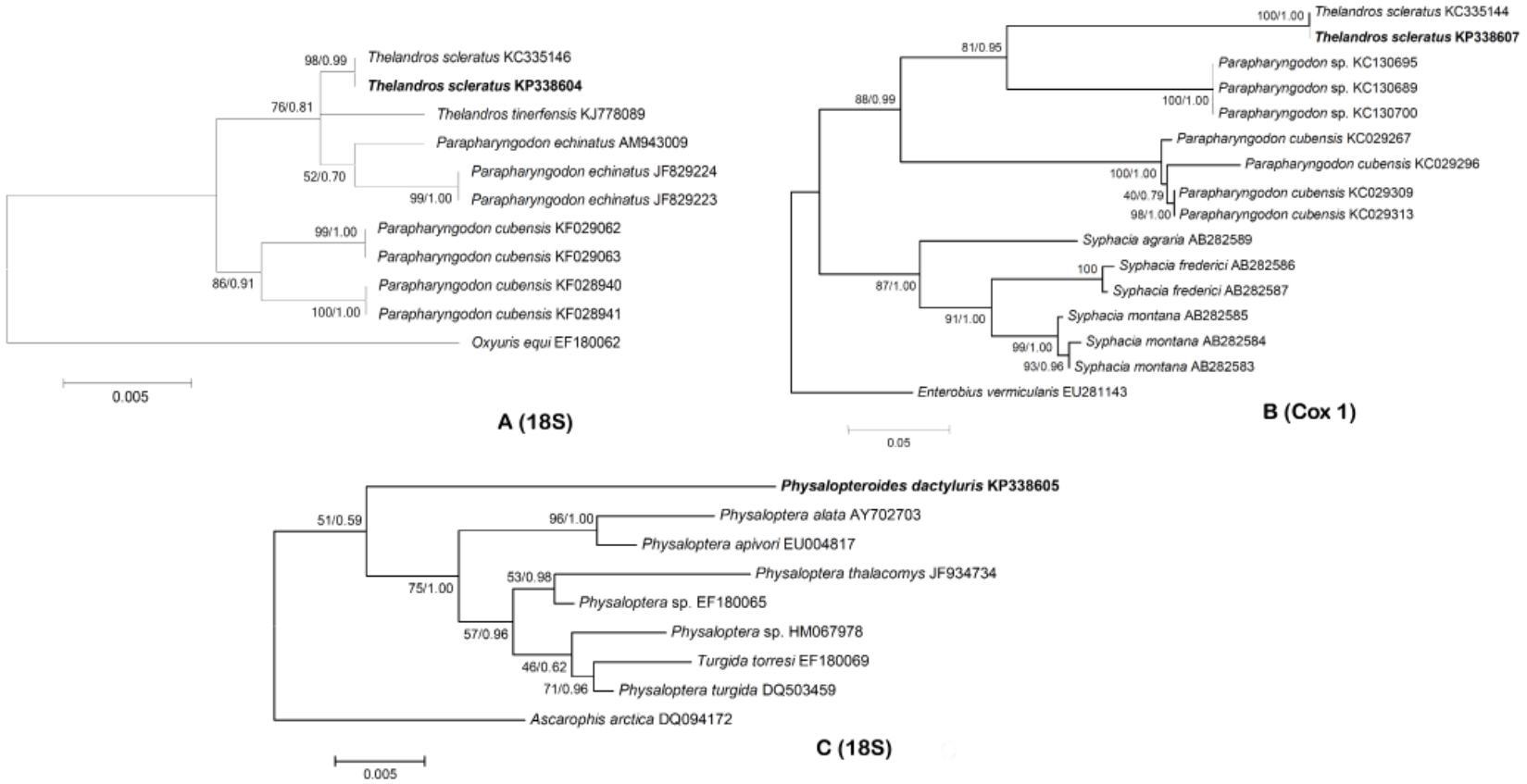

Fig. 2. Phylogenetic trees generated by maximum likelihood (ML) and Bayesian Inference (BI) analyses. Numbers at nodes indicated bootstrap values (ML) and posterior probabilities (BI). Phylogenetic tree A and B shows, respectively, 18s and Cox 1 results for *T. scleratus* and C shows 18s results for *P. dactyluris*. *Oxyuris equi*, *Enterobius vermicularis* and *Ascarophis arctica* were used as outgroups

In males, caudal papillae pedunculated, spicules simple, unequal, chitinized. Right spicule is smaller than the left one. Caudal end consist of curved pointed tail.

Host: Hemidactylus brooki (Brook's house gecko); *Site:* Intestine; *Locality:* Hastinapur (29°01'N and 77°45'E), Meerut, Uttar Pradesh, India; *Reference material: Physalopteroides dactyluris*; Family: Physalopteridae Railliet, 1893; Prepared slides of this parasite were deposited in the Museum of the Department of Zoology, Chaudhary Charan Singh University, Meerut, Uttar Pradesh, India, under the voucher number Nem/2014/02.

Molecular characterization: The 18S sequence obtained from *P. dactyluris* was deposited in GenBank under the accession number KP338605. Unfortunately, we failed to amplify the mt Cox 1 region of *P. dactyluris*. Maximum likelihood (ML) and Bayesian inferences (BI) placed *P. dactyluris* on a basal position within the genus *Physaloptera* (94 – 96 %) along with *Turgida*. Both ML and BI methods conferred similar topologies (Figure 2 C). The 18S sequence of *P. dactyluris* (930 bp) was closely related to the sequence available for *P. turgida* (96 %) and it was also closely related to the *Turgida torresi* and other *Physaloptera* sp. (94 – 95 %; Fig. 2 C).

The present study provides the first molecular identification of *T. scleratus* and *P. dactyluris.* This is the first report of molecular identification of *P. dactyluris* based on 18S ribosomal RNA gene data (Fig. 2 C). The phylogenetic tree based on ML and BI analyses for the 18S (Fig. 2 A) and Cox 1 (Fig. 2 B) gene shows that *T. scleratus* clusters with high bootstrap support (98 – 100 %) together with other representative of the same species collected from the same host (*H. brooki*). The phylogenetic tree also groups closely *T. scleratus* and *Parapharyngodon.* This supports the previous findings which already pointed on the closeness of these two genera (Chaudhary *et al.,* 2014). In fact, the two genera are morphologically very similar (Mašová *et al.,* 2008). Sequences of *T. scleratus* and *Parapharyngodon* spp. showed only 0.01 – 0.02 % divergence. This lower sequence divergence supports that they are sister taxa. This is also supported by similar topology for both the 18S and Cox 1 gene sequences (Fig. 2 A and B).

With regards to the genus *Physalopteroides* our analysis unambiguously places *P. dactyluris* as closely related to the *Physaloptera*. *P. dactyluris* shows morphological similarities with the species of *Physaloptera* but both these genera are morphologically different on the basis of an anterior region, pre-equatorial vulva, conical tail, thick shelled egg, unequal-chitinized spicules and pedunculated caudal papillae. Moreover, the 18S rDNA sequence of the *P. dactyluris* described here is clustered in the same clade of *Physaloptera* (Fig. 2 C). So far, there were no reports available providing the SEM images for *T. scleratus* and *P. dactyluris* from India. In biological research, the DNA based taxonomy is a reliable tool for identification of species. However, for the delineation of future phylogenetic inferences an addition of more molecular data is needed for the *Thelandros* and *Physalopteroides* species.

In summary, further morphological studies along with the SEM and molecular analyses are needed to elucidate the stability of the morphological characters used so far for the identification. Moreover, our analyses confirm the necessity of getting additional molecular data for other representatives of genus *Thelandros* and *Physalopteroides.*

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