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RESEARCH ARTICLE

TAXONOMICAL AND PHYLOGENETIC CHARACTERIZATIONS OF A NEW INTESTINAL PARASITE PROENENTERUM MYRIPRISTIAE (DIGENEA, LEPOCREADIIDAE) INFECTING PINECONE SOLDIER FISH MYRIPRISTISMURDJAN (BERYCIFORMES, HOLOCENTRIDAE) FROM RED SEA, EGYPT

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ABSTRACT

Members of superfamily Lepocreadioidea are of considerable interest biologically as they comprise important groups of worms in range of marine habitats. In the present study, morphological characterization of *Proenenterum myripristiae* (Family: Lepocreadiidae), a new digenetic trematode infecting the intestine of Pinecone soldierfish *Myripristismurdjan,* was described using light microscopy for the first time from Coasts of Red Sea at Hurghada City, Egypt. Fish samples were trapped alive during the period of August 2014–May 2015 and necropsied for any helminth parasites. Out of 50, examined fish specimens, only 16 (32.0%) were found to be naturally infected. The large-sized fish reaching >30 cm and >110 g were more intensively infected than the smaller ones. The present species has all the characteristic features of the genus *Proenenterum* and characterized by elongated body with anterior pointed and posterior broad ends, two well-developed muscular suckers with ventral larger than oral one, caeca united posteriorly to form cyclocoel, two lobed testes, cirrus sac largely pre-acetabular, lobed ovary, vitelline fields reach to the ventral sucker and excretory vesicle is I-shaped. The present species morphology resembles the previous recorded species from Beryciformes, but with less dimensions of different body parts. Molecular characterization based on 28S large subunit ribosomal DNA gene was done to confirm the obtained morphological and morphometric results. A preliminary genetic comparison between LSU rDNA of this parasite and other species of Lepocreadioidea places the present specimen as a putative sister taxon to *P. ericotylum* and *P. isocotylum.*

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INTRODUCTION

Pinecone soldierfish, *Myripristismurdjan* Forskål (1775), is a common coral-reef species usually found in shallow lagoons and seaward reefs, nocturnal and feeds mainly on larger animals of zooplankton (El-Shahawy and Desouky, 2010). It is widely distributed from Red Sea, including Gulf of Oman and East of Africa, to Oceania, being one of the most important commercial species in the Egyptian markets (El-Shahawy and Desouky, 2010). In aquatic systems parasites play an important role in the ecology of coastal and marine ecosystems as well as in mariculture (Hoffman, 1999; Abdel-Gaber *et al*., 2015a). Helminth parasites in marine system considered generalists, lacking host specificity for both intermediate and definitive hosts (Koskivaara *et al*., 1992).

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In addition, many parasites in marine water possess life cycles consisting of long-lived larval stages residing in intermediate and paratenic host (Williams and Jones, 1967, 1994). Most helminth parasites carried by fish were trematodes, cestodes, nematodes and Acanthocephala (Abdou, 2001).During the last ten years, digenetic trematodes of marine fish from Red Sea received a great attention from several workers (Nagaty, 1957; Nagaty and Abdel-Aal, 1972; Saoud, 1985; Gibson, 2002; Abdel-Gaber *et al*., 2015b). Recently, continuous works on the description of several new digenea species from Red Sea fish and revision of the taxonomy for the others (Gibson, 2002; Abdel-Gaber *et al*., 2015b; Morsy *et al*., 2011; Abdel-Ghaffar *et al*., 2013, 2015). The Lepocreadioidea Odhner (1905) is one of the complex and problematic digenean superfamilies. Ten families and 137 genera are recognized (Toledo and Fried, 2014) but molecular studies have demonstrated that three of these families (Acanthocolpidae, Apocreadiidae and Brachycladiidae) are not closely related to the Lepocreadiidae (Bray and Cribb, 2002; Bray, 2005).

Bray, Cribb and their colleagues devoted a comprehensive series of studies on the diversity of the Lepocreadioidea in marine teleosts, predominantly in the Indo-West Pacific and the North-East Atlantic, which resulted in detailed descriptions of a vast number of species, erection of new and/or reassessment of the existing genera and construction of identification keys to species and parasite-host and host-parasite lists. These data provided a sound basis for revisory work (Bray *et al*., 2009). On the other hand, extensive sampling for molecular studies carried out in parallel with morphological assessments has supplied an admirable number of sequences for species from a wide range of genera. Bray *et al*. (2009) assessed the phylogenetic relationships of representative species of the superfamily Lepocreadioidea using partial 28LSU rDNA and NAD1 sequences for members of the families Lepocreadiidae (42 species), Enenteridae (6 species), Gyliauchenidae (6 species) and Gorgocephalidae (1 species), along with 22 species representing eight other digenean families. Lepocreadioidea considered as monophyletic in origin, comprising six groups: three well-recognized families (Enenteridae, Gorgocephalidae and Gyliauchenidae) and three groups from the partitioning of the Lepocreadiidae in the phylogenetic tree (Bray *et al*., 2009).

In the present study, natural prevalence, morphological, as well as molecular analyses of 28LSU rDNA of *Proenenterum myripristiae*sp. nov. infecting Pinecone soldierfish *Myripristismurdjan* were carried out to determine the exact taxonomic and phylogenetic position of this parasite species within Lepocreadiidae family.

MATERIALS AND METHODS

Experimental animals and parasitological examination

Fifty specimens of the Pinecone soldierfish *Myripristismurdjan* belonged to family Holocentridae were trapped alive during the period of August 2014–May 2015 from the commercial fishermen from Coasts of Red Sea at Hurghada City, Egypt. All specimens ranged between 15 to 45 cm (total length) and 80 to 130 g (body weight). They were transferred under good aeration to Laboratory of Parasitology Research in Department of Zoology, Faculty of Science, Cairo University, Egypt. Fish were dissected and their body cavities and internal organs were examined under a stereomicroscope for the presence of any parasitic infections.

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Live digenea parasites were slightly compressed between a slide and a coverslip prior to examination under light microscope. Digenea parasites were washed in running water, soaked in semichon's aceto–carmine for 3 hrs, washed in distilled water, passed through ascending grades of ethyl alcohol 50, 70, 90 and 100%, transferred into xylol, clove oil and then mounted in Canada balsam. Slides were then incubated at 60ºC for 24 hrs to driving the air bubbles according to Schmidt (1992). Taxonomic identifications of the recovered digenean worms were based on Yamaguti (1971). Photomicrographs were taken using Zeiss Axiovert 135 microscope supplied with a Canon Digital Camera. All drawings were made with the aid of camera Lucida (Weesner, 1965). Measurements are taken in millimeters and presented as a range followed by mean ± SD in parentheses.

Molecular analysis

Total genomic DNA (gDNA) was extracted from ethanol preserved specimens using the DNeasy tissue kit (QIAGEN, Gmbh, Germany) following the manufacturer's instructions. The eluate was concentrated to a volume of 20 µl using Microcon YM–100 (Millipore) columns. PCR reactions were carried out in 25 µl volumes using illustraTMpuReTaq Ready-To-GoTM PCR beads (GE Healthcare), 10 µM of each primer and 1–2 µl gDNA. Partial LSU rDNA (600-650 bp) was amplified using forward primers (LSU5: TAGGTCGAC CCGCTGAAYTTAAGCA and ZX-1: ACCCGCTGAATT TAAGCATAT) and reverse one (1500R: GCTATCCTGA GGGAAACTTCG) according to Bray *et al*. (2009). Cycling conditions for partial LSU rDNA were as follows: denaturation for 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min 30 s at 72°C, and 7 min for extension at 72°C. PCR amplicons were gel-excised using a QIAquickTM Gel Extraction Kit (QIAGEN) following the standard manufacturer recommended protocol.

Cycle-sequencing from both strands was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) with 310 Automated DNA Sequencer (Applied Biosystems, USA) and the same primers for annealing. Contiguous sequences were assembled and edited using Sequencher^{TM} (GeneCodes Corp., Ver. 4.6) and sequence identity checked using the Basic Local Alignment Search Tool (BLAST server) (www.ncbi.nih.gov/BLAST/). Alignments were performed using CLUSTAL-X multiple sequence alignment (Thompson *et al*., 1997) with default settings and penalties. The alignment was adjusted by eye in MacClade (Maddison and Maddison, 2005). Regions that could not be aligned unambiguously were excluded from the analysis. Phylogenetic tree was constructed by Bayesian inference (BI) and neighbor-joining method using MEGALIGN (DNASTAR, Window version 3.12e).

RESULTS

Sixteen out of fifty specimens of Pinecone soldier fish *M. murdjan* with 32.0% and 1.87 as a prevalence and mean intensity of infection were found to be infected with *P. myripristiae* sp. nov. The infection was recorded in the intestine of fish with >30 cm and >110 g. The infection was recorded only during winter season to be 64.0% (16 out of 25).

Microscopic examination (Figs. 1,2)

The body of the adult worm is elongated, dorso-ventrally flattened with more or less a narrow anterior and broadly rounded posterior end. It measured 1.98–3.21 (2.54±0.1) mm long and 0.39–0.49 (0.37±0.01) mm wide. Two well-developed muscular suckers present. Oral sucker is oval in shape and measured 0.15–0.21 (0.19±0.01) mm in diameter, ventral sucker is spherical in shape and larger than the oral one measuring 0.20–0.32 (0.27±0.01) mm in diameter. Pharynx spherical in shape, measured 0.10–0.21 (0.18±0.01) mm long

and 0.13-0.23 (0.20±0.01) mm wide, while the esophagus very short, looped and bifurcating into two long narrow intestinal caeca. Two tandem testes are located in the middle third of the body, separate by vitelline follicles, and equal in size measuring 0.26–0.39 (0.32±0.01) mm long and 0.11–0.25 (0.19±0.01) mm wide. The cirrus sac in the form of a large ovoid structure filling the space between the ventral sucker and intestinal bifurcation containing tubular coiled seminal vesicle and measuring 0.27–0.42 (0.38±0.01) mm long. The ejaculatory duct is short, narrow and the genital atrium indistinct. Ovary is found immediately in the pre-testicular region measuring 0.16–0.35 (0.21±0.01) mm in diameter. Seminal receptacle present at right posterior edge of the ovary. Laurer's canal present, opening dorsal to ovary. Mehlis' gland consists of very extensive glandular cells anterior to the ovary. Uterus usually coils intercaecally between the anterior margin of the ovary and the posterior expanded portion of the seminal

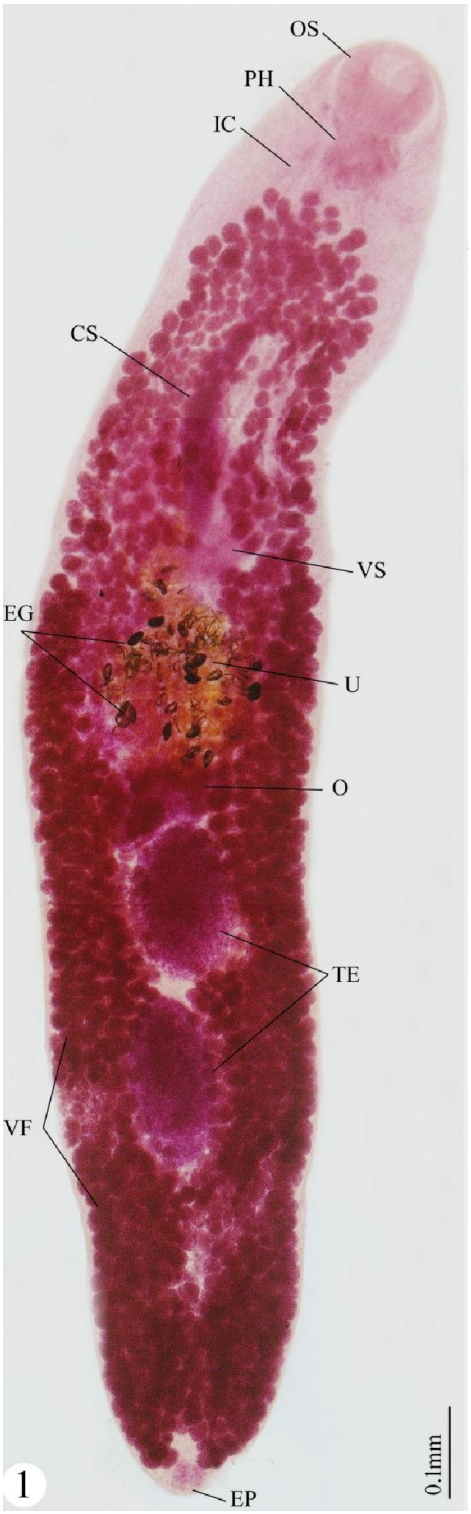

Fig. 1. Photomicrographs of the adult *P. myripristiae* sp. nov.infecting Pinecone soldierfish *Myripristismurdjan* fish showing oral sucker (OS), pharynx (PH), intestinal caeca (IC), ventral sucker (VS), cirrus sac (CS), ovary (OV), uterus (U), eggs (EG), testis (TE), vitelline follicles (VT), excretory bladder (EB), excretory pore (EP)

vesicle and then it passes to the genital aperture with little or no coiling and metraterm lacking. Vitellaria extend from the level of the intestinal bifurcation to the posterior end of the body locating laterally and dorso-ventral to the caeca filling the post–testicular space. It is clearly visible at the body constrictions and unites with a tubular, I–shaped excretory vesicle near the posterior end of the body to form uroproct, while the excretory vesicle passes anteriorly terminating at a point just anterior to the posterior margin of the ovary.

Eggs with short pointed knob at anopercular end, thin-shelled and measured 0.03–0.05 (0.04±0.001) mm long and 0.01–0.03 (0.02±0.001) mm wide. Excretory pore is terminal in position. Excretory vesicle I-shaped, extending ventral to the caecal union and dorsal to the testes near the ovary.

Taxonomic summary

Parasite name: *Proenenterummyripristiae* sp. nov. (F:Lepocreadiidae).

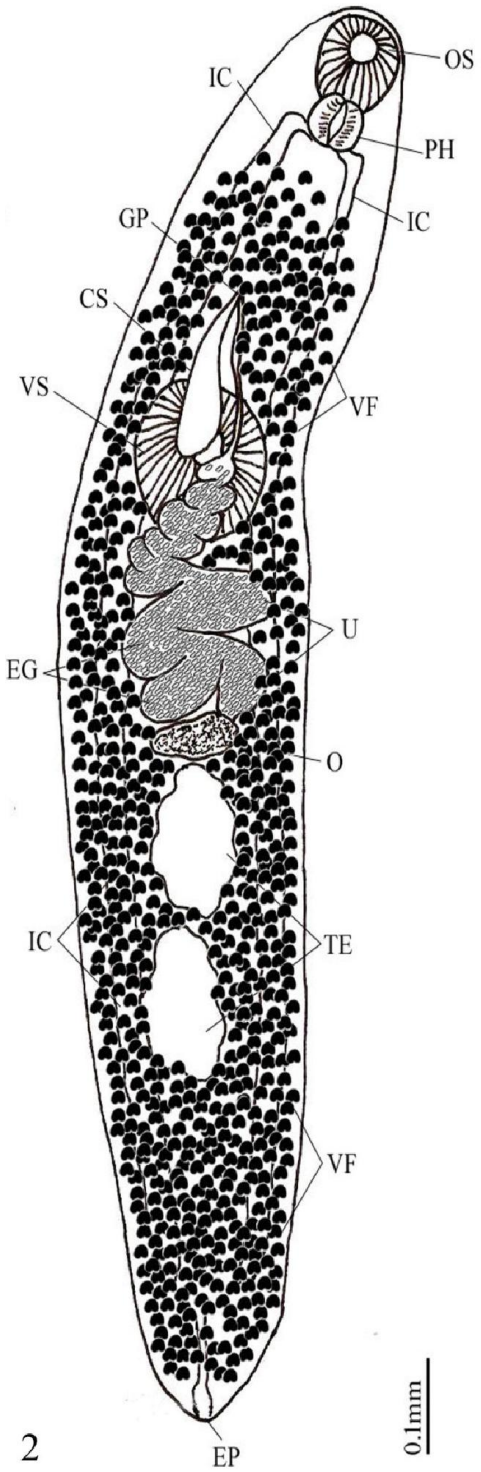

Fig. 2. Line diagram with camera Lucida of the adult *P. myripristiae* sp. nov

Type-Host: Pinecone soldierfish *Myripristismurdjan* Forsskål (1775) (F: Holocentridae).

Site of infection: Intestine of the infected fish.

Type–Locality: Coasts of Red Sea at Hurghada City, Egypt.

Prevalence and mean intensity: 32.0% and 1.87, respectively.

Specimen deposition: Specimens deposited in Department of Zoology, Faculty of Science, Cairo University, Cairo, Egypt.

Etymology: The species is named in reference to the fish name where the parasite was discovered and described for the first time.

Molecular analysis

A total of 630 bp was deposited in GenBank under the accession no. KT943447 with 55.87% GC content for LSU rDNA gene sequences of the present Lepocreadiid species. Pairwise comparison of the isolated genomic DNA sequence with a range of other species belonged to superfamily Lepocreadioidea within order Plagiorchiida and genotypes revealed a unique sequence. By calculating the percentage of identity between this novel sequences with other retrieved from Genbank demonstrated a high degree of similarity (>88%). Comparison of the nucleotide sequences and divergence showed that LSU rDNA of the present Lepocreadiid species revealed the highest blast scores with small number of nucleotide differences with FJ788499 of *P.ericotylum*, FJ788500 of *P. isocotylum*, AY222232 of *Enenterumaureum*, and AY222233 of *Koseiriaxishaense*.

Phylogenetic analysis produced a neighbor-joining tree constructed with partial sequences and consistently formed two clades (Fig. 3). The major one representing five families of superfamily Lepocreadioidea within order Plagiorchiida which are Enenteridae, Brachycladiidae, Acanthocolpidae, Leptocreadiidae, Gyliauchenidae with sequence similarity between 97-88%.

While, the other minor clade represents EU025874 *Fasciola hepatica* (Fasciolidae) and AY222246 *Echinostomarevolutum* (Echinostomatidae) as outgroup species. This sequence in conjunction with existing data investigates the placement of the recovered species within family Lepocreadiidae and deeply embedded in genus *Proenenterum* with close relationships to *P. ericotylum*and *P. isocotylum*as putative sister taxons.

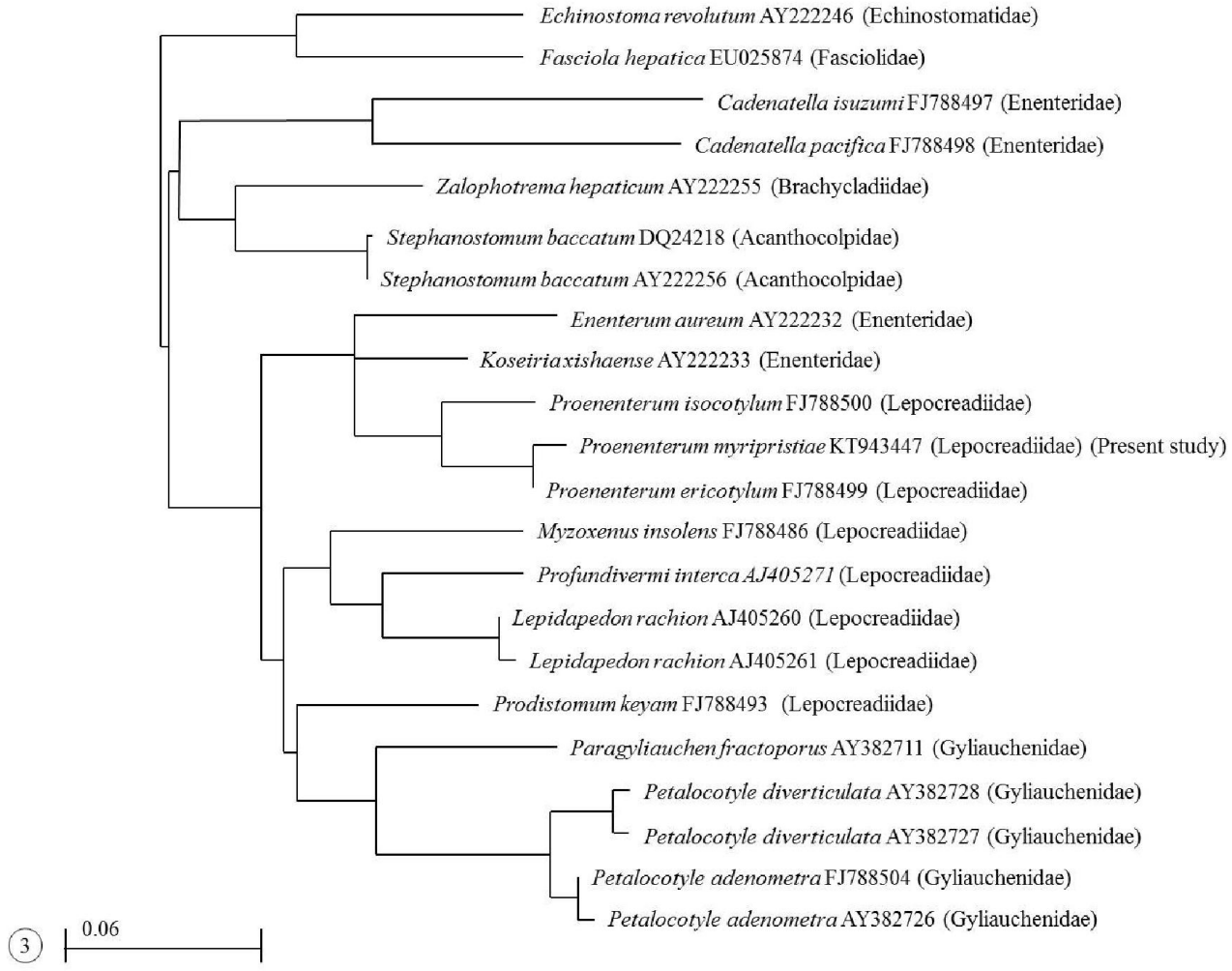

Fig. 3. Dendrogram based on LSU rDNA gene sequences showing the phylogenetic relationship between *P. myripristiae* sp. nov. and other Plagiorchiida species

SSCCION

Proenentrum is a genus of the digenetic trematodes belonging to family Lepocreadiidae established by Manter (1954). Parasites within this genus are characterized by the presence of elongated spinose body with no eye spot pigment, un–lobed and sub–terminal oral sucker, with pre–pharynx, posteriorly united caeca to form a blind cyclocoel, lacking of anus, presence of two lobed testes with tandem position in the third part of the worm body, cirrus sac largely pre–acetabular, lacking of the external seminal vesicle, ovary is oval or lobed–shaped, vitelline fields reach to the ventral sucker or into the forebody, uterus pre–ovarian, seminal receptacle is present, eggs thin–shelled and the excretory vesicle is I–shaped as stated by Bray (2005).

According to all above mentioned characters the present species have all characteristic features of the genus *Proenentrum*, and identified as a new species of *P. myripristiae* and recorded with 32.0% in the intestine of *M. murdjan*, these results coincided with data obtained by Morsy *et al*. (2011) who stated that *Pagrus pagrus* was found to be naturally infected with *Proenentrum* sp. with 32.8%. From previous literatures, there are three species of *Proenenterum* were reported, two of them recorded from the intestine of *Nototheriamacrocephala* from New Zealand, which are *P. isocotylum* Manter (1954) and *P. ericotylum* Manter (1954), while the third one is *Proenenterum* sp. from the pyloric portion of stomach and the middle part of the intestine of *P. pagrus* from Egypt.

Table 1. Comparative measurements (in millimeters) of the present *P. myripristiae* sp. nov. with other described previously

Related species / Parameters	*P.isocotylum* Manter (1954)	*P. ericotylum* Manter (1954)	*Proenenterum* sp. Morsy *et al*. (2011)	*P. myripristiae* (Present study)
Host	*Nototheriamacrocephala*	*N. macrocephala*	*Pagrus pagrus*	*Myripristismurdjan*
Locality	Wellington, New Zealand	Wellington, New Zealand	Red Sea, Egypt	Red Sea, Egypt
Body length	2.310–4.046	3.556–4.844	2.30-3.84 (3.25±0.20)	1.98–3.21 (2.54±0.1)
Body width	0.588–0.990	0.798–1.162	0.42-0.54 (0.48±0.02)	0.39–0.49 (0.37±0.01)
Pharynx length	0.107–0.192	0.138–0.323	----	0.10–0.21 (0.18±0.01)
Pharynx width	0.123–0.231	0.146–0.262	0.12-0.25 (0.17±0.02)	0.13-0.23 (0.20±0.01)
Oral sucker diameter	0.207–0.285	0.277–0.400	0.17-0.28 (0.20±0.02)	0.15–0.21 (0.19±0.01)
Ventral sucker diameter	0.200–0.300	0.515–0.708	0.26-0.39 (0.31±0.02)	0.20–0.32 (0.27±0.01)
Testes length	0.154–0.323	----	----	0.26–0.39 (0.32±0.01)
Testes width	0.138–0.300	----	0.32-0.48 (0.37±0.02)	0.11–0.25 (0.19±0.01)
Cirrus sac length	----	----	----	0.27–0.42 (0.38±0.01)
Ovary diameter	----	----	----	0.16–0.35 (0.21±0.01)
Egg length	0.054-0.065	0.042–0.053	----	0.03–0.05 (0.04±0.001)
Egg width	0.034–0.038	0.030–0.038	----	0.01–0.03 (0.02±0.001)
Excretory pore	Sub-terminal	Terminal	Terminal	Terminal

The present species morphology resembles the previous recorded species, but with lower dimensions of the body parts (Table 1). *P. isocotylum* and *Proenenterum* sp. is characterized by the presence of two lobed testes which is similar to the finding of the present study, but the ventral sucker is equal in dimension to the oral sucker instead of a large ventral sucker in our parasite species. *P. ericotylum* differs in presence of un–lobed testis but it supports the parasite under discussion in possessing a larger ventral sucker. The infection was recorded in the intestine of bigger fish with >30 cm and >110 g due to the fact that larger fish gave greater surface area for infection than smaller fish. This result coincided with Abdel-Gaber *et al*. (2015a) who reported that the juvenile fish had lower prevalence values while sub-adults and adults had higher prevalence of parasitic infections. Also, higher parasitism in sub-adults over juveniles may be due to activity. Sub-adults would be more active than the juveniles and probably even adults, as such, they are able to compete better than other age groups which means more contact with food and hence a higher tendency of getting infection with parasites. The seasonal prevalence for parasitic infection was recorded during winter season only to be 64.0% agreed with Schludermann *et al*. (2003), Silva *et al*. (2011) and Madanire-Moyo and Avenant-Oldewage (2013) reported that metal residues were higher in bottom sediments than in water and since the cichlid host fish aggregate in large pits on the river bed during winter. Therefore, it is possible that the impacts of these pollutants on the adult parasites which lead to increase the prevalence of infection.

Morphological systematics has encountered a problem resulting from the great similarity of many digenean parasites of teleost fish that called 'allocreadioid problem'. Several hundred current genera can be included in this somewhat homogeneous group, many of which were, in the first half of the 20th century, placed in the superfamily Allocreadioidea (Cribb, 2005a,b). More recently, the superfamily Lepocreadioidea has generally been considered the best depository for those taxa with a spiny tegument which are involved in this problem. The morphology in addition to phylogenetic analysis using LSU rDNA genes enhances significantly the chance for an accurate differentiation between the different species (Abdel-Ghaffar *et al*., 2015; Vilas *et al*., 2005).

This method offers essential criteria for the identification of new species and for re-description of an inadequately described species (Testini *et al*., 2011). The general structure of the dendrogram obtained in the present study is consistent with previous analyses by Bray and Cribb (2002), which were constructed using neighbor-joining method revealing the same gross topology and showing that our phylogenetic relationship demonstrated by two clades the major one represented by Enenteridae, Brachycladiidae, Acanthocolpidae, Lepocreadiidae, Gyliauchenidae as five families belonged to Lepocreadioidea while the minor one by Fasciolidae and Echinostomatidae as outgroup species. From the data obtained herein, Lepocreadiidae species revealed a separate line in the Lepocreadioidea as it strongly supported by the obtained

molecular data. Some clades, which are strongly supported by molecular data lack corresponding morphological synapomorphies. This leads to the conclusion that both kinds of data are valuable to describe the relationships among the Digenea (Abdel-Ghaffar *et al*., 2015; Testini *et al*., 2011). It was observed that Enenterinae and Lepocreadiidae are more related to each other than other families and the former included three genera which are, *Enenterum*, *Koseiria* and *Cadenatella* while the second included *Proenenterum*, *Myzoxenus*, *Profundivermi*, *Lepidapedon*, *Prodistomum* as stated by Bray *et al*. (2009) followed by Brooks *et al*. (2000).

Previous molecular phylogenetic studies have demonstrated a high degree of sequence similarity between the subset of *Proenenterum* species (Toledo and Fried, 2014; Bray and Cribb, 2001). The present investigation showed at least 88% sequence similarity to all Lepocreadioidea species described previously. Moreover, the present analysis revealed that the present parasite species was found to be deeply embedded in the genus *Proenenterum* with close relation to *P. ericotylum* and *P. isocotylum*as a more related sister taxons. Bray and Cribb (2001, 2012) included the genus of *Proenenterum* Manter (1954) within family Ententerinae due to the presence of two large testes and cirrus sac suggests that this parasite belongs to genus *Enenterum* Linton (1910).

Our data demonstrate some important differences between *Proenenterum* and enenterids, as the latter characterized by having oval body, spinose tegument, usually with eye spot pigment, the oral sucker is often lobed, the ventral sucker is rounded, pre-equatorial, external seminal vesicle is absent, and caeca may unite to form a blind cyclocoel which opens through an anus. Therefore, *Proenenterum* and its related species were grouped as a separate family Leptocreadiidae with monophyletic in origin.

Conclusion

According to these observations we consider that the present parasite species has first host record in Pinecone soldierfish *M. murdjan* and termed as *P. myripristiae* sp. nov. Also, the addition of new sequences from this study identifies the ancestral marine origin of the present species and it strongly aids to understand the cladistic arrangement within the more recent clade due to the addition of new species belonging to the previous genera.

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