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Molecular-Based Identification of *Polystoma integerrimum* by 28S rDNA, Phylogenetic and Secondary Structure Analysis

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Abstract

The present study was carried out to molecular identification, phylogenetic evolutionary and secondary structure prediction of the monogenean, *Polystoma integerrimum* which was isolated from amphibians *Bufo viridis* and *B. regularis*, collected from various regions in Erbil City, Iraq. After the morphological examination of the parasite, molecular identification and phylogenetic relationships were carried out using 28S ribosomal DNA (rDNA) sequence marker. The result indicated that the query sequence (sample sequence) was 100% identical to this species of the parasite and the phylogenetic tree showed 97-99% relationships in the sequence of *P. integerrimum* and 28S rDNA regions for other species of *Polystoma*. In addition, the present finding was confirmed by the molecular morphometrics, according to the secondary structure of 28S rDNA region. The topology analysis produced the same data as the acquired tree. In conclusion, the primary sequence analysis showed the validity of *P. integerrimum*. Phylogenetic tree and secondary structure analysis can be considered as a valuable method for separating species of *Polystoma*.

Keywords: *Polystoma integerrimum*, 28S rDNA marker, Molecular identification, Phylogeny, Secondary structure.

1. Introduction

Polystoma integerrimum (Froelich, 1791) belongs to Phylum: Platyhelminthes, Class: Monogenea, Order: Polyopisthocotylea. Mainly infected amphibian hosts (a parasite of the excretory bladder of frog) freshwater terrapins. It has been also recorded in the Australian lungfish and the African hippopotamus (Du Preez, 2015). The parasite has a direct life cycle, typically; eggs are laid and hatch into water and become a free-swimming stage, then infect host (Koprivnikar *et al*., 2012).

Polymerase Chain Reaction- (PCR-)based techniques utilizing the 28S rDNA regions have proven to be a reliable tool for identifying the platyhelminthes species including *Polystoma* spp. and their phylogenetic relationships (Mollaret *et al*., 2000).

Phylogenetic relationships among members of Polystomatidae are controversial (Mollaret *et al.*, 2000). Resolving the interrelationships (phylogeny) of *P. integerrimum* and other members of Polystomatidae are especially significant issues in development, since the basal resolution critically affects our knowledge of primitive platyhelminthes. Study of the parasite morphology did not resolve a terminal polytomy (Du Preez, 2010) and it has been rejected in molecular analysis by Mollaret (1997).

Secondary structures of RNA are specially beneficial in the classification of organisms because they consist of certain properties that are not present in the primary region sequence, which represents morphological information; and the source of dependable secondary structure framework for 28S rDNA sequence would exhibit a basic step that directs a detailed knowledge of their biological functions, which gives a strength method for determining biologically relevant looping patterns in RNA structure (Chandni *et al*., 2012).

The first record of *P. integerrimum* in Iraq was done by Dauood (1974) from the green toads, *B. viridis*. Most studies on the monogenic members, especially *Polystoma*, were based on the morphological descriptions and diagnostic keys, in which the family Polystomatidae is

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distinguished by a well-evolved opisthaptor with six cup-like suckers.

In the present study, the first objective is to confirm the presence of *P. integerrimum* in amphibians collected in Erbil City (Iraq) using 28S rDNA sequence, then to establish the evolutionary relationships with other species of the *Polystoma* and provide a secondary structure modeling as a support of the primary molecular finding.

2. Materials and Methods

2.1. Description of the Study Area

Erbil City is the capital of Kurdistan Region, in the north of Iraq. The latitude of Erbil is 36.20, and the longitude is 44.01 (Figure 1). The Sami Abdul-Rahman Park lies near the center of the city. It is covered with different kinds of trees and grasses as the main green zone park in the city.

Various vertebrate animals live at the park, like pigeons, ducks, some reptiles, like turtles, and some amphibians, as frogs, in addition to different kinds of invertebrates, such as snails, crustaceans and aquatic insects.

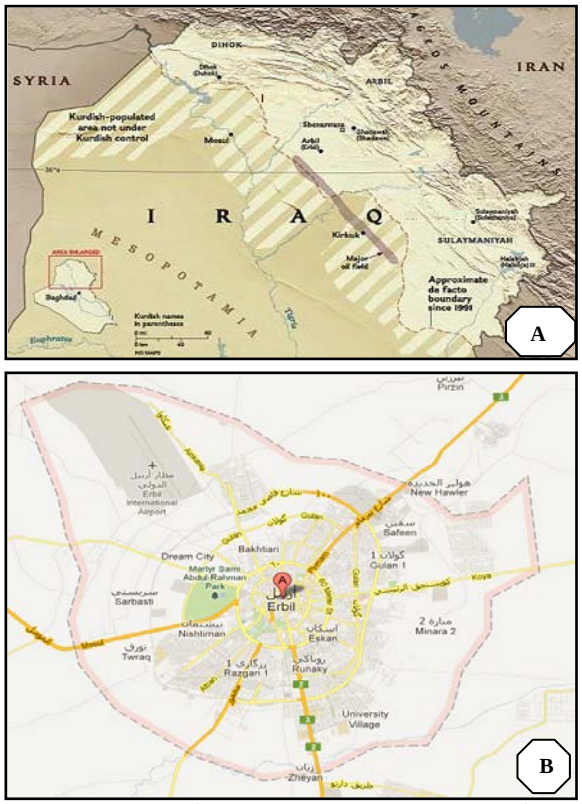


Figure 1: A- Map of Iraq showing northern part.B- Map of Erbil City showing studied area.

Shows the point of sample collection (red concentric circles).

2.2. Parasite Materials

A total of 33 frogs (21 of *Bufo viridis* and 12 of *B. regularis*) were collected by hand from different regions in Erbil City during the period of November 2014 to the end of April 2015. The frogs were anesthetized intra peritoneally with 2 ml of ketamine hydrochloride (50 mg/kg), and then the frogs were dissected as soon as possible. All viscera were removed and each of them was placed in a suitable Petri dish containing normal physiological saline 0.9% (Maulood, 2005). The following morphological descriptions and diagnostic keys were consulted for identification aid in the analysis of the distinctive features and finally the most determination of parasitic forms encountered (Yamaguti, 1963; Kudo, 1971; Smyth and Smyth, 1980).

2.3. DNA Extraction

Genomic DNA from *Polystoma* samples were obtained by employing kit of extraction (BIONEER, KOREA) according to the manufacture's instruction with few modifications (incubation time of tissue lyses step was extended into 3 hours and utilized absolute ethanol instead of isopropanol for DNA precipitation). The samples were macerated in mortar and pestle, and the contents were transferred into sterile tube containing 200 µl tissue lysis buffer and kept in incubator for 3 hrs. Qualification and quantification of DNA concentration were performed by using NanoDrop (ND- 1000, USA). Samples of DNA genomic with (A260–A320) / (A280–A320) ratio more than 1.7 and outputs more than 0.5µg were obtained.

2.4. DNA Amplification and Sequencing

A region of 28S rDNA was amplified by Polymerase Chain Reaction (PCR). The primers were universal, forward primer C1 (ACCCGCTGAATTTAAGCAT at position 25), and reverse primer C3 (CTCTTCAGAGTACTTTTCAAC at position 390), they were designed and selected by Mollaret *et al*. (2000) and expected to be specific to Platyhelminthes. PCR reaction and condition were performed using MJ Research, Applied Biosystem (AB) thermal cycler. Fifty µl reaction mixture was prepared in PCR tubes containing 2 µl DNA template, 25 µl OnePCR^{TM} master mix (GENEDIREX, KOREA), 1 µl for each primer and 21 µl double demonized water (ddH_2O). The cycling conditions comprised of initial denaturation at 94˚C for 5 min, 35 cycles of denaturation at 94˚C for 45 sec, annealing temperatures at 51˚C for 45 sec and extension at 72˚C for 45 sec, and final extension at 72˚C for 5 min. Agarose gel electrophoresis was employed to check the efficiency of PCR reactions. The samples were prepared and run in 2% gel of agarose then stained with ethidium bromide that makes the DNA visible under UV light, with the expected size of the PCR product was 365 bps.

In the present study, ABI 3130X nucleotide sequence analyzer (SINGAPORE) was used to find the nucleotides order of 28S rDNA from *P. integerrimum*. The PCR fragments of the *P. integerrimum* were excised from the agarose gel and used as a source of DNA template for sequence specific PCR amplification.

2.5. Phylogenetic and Secondary Structure Analysis

The regions of sequences were aligned employing muscle multiple alignment with the neglectful gap and expansion penalties employed by this program. The results of 28S rDNA regions were entered in the MEGA 6.0 (Barry, 2013) for constructing the evolutionary developmental trees. The phylogenetic trees of the *P.*

integerrimum were constructed using character state method (maximum likelihood). Branch support was given employing 1000 bootstrap replicates. Secondary structures of 28S rDNA regions of *P. integerrimum* were expected by the online MFold package (version 3.5) (Chandni and Hridaya, 2012). MFold is the broad employed algorithms for secondary structure of RNA expectation that are dependent on a search for the minimal free energy state.

3. Results

3.1. Molecular Based Identification

The sequence from DNA of *P. integerrimum* was 28S rDNA of 315bp (amplified fragment was 365bp, while after sequencing 50 miss-nucleotides were excluded, related to quality of sequencing analysis) put to BLAST then compared with other stored species of *Polystoma* sequences from GenBank. The BLAST results indicated that the query sequence was 100% identical to *P. integerrimum (*Figure 2).

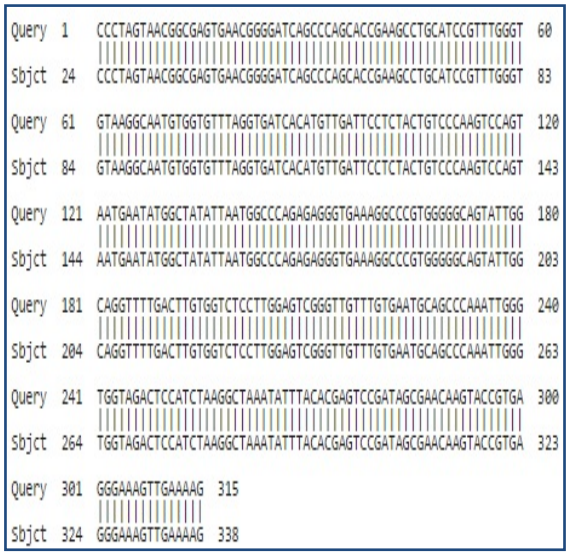
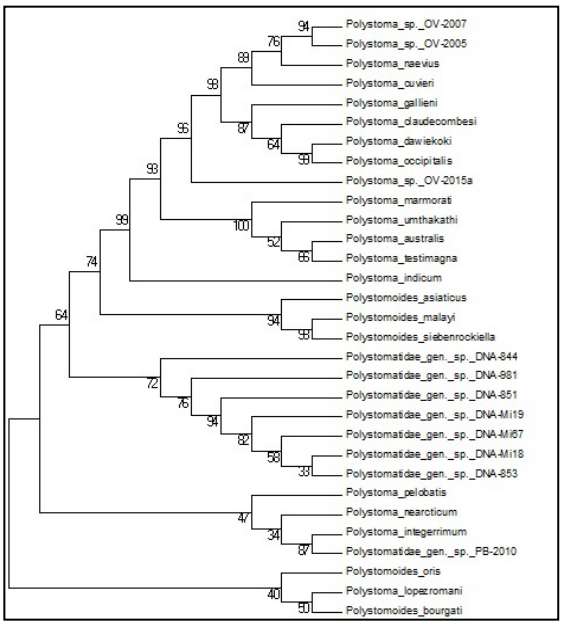


Figure 2. Pairwise alignment of 28S rDNA sequence of *P. integerrimum*. Query is the study or sample sequence and Sbjct is the GenBank sequence.

3.2. Phylogenetic Tree Analysis

Phylogenetic tree showed 97-99% relationships in comparing the sequence of *P. integerrimum* and stored 28S rDNA regions for the other species of *Polystoma*. Phylogenetic analysis employing the diverse method, maximum likelihood method as mentioned in Figure 3. It was revealed and indicated that the topology was the same among acquired trees with significant bootstrap support for the clades. For the bootstrap analysis, the values of 70% and above represented the accuracy of evolutionary development and showed reliable grouping among various species of *Polystoma*.

Figure 3. Phylogenetic positioning of P. integerrimum according to sequences of 28S rDNA employing maximum likelihood method, (Tamura 3-parameter model with invariant sites).



3.3. Secondary Structure Analysis

Predicted 28S rDNA secondary structural properties with the highest negative free energy ΔG= -113.00 Kcal/mol of *P. integerrimum* to provide the principal information for the evolutionary developmental analysis. The secondary sequence properties of 28S rDNA structure as represented in Figure 4 were analyzed according to loops and conserved stems. In the structure of *P. integerrimum*, the loops arrangements exhibit according to their numbers and classified into: hairpin loop, interior loop, multi loop, bulge loop and exterior loop (Figure 5). The topology was only based on the expected RNA secondary structure of 28S rDNA sequence, which determined most associations among the species researched.

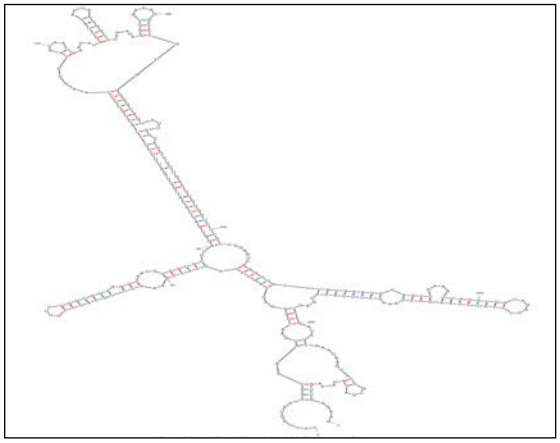


Figure 4: Schematic representation of the 28S rRNA expected secondary sequence of *P. integerrimum.*

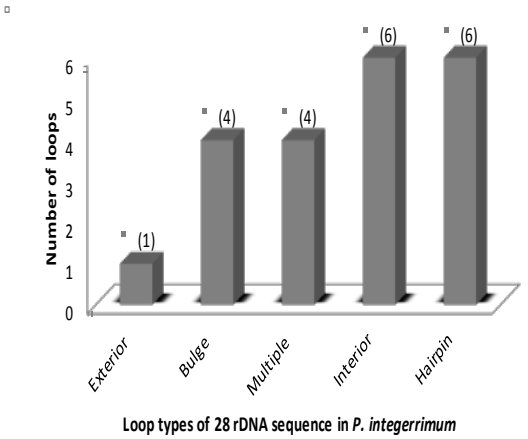


Figure 5: Arrangement of various loops of 28S rDNA sequence region of *P. integerrimum.*

4. Discussion

Polystoma is a cosmopolitan and diverse genus (Raharivololoniaina *et al*., 2011). Its-diversification might be related to host-specificity and host diversification by parallel evolution (Bentz *et al*., 2001). The present study includes several steps based on molecular characterization; phylogeny and secondary structure analysis of *P. integerrimum*.

P. inegerrimum species genetically well distinguished from the other available species of *polystoma* previously identified with the same rDNA sequence fragment marker, available at the National Center for Biotechnology Information (NCBI). The primary sequence analysis, using universal primers of study sample, revealed that the parasite belongs to species *P. inegerrimum* (Figure 2).

If the bootstrap value for a given interior branch of a phylogenetic tree is 70% or higher, then the topology at that branch is considered reliable according to the phylogenetic analysis rule (Chandni *et al*., 2012). The current data revealed that the bootstrap value was more than 70% for the acquired tree. The tree topologies according to phylogenetic analysis showed *P. integerrimum* as molecularly closely related with *P. pelobatis* and *P. nearcticum* species (Figure 3).

In addition, phylogenetic findings were confirmed using secondary structure analysis as a tool of bioinformatics, RNA loops were employed for rectification the alignment. Molecular morphometrics is intended to analyze phylogenetic relationships based on similarities among some structural characteristics of folded nucleotide molecules (Bernard *et al*., 2000). Molecular morphometrics has been reported to be the strongest tool in comparison to classical primary structure analysis, due to the only consideration of the size variations for homologous structural segments, whereas molecular morphometrics represents the folding pattern of RNA molecule (Bernard *et al.*, 2000). The topology of the present study, based on the expected RNA secondary sequence of the 28S rDNA structure, showed and determined most relationships among the species studied; for instance the structure of *P. integerrimum*, total number loops preference were 21 loops, types of the loops according to their numbers were exterior loop (1), bulge loop (4), multi loop (4), interior loop (6) and hairpin loop (6), respectively, as presented in Figure 5. RNA loops and conserved stems appeared to be complementary to classical primary sequence analysis in phylogenetic studies. Incorporation of secondary structure information allows improved estimates of phylogeny among several *Polystoma* species.

PCR-based techniques utilizing the 28S rDNA sequences have proven to be a reliable tool to identify the helminth species and their phylogenetic (Chandni *et al*., 2012). In platyhelminth systematics, rDNA genes, in general, have been used successfully and 28S rDNA, in particular, to estimate the relationships existing among the platyhelminthes (Morand *et al*., 2015). Therefore, many previous studies on phylogeny of the Polystamatidae members have been achieved using 28S rDNA and 18S rDNA. Mollaret *et al*. (2000) reported the phylogeny of Monopisthocotylea and Polyopisthocotylea (platyhelminthes) that were obtained from using 28S rDNA sequence analysis. The phylogenetic relationships of the families Polystomatidae and Sphyranuridae (subclass Polystomatoinea) within tetrapod monogenean parasites were investigated by using partial 18S rDNA sequences (Sinnappah *et al*., 2001). *Polystoma floridana* was described by Du Preez *et al*. (2007) as a new species of the Polystomatidae parasitic in the urinary bladder of *Hyla cinerea* by using 28S rDNA (analysis).

5. Conclusions

The results of the present study were concluded that the genus *polystoma* is the most diverse genus. Therefore, it is difficult to separate the species and sub-species from any phases in their life cycle only by relying on the morphology. For this reason, a dozen presumably distinct species remain unnamed. Primary sequence analysis revealed the validity of *P. integerrimum*. Phylogenetic tree and secondary sequence analysis could be a valuable method for separating species of *Polystoma*. The phylogenetic position of *P. integerrimum* within subfamily Polystomatinae renews interest in the facultative alternation of generations observed in anuran polystomatids.

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