Research Note

Phylogenetic position of *Pleurogenoides* species (Plagiorchiida: Pleurogenidae) from the duodenum of Indian skipper frog, *Euphlyctis cyanophlyctis* (Amphibia: Dicroglossidae) inhabiting the Western Ghats, India

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Summary

Two species of digenetic trematodes of the genus *Pleurogenoides* viz., *P. cyanophlycti* Shinad & Prasad (2018a) and *P. euphlycti* Shinad & Prasad (2018b) have been described from India. Information regarding the molecular data of various species of the genus *Pleurogenoides* Travassos, 1921 is virtually lacking. This study addresses the application of molecular markers to validate the phylogenetic position of *P. cyanophlycti* and *P. euphlycti*. In the present study, two species *P. cyanophlycti* and *P. euphlycti* were collected between January 2016 to October 2017, infecting the freshwater frogs inhabiting the Western Ghats, India. In the present study, the two species were identified morphologically and by PCR amplification of the 28S ribosomal RNA gene. Phylogenetic tree results clearly demonstrate that both *P. cyanophlycti* and *P. euphlycti* belongs to the family Pleurogenidae Looss, 1899. Based on these results, we presented and discussed the phylogenetic relationships of *P. cyanophlycti* and *P. euphlycti* within family Pleurogenidae from India. Phylogenetic analyses showed that *P. cyanophlycti* and *P. euphlycti* cluster according to their vertebrate host and revealed an important congruence between the phylogenetic trees of *Pleurogenoides* and of their vertebrate hosts. *P. cyanophlycti* and *P. euphlycti* clearly constitute a separate, sister branch with other species of the genera, *Pleurogenoides*, *Pleurogenes* (= *Candidotrema*), *Prosotocus* and *Brandesia*. The present study firstly provides important information about the molecular study and phylogenetic analysis of *P. cyanophlycti* and *P. euphlycti*. This study will also serve as a baseline for *Pleurogenoides* species identification for further studies.

Keywords: *Pleurogenoides*; *P. cyanophlycti*; *P. euphlycti*; 28S; frogs; India

Introduction

Studies on the metazoan parasites of amphibians in the Indian subcontinent are fragmentary. Darrel (2013) suggested that like other vertebrates a considerable range of metazoan parasites harbors frogs as preferred vertebrate hosts. The genus *Pleurogenoides* was proposed by Travassos, 1921 to accommodate type-species *P. tener* that was described by Looss, 1898 as *Pro-*

sotocus tener. The genus comprises species described from frogs worldwide. Brinsh & Janardanan (2014) documented nine species reported from Indian frogs. Recently, three new species of *Pleurogenoides* were described including *P. cyanophlycti* Shinad & Prasad (2018a), *P. euphlycti* Shinad & Prasad (2018b) and *P. wayanadensis* Shinad & Prasad (2018b) from *Euphlyctis cyanophlyctis* of the Wayanad region of the Western Ghats. During a parasitological survey of trematode parasites of frogs from

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the Wayanad region of the Western Ghats, India, *Pleurogenoides cyanophlycti* and *P. euphlycti* were recovered from the gastrointestinal tracts of *Euphlyctis cyanophlyctis* Schneider, 1799. Both the species of *Pleurogenoides* collected in this study were characterized on the basis of their morphology and molecular characteristics. The systematic position of *Pleurogenoides* species within the family Pleurogenidae has also been worked out. The main aim of this study was the molecular identification of *Pleurogenoides* species collected from Indian frog and a comparison of the resulting data with that of the available species in the GenBank database.

Material and Methods

The hosts were collected from the Wayanad region of the Western Ghats, which stands second only to the Eastern Himalayas as a treasure trove of biological diversity in India. The map of the study area (Fig. 1) was prepared using QGIS2.16.1 software. Information on the collections of specimens of *Pleurogenoides cyanophlycti* and *P. euphlycti* is presented in Table 1.

Specimens of *Euphlyctis cyanophlyctis* collected during the period from January 2016 to October 2017 from various water bodies using sweep hand net were brought to the Laboratory, maintained in cement cisterns and fed occasionally with insects. The hosts were narcotized with chloroform, dissected and their body parts were examined for digenetic trematodes under a stereozoom dissecting microscope (Labomed Luxeo 4Z, USA). Internal organs were also dissected out from each frog, placed in separate Petri dishes containing 0.75 % saline, macerated and examined under the stereo zoom microscope. Adults trematodes were carefully removed from the duodenum, transferred to 0.75 % saline in separate watch glasses and studied under Nikon ECLIPSE Ni-U phase contrast research microscope (Nikon Ni-SS 935179, Japan) without supra vital staining or after staining with neutral red. Permanent slides of adult parasites were prepared after fixing them in 5 % formalin under slight cover glass pressure and staining with acetocarmine, following the procedure outlined by Cantwell (1981). Prevalence of infection was measured following Bush *et al.*, (1997). Prevalence is the number (%) of hosts infected with one or more individuals of

Fig. 1. Study area – Western Ghats – Wayanad region, Localities – Chundel (11°34'26.2"N 76°03'32.5"E) and Panamaram (11°45'03.6"N 76°04'01.3"E).

Table 1. *Pleurogenoides* species collected from January 2016 to October 2017 from duodenum of host from Wayanad District, India.

Species	<i>P. cyanophlycti</i>	<i>P. euphlycti</i>
Sample no.	PC1 and PC2	PE1 and PE2
Host	<i>Euphlyctis cyanophlyctis</i>	<i>Euphlyctis cyanophlyctis</i>
Infected organ	Duodenum	Duodenum
Location	Chundel	Panamaram
Coordinates	11°34'26.2"N 76°03'32.5"E	11°45'03.6"N 76°04'01.3"E
Prevalence (Parasite no. per host) (%)	16 out of 100 frogs (16%)	10 out of 100 frogs (10%)
Voucher no.	Z-P/H-F 101*	Z-P/H-F 109*
GenBank accession nos.	MN218390 and MN218388	MN218391 and MN218389

*Voucher specimens deposited in the Helminth parasite collections, Ecological Parasitology and Tropical Biodiversity Laboratory, Department of Zoology, Kannur University, Mananthavady Campus, Wayanad-670645, Kerala, India.

a particular parasite species (or taxonomic group) divided by the number of hosts examined for that parasite species. Information about the collections of specimens of *Pleurogenoides* species is presented in Table 1.

DNA from *Pleurogenoides* species was extracted from two different individuals of both species using the DNeasy™ Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. 28S gene region of the rDNA was amplified using primers, Ancy55F (5'-GAGATTAGCCCATCACCGAAG-3') (Plaisance *et al.*, 2005); LSU1200R (5'-GCATAGTTCACCATCTTTCGG-3') (Littlewood *et al.*, 2000) and L300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'); ECD2 (5'-CCTTGGTCCGTGTTTCAAGACGGG-3') (Littlewood *et al.*, 2000) respectively. Polymerase chain reaction (PCR) was carried out in a total volume of 25 µl consisting of 2.5 µL 10× PCR buffer, 4 µL 1 mM deoxyribonucleotide triphosphates (dNTPs) mix, 0.8 µL of each primer, 1 U Taq polymerase (1U; Biotools) and 4 µl genomic DNA. The cycling conditions were as follows: one cycle of initial denaturation at 94 °C for 3 min; 35 cycles at 94 °C for 40 s, 55 °C for 1 min, and 72 °C for 1 min; with a final extension at 72 °C for 7 min. Negative sample with no DNA was used in per amplification run to exclude contamination. Amplified PCR products were analyzed by electrophoresis in agarose gel stained with ethidium bromide, purified using the Purelink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) and sequenced with the above primers using Big Dye Terminator vr. 3.1 cycle sequencing kit in ABI 3130 Genetic Analyzer, Applied Biosystems.

Obtained contigs were assembled and compared for similarity by searching the GenBank database using the BLAST search (www.ncbi.nlm.nih.gov/blast). Sequences obtained for 28S were retrieved and aligned with sequences from other related species downloaded from GenBank using ClustalW with default parameters implemented in MEGA version 7.0 (Kumar *et al.*, 2016). For 28S gene, GTR + G + I were estimated as the best-fitting nucleotide substitution model using the Akaike Information Criterion (AIC). For phylogenetic analyses, maximum likelihood (ML) and Bayesian inference (BI) analyses were performed using MEGA v. 7.0 (Kumar *et al.*, 2016) and TOPALI 2.5 (Milne *et al.*, 2009)

respectively. 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. For the ML analysis, the bootstrap values based on 1,000 resampled datasets were generated. The genetic divergence among taxa was estimated using uncorrected 'p' distances in MEGA version 7.0. In addition, the sequence of *Fasciola hepatica* (AY222244) was used as the out group. The obtained sequences of the 28S gene for both species were submitted to GenBank for accession numbers (Table 1).

Ethical Approval and/or Informed Consent

The research related to the experiments and handling of frog in the present study has been conducted with all the relevant national regulations and institutional policies for the care and use of animals.

Results and Discussion

During the study, *Pleurogenoides* Travassos, 1921 species were found from the duodenum of freshwater frog *E. cyanophlyctis*. On the basis of morphological characteristics, the two species were identified as *P. cyanophlycti* Shinad & Prasad (2018a) and *P. euphlycti* Shinad & Prasad (2018a) respectively.

We have generated partial 28S sequences of ribosomal RNA for the two species of *Pleurogenoides* recovered from the Indian skipper freshwater frog of the Western Ghats, India. These sequences were analyzed together with other sequences of order Plagiorchiida under which the present studied species fall. ML and BI analyses produced similar topological tree, with somewhat different support values at some nodes, therefore, only the ML tree was presented here. The resulting tree branch topologies from both, ML and BI analyses, were in consensus and representing species genetic lineages (Fig. 2). Moreover, the newly generated sequences of *Pleurogenoides* isolate clustered within family Pleurogenidae with well-supported clade A that representing parasite infecting frogs (Fig. 2). Both the two *Pleurogenoides* species (*P. cyan-*

Fig. 2. Phylogenetic tree based on partial sequences of 28S rDNA gene. Nodal support from maximum likelihood (ML) and Bayesian Inference (BI) analyses is indicated as ML/BI. Hyphen indicates node unsupported by BI. GenBank accession numbers are provided alongside the species names. The scale-bar indicates the expected number of substitutions per site.

Table 2. Genetic distances (below diagonal) and sequence similarities (above diagonal) of Pleurogenidae species infecting frogs. Taxa are listed as they appear in Fig. 2.

	1	2	3	4	5	6	7	8	9	10
1. <i>Pleurogenoides cyanophlycti</i> PC1 MN218390		100	75.0	75.3	69.6	69.2	69.5	67.6	69.8	70.4
2. <i>Pleurogenoides cyanophlycti</i> PC2 MN218388	0.00		75.0	75.3	69.6	69.2	69.5	67.9	69.8	70.4
3. <i>Pleurogenoides euphlycti</i> PE1 MN218391	0.23	0.23		100	78.4	78.2	78.4	77.9	79.4	79.7
4. <i>Pleurogenoides euphlycti</i> PE1 MN218389	0.23	0.23	0.00		78.7	78.5	78.7	78.1	79.4	80.0
5. <i>Pleurogenoides medians</i> AF433670	0.28	0.28	0.21	0.21		97.8	97.8	95.8	95.4	96.0
6. <i>Pleurogenes claviger</i> AF151925	0.28	0.28	0.21	0.21	0.02		99.0	95.9	94.9	95.5
7. <i>Candidotrema loossi</i> AY220621	0.28	0.28	0.21	0.21	0.02	0.01		95.8	95.0	95.4
8. <i>Pleurogenes</i> sp. Lb1P K342571	0.28	0.28	0.21	0.21	0.04	0.04	0.04		95.7	96.6
9. <i>Brandesia turgida</i> AY220622	0.28	0.28	0.21	0.21	0.04	0.05	0.05	0.04		98.7
10. <i>Prostocus confusus</i> AY220623	0.29	0.29	0.21	0.21	0.04	0.04	0.04	0.03	0.01	

ophlycti and *P. euphlycti*) were resolved as the 'Lineage II' representing them with well supported bootstrap values and formed a sister relationship with other species of Pleurogenidae belongs to different genera in 'Lineage II': *Pleurogenes* Looss, 1896 (Synm. *Candidotrema* Dollfus, 1951); *Prostocus* Looss, 1899; *Brandesia* Stossich, 1899 and all parasites of amphibians (Fig. 2). Table 2 represents the pairwise distance (uncorrected p-distance range) and identity values of both *Pleurogenoides* species with closely related species. The intraspecific divergence observed within the 28S sequences of isolates of *P. cyanophlycti* and *P. euphlycti* shows no differences while interspecific divergence between *P. cyanophlycti* and *P. euphlycti*, was found 0.23 %, and with other species of 'Lineage II' ranged between 0.21 – 0.29 % (Table 2). Travassos, 1921 erected the genus *Pleurogenoides* with type-species *P. tener* that was earlier described by Looss, 1898 as *Prostocus tener*. This genus comprises more than 26 species worldwide that infect frogs (Brinsh & Janardanan, 2014). In India, about 12 species of genus *Pleurogenoides* have been reported from frogs to date viz., *P. gastroporus* (Luhe, 1901) Travassos, 1921; *P. sphaericus* Klein, 1905; *P. sitapurii* Srivastava, 1934; *P. orientalis* Srivastava, 1934; *P. bufonis* Kaw, 1943; *P. ovatus* Rao, 1977; *P. jamshedpurensis* Hasnain & Sahay, 1987; *P. ranchiensis* Dan & Hasnain, 1991; *P. malampuzhensis* Brinsh & Janardanan, 2014; *P. cyanophlycti* Shinad & Prasad, 2018a; *P. euphlycti* Shinad & Prasad, 2018b & *P. wayanadensis* Shinad and Prasad, 2018b. Description and characterization of all the above species were made on the basis of morphological characteristics; however, additional molecular work is needed to support their taxonomic validity that is also important in the case where several species of the same genus are reported from the same host.

A nuclear gene (28S) was used as molecular marker for *Pleurogenoides cyanophlycti* and *P. euphlycti* for the present study. The only member of *Pleurogenoides* with a DNA sequence available is *P. medians* (AF433670) from *Rana lessonae* which demonstrates the scarcity of molecular data of this genus. The results of the present study demonstrate the need for DNA sequence data of

other congeneric species distributed worldwide to understand the evolution and taxonomy of this group of parasites. Remarkably, *Pleurogenoides* Travassos, 1921; *Pleurogenes* Looss, 1896 (Synm. *Candidotrema* Dollfus, 1951); *Prostocus* Looss, 1899 and *Brandesia* Stossich, 1899 are morphologically differentiated with each other by the variability of the position of the genital atrium (Sharpilo & Iskova, 1989; Lotz & Font, 2008). In *Pleurogenoides* it is antero-lateral and situated distantly from the ventral sucker; in *Pleurogenes* (= *Candidotrema*) it is antero-lateral in position and located close to the oral sucker; in *Brandesia* it is placed in a lateral position and situated close to the posterior part of the body; while in *Prostocus* it is positioned laterally and distantly placed from oral sucker. Tkach *et al.*, 2003 in a study on molecular phylogeny and morphological data of the Microphalloidea Ward, 1901 synonymized *Candidotrema* Dollfus, 1951 with *Pleurogenes* Looss, 1896.

Our study has revealed the phylogenetic similarity of *P. cyanophlycti* and *P. euphlycti* and allocated their correct systematic position that they belongs to the family Pleurogenidae within the superfamily Microphalloidea, that corresponds to the trees generated in other studies (Kanarek *et al.*, 2014, 2015, 2017; Bella *et al.*, 2018; Tkach *et al.*, 2019). Though, for molecular study of *Pleurogenoides* we have only one representative species, *P. medians* and more data are required for a more congruent phylogeny. In contrast, *P. medians* seem to occur in 'Lineage II' that results a key question is the genus *Pleurogenoides* is not monophyletic, but it is very early to predict anything regarding paraphyly without addition and analyzing of other congeneric species data of this genus should be revised. Although, molecular sequences of the various genera included in the Pleurogenidae in future studies will clarify their phylogenetic affinities and systematic positions.

In the phylogenetic tree, *Pleurogenoides cyanophlycti* and *P. euphlycti* were nested in a 100 % supported clade formed for Indian species and suggested that the status of *Pleurogenoides* species needs to be re-evaluated in light of more molecular sequences and included in future phylogenetic analyses.

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Conflict of interest

The authors declare that they have no conflict of interest.

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