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First molecular identification and phylogenetic tree of *Petasiger exaeretus* Dietz, 1909 (Digenea: Echinostomatidae) from an intermediate host *Radix auricularia* (L., 1758) in Greater Zab river, Iraq

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Abstract

Introduction. *Radix auricularia* (Linnaeus, 1758) is a freshwater gastropod belongs to the Lymnaeidae (pond snails) family which act as intermediate hosts or vectors of various parasitic flukes. No study has yet been undertaken on the prevalence of *Petasiger* spp. infection in *R. auricularia*. Species of *Petasiger* (Dietz, 1909) are a cosmopolitan parasite that utilize snails as the first intermediate host, with vertebrates like amphibians larvae and fish as the second intermediate host, followed by fish-eating birds. The current paper is considered to be the first report of *Petasiger exaeretus* parasitized *R. auricularia* in Iraq, which is supported with molecular and phylogenetic analysis.

Materials and method. Freshwater snails *R. auricularia* were collected during October 2016 – September 2017 from different locations of Sufaia village on the Greater Zab river, Erbil province, Iraq.

Results. A total of 307 freshwater snails *R. auricularia* were collected, only five of them were infected with a prevalence of *Petasiger exaeretus* (1.62%).

Conclusions. The current study agrees with the opinion of Selbach, Soldánová (26), which suggested the possibility of a much higher morphological diversity within *Petasiger* species, based on the number of described cercariae, compared with adult forms.It is clear that *P. phalacrocoracis* specimens have often been erroneously designated as *P. exaeretus* by many authors (Našincová et al., 1994). Certain morphological similarities and dissimilarities between *P. exaeretus* and *P. phalacrocoracis* can be detected: the pear-shaped body resembles *P. exaeretus*, whereas, *P. phalacrocoracis* have an elongated body.

Key words

Petasiger, Cercariae, *Radix*, Snail, Iraq

INTRODUCTION

Radix auricularia (Linnaeus, 1758) is a freshwater gastropod belonging to the family of Lymnaeidae (pond snails) which act as intermediate hosts or vectors of various parasitic flukes. This snail favours inhabiting stagnant, slow flowing water heavy with vegetation [1, 2]. At least 71 species of digenetic trematodes belonging to 13 families use this species as obligatory intermediate host in their life cycle, which can cause severe debilitating pathogenicity in many hosts, for instance, parasitized on birds and mammals as definitive hosts [3, 4]. In Iraq, different species of Lymnaeid snails were recorded, the most important being *Radix auricularia* (Eared pond snail), numerous investigations have been carried out on the abundance of cercariae infection and diversity in Lymnaeid snails, particularly *R. auricularia*, among them: [5–12]. To our knowledge, no study has yet been undertaken on the prevalence of *Petasiger* species infection in *R. auricularia*.

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Species of *Petasiger* (Dietz, 1909) are a cosmopolitan parasite that utilizes snails as the first intermediate host and vertebrates, like amphibian larvae and fish as the second intermediate host, followed by fish-eating birds (cormorants, grebes, herons, etc.) as a final host, occurring usually in the Palearctic region [13, 14, 15]. The main diagnostic features for identifyinjg *Petasiger* among species are the number of collar spines, and the arrangement for adult and developing stages. The numbers range between 19–27 with lateral and dorsal spines in a single row, and two groups of 3–4 angle spines which are longer than the lateral spines [16]. This constitute a relatively 33 nominal species (23 species recorded from the Palaearctic) and the most recent revision based on a comparative morphological study of the genus recognized a total of 18 valid species [17]. Seven species have been recorded in Europe: two species possessing 27 collar spines *Petasiger exaeretus* (Dietz, 1909) and *Petasiger phalacrocoracis* (Yamaguti, 1939) and five species with 19 collar spines (*P. grandivesicularis* Ishii, 1935, *P. islandicus* Kostadinova and Skı´rnisson, 2007, *P. megacanthus* (Kotla´n, 1922), *P. neocomense* Fuhrmann, 1927 and *P. pungens* (Linstow, 1893) [16]. In addition, it can be difficult to identify the infection at the species level using these methods, since the larval morphology is similar to each other [18].

Consequently, molecular approaches have been applied for accurate identification of the parasite at larval stages [19].

Despite the diversity of species of *Petasiger* in Europe and North America, only two species have been identified in birds from South America: *P. novemdecim* (Lutz, 1928), *P. argentinensis* (Lunaschi and Drago, 2010)) described in both the Great Grebe, *Podiceps major* (Boddaert, 1783), and the White-Tufted Grebe, *Rollandia rolland* (Quoy and Gaimard, 1824) in Argentina [16]. In addition, two undetermined species of Petasiger were reported in Argentina: *Petasiger* sp. 1 and *Petasiger* sp. 2 [20]. Information regarding the occurrence of larval stages of *Petasiger* in South America is scarce [21].

Moreover, all the above-mentioned researchers indicate that this species have not recorded outside its present location. The current study is therefore considered to be the first to report of *Petasiger exaeretus* parasitized *R. auricularia* in Iraq, which is supported by molecular and phylogenetic analysis.

MATERIALS AND METHOD

Study area. The freshwater snails *R. auricularia* were collected during October 2016 – September 2017 from different locations of Sufaia village on the Greater Zab river, Erbil Province, Iraq (36°11'49.3"N 43°35'02.8"E). The snails were examined for cercariae infection (Fig. 1).

Figure 1. Map of Greater Zab river in north Iraq showing the studied area

Collection of Lymnaeid snails and digenean cercariae identification. The collected *R. auricularia* snails were kept in a plastic screw-capped container supplied with water from their habitat, then directly transferred to the Advanced Parasitology Laboratory. The species were identified by using an identification key [22]. The snails were kept alive in an aquarium with optimal temperature and O_2 pressure to study cercariae infection [23]. In order to isolate the larval trematodes from snails, a conventional method was used to examine cercarial infections by exposing the snails to light (shedding) and/or by dissection (Rediae were obtained by crushing infected snails between glass slides). The snails were individually placed in glass Petri-dishes containing 50 ml dechlorinated tap water. Each Petri-dish was lit for 4–6 hrs. with a 100-W light bulb at a distance of 15 cm to increase water temperature and induce the expulsion of cercariae. If no cercariae shedding observed, snails were crushed under a stereomicroscope by forcep in order to find immature cercariae, sporocyst and/or redia. Cercariae or other stages were fixed in 70% molecular grade ethanol for DNA extraction and sequencing.

DNA extraction and 28S amplification. DNA was extracted from the redia and cercariae isolated from the snails, preserved in ethanol and centrifuged at 8,000 RPM for 5 min.; the ethanol was then removed. The DNA was extracted using a *PrimePrep* TM Genomic DNA Extraction Kit (GeNetBio, Korea) according to the manufacturer's instructions. Quantification of DNA concentration was performed by using NanoDrop (ND-1000, USA). An area of 28S rDNA was amplified by applying a universal primer which expected to be specific to Platyhelminthes. The forward primer C1 (ACCCGCTGAATTTAAGCAT at position 25), and the reverse primer C3 (CTCTTCAGAGTACTTTTCAAC at position 390), which were designed by [24]. The expected size of PCR product was 365 bps. A standard PCR reagent was used (One PCRPTMP master mix, GeNetBio, Korea). The PCR mixture (20 µl) contained: 10 µl master mix, 1.5 µl of each of the 2 primers, 2 µl DNA template and 5 µl double-demonized water (ddH_2O). PCR was performed in a MJ Research, Applied Biosystem (AB) thermocycler under the following conditions: 94 C°/5 min; 35 cycles of 94 C°/45 sec; 50 C°/45 sec; 72 C°/45 sec, followed by one cycle of 72 C°/7 min. PCR products were resolved in 2% agarose gel stained with Safe dye or ethidium bromide for UV light visualization of DNA. DNA sequencing was performed using forward primer of DNA amplicons by using ABI 3730XLs nucleotide sequence analyzer (Macrogen-Korea). The evolutionary history was inferred by applying the Maximum Likelihood method based on the Jukes-Cantor model [25]. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 333 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [26].

TAXONOMIC

Evaluation analysis assisted in classifying the trematodes as:
Family Echinostomatidae Loss, 1899.
Subfamily Echinostomatinae Loss, 1899.
Genus *Petasiger* Dietz, 1909.
Petasiger exaeretus Dietz, 1909.
Syn. *P. baschkirovi* Ablassov and Iksanov, 1958

RESULTS

A total of 307 freshwater snails *R. auricularia* were collected, only 5 of which were infected with *Petasiger exaeretus*, with a prevalence (1.62 %). The examined parasite includes redia (mother and daughter) and cercariae. The Blast result in GenBank of DNA (28S rDNA) sequence of *Petasiger exaeretus* indicated 99% similarity to this species with Accession No. KT956923.1) (Fig. 2).

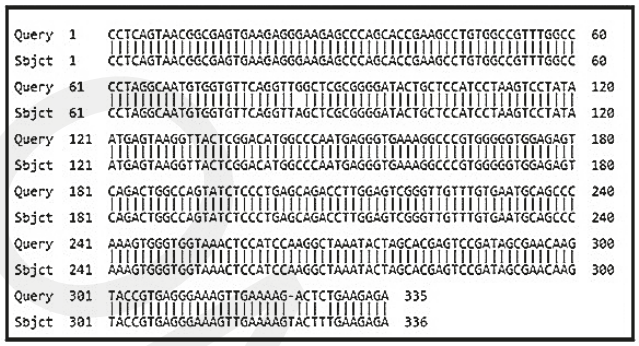

Figure 2. Pairwise alignment of 28S rDNA sequence of *Petasiger exaeretus*. Query is the study or sample sequence and Subject is the GenBank sequence.

DISCUSSION

Genus *Petasiger* was first named by Dietz in 1909. Members of this genus are common parasites of fish-eating birds and few others. The genus was previously reported in Europe, Asia, America, Africa and Australia [14, 17]. *Petasiger exaeretus* Dietz, 1909, is commonly found in birds belonging to the family Phalacrocoracidae [27]. The result obtained showed a 99% similarity to the partial 28S rDNA region of sporocyst, redia and cercaria sequences of *P. exaeretus* isolated from the *Radix auricularia* freshwater snail, recorded in Ukraine (KT956923.1) [28] and Hungary (KY284001.1- KY284007.1) [29] (Fig. 3), and were identical or nearly identical with the deposited sequences of *P. phalacrocoracis* (KY283999.1) described by Tkach, Kudlai [28], *P. phalacrocoracis* (KT956926.1) and *Paryphostomum radiatum* (KT956927.1) by Cech, Molnár [29]. Sequences of *P. exaeretus* with *P. phalacrocoracis*, *Pa. radiatum* did not match any of them and these differences can be attributed to host ecology and geography [29].

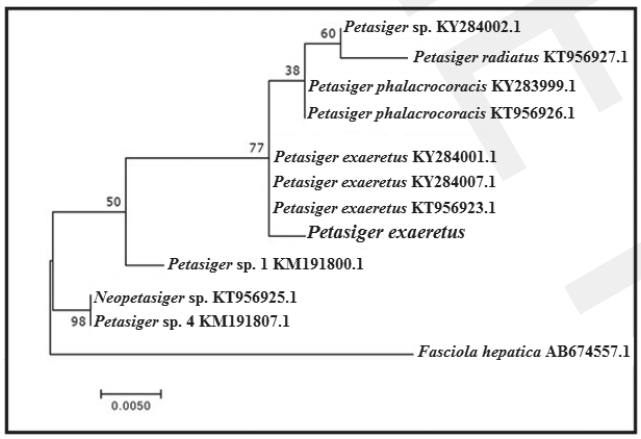

Figure 3. Maximum Likelihood tree 28S rDNA of the *P. exaeretus* isolated in *Lymnaea auricularia* in relation to echinostomid deposited in GenBank. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There was a total of 333 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

It is clear that *P. phalacrocoracis* specimens have often been erroneously designated by many authors as *P. exaeretus* [13]. Certain morphological similarities and dissimilarities between *P. exaeretus* and *P. phalacrocoracis* can be detected: the pear-shaped body resembles *P. exaeretus*, whereas *P. phalacrocoracis* have an elongated body. However, present trematodes closely resemble *P. exaeretus,* with slight variations [27]. *Neopetasiger* (KT956925.1) by Tkach, Kudlai [28], *Petasigr* sp. 4 (KM191807.1) by Selbach, Soldánová [30], which represent a molecular difference from the results of the current study, and dissimilarity with the outline specimen *Fasciola hepatica* (AB674557.1) [31]. Tkach, Kudlai [28], also proposed that *Pa. radiatum* should be transferred from the genus *Paryphostomum* to *Petasiger.* The current study agrees with the opinion of Selbach, Soldánová [30], which suggested the possibility of presenting a much higher morphological diversity within *Petasiger* species, based on the number of described cercariae, compared with adult forms. There are other *Petasiger* species described in Europe [32] for which their sequence data are not yet available, including *P. grandivesicularis* Ishii, 1935, *P. megacanthus* (Kotlán, 1922) and *P. pungens* (Linstow, 1893). Faltýnková, Gibson [17]{Faltýnková, 2008 #163;Faltýnková, 2008 #163} considered *P. caribbensis*, *P. novemdecim P. tientsinensis* and *P. baschkirovi* as synonymous of *P. exaeretus*. Recently, 2 studies used molecular markers on species of the *Petasiger* [33], and recorded 4 new cercariae of *Petasiger* species based on morphological and 28S rDNA, and nadI sequence analysis. Recently, using the 28S rDNA sequence, a full molecular phylogeny of Echinostomatoidea Looss, 1899, have been provided that involvedthe *Petasiger* species. In platyhelminth systematics, rDNA genes, in general, have been used successfully and 28S rDNA, in particular, to value the relationships existing among the Platyhelminthes [34].

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