Polish Journal of Veterinary Sciences Vol. 22, No. 4 (2019), 667–675

DOI 10.24425/pjvs.2019.129979

Original article

Phylogenetic and secondary RNA structure analysis of monogenean gill ectoparasites (*Dactylogyrus* spp.) parasitizing certain freshwater fishes

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Abstract

The current study is the first phylogenetic and secondary RNA structure analysis of *Dactylogyrus* species parasitising gill filaments of Iraqi cyprinid fishes. Most previous phylogenetic studies have targeted on primary DNA sequence data. Nevertheless, RNA secondary configuration is principally helpful in systematics since they comprise features that do not appear in the primary sequence and provide morphological information. The primary objective was molecular-based identification of Dactylogyrids species using evolutionary tree and secondary RNA structure prediction. A total of 681 fish were collected from the Lesser Zab River in the northeast of Iraq in the sub-district of Altun-Kopru from August 2016 to September 2017 and brought to the Zoology Research Laboratory, Salahaddin University-Erbil, Iraq. All fish were classified as 18 cyprinid species. The species of *Dactylogyrus* were identified by the 28S rDNA subunit using PCR and sequencing methods, and the obtained nucleotide sequences were then compared with the available GenBank sequences. Phylogenetic relationships were concluded using Neighbour-Joining (NJ), Maximum Likelihood (ML), and Minimum Evolution (ME) methods. The results justify the validation of 11 *Dactylogyrus* species (three of them were newly recorded in Iraq). Additionally, out of nine infected fish species, seven of them were regarded as a new host for *Dactylogyrus* species. Secondary RNA configuration prediction using minimum free energy was considered as a hopeful tool for species identification. This was considered the first comprehensive phylogenetic study in the area. It was concluded that PCR sequencing, phylogenetic and secondary RNA analysis were proper molecular methods for identifying Dactylogyrids species on the gills of fishes.

Key words: evolutionary tree, secondary RNA structure, Dactylogyrus spp., cyprinids, Iraq

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Introduction

Cyprinidae is the most common freshwater fish family. It consists of more than 3000 species which dwell naturally in all types of aquatic habitats. This family covers the most abundant species in Iraqi freshwaters where natural fish reside in more than 50% of the total fish inhabitants (Coad 2010, Reid et al. 2013, Nelson et al. 2016). Cyprinids have been adapted to carry species of *Dactylogyrus* Diesing, 1850. This monogenean gill inhabitant is one of the highest critical genera which involve more than 900 species (Šimková et al. 2017).

Accurate identification of parasites is a critical initial step in understand their biology, ecology, geography, specificity and transmission methods. The ribosomal DNA (rDNA) reappearance unit is the best choice for identification since it exists as a multi-copy unit repeat in the genetic materials of most eukaryotes. The rDNA covers highly conserved areas and possibly highly variable regions (Susurluk et al. 2007). Application of molecular markers in the phylogenetic research of several organisms has witnessed significant progress in recent years. On the other hand, conventional systems of organism identification based on morphology have some limits in classification. However, the use of DNA markers has become popular in recent years, but it is not entirely free of errors (Patwardhan et al. 2014).

Occasionally, molecular techniques are used in phylogeny and have developed as valuable supplementary tools in reliable and clear proof of taxa identification. The gene fragment of eukaryotic rDNA embraces highly conserved, 5.8S, 18S, and 28S regions and forms a repetitive tandem group to profoundly different, transcribed and intergenic spacer areas. It is worth mentioning that nuclear rDNA is being used to resolve taxonomic issues of helminthic parasites (Prasad et al. 2011, Dodangeh et al. 2017, Dutra Vieira et al. 2017, Mohanta and Itagaki 2017). So far, in Platyhelminthes systematics, including monogenean parasites, Polymerase Chain Reaction (PCR)- based methods utilising 28S rDNA areas have been successfully established to be a dependable tool (Rahmouni et al. 2017, Šimková et al. 2017, Benovics et al. 2018). Moreover, the partial sequences of the 28S rDNA region have been used effectively for monogenean phylogenetic tree reconstruction study (Singh and Chaudhary 2010). Having said that, the prediction of RNA secondary structure could be a complement to the phylogenetic monogenean classification, since they comprise particular characteristics that do not exist in the primary DNA sequence. The fragments of 28S rDNA are convenient for evaluating various divergence levels of taxonomy as they are a model for phylogenetic markers (Gillespie et al. 2005, Verma et al. 2012, Choudhary and Agrawal 2017).

Epidemiologically, more than 80 species belonging to the genus *Dactylogyrus* have been described from farmed and wild freshwater fish of Iraq (Mhaisen and Abdul-Ameer 2019). However, available data on the molecular phylogeny of these species are inadequate. Furthermore, 28S rDNA has been effectively used for determining the variations of DNA sequence among species of *Dactylogyrus* isolates (Singh and Chaudhary 2010, Ahmadi et al. 2017). Therefore; the first objective was to confirm the presence of *Dactylogyrus* spp. in cyprinid fish species exploiting molecular assays in the Northern part of Iraq. The second objective was to find phylogenetic trees of Dactylogyrids isolates as well as the prediction of secondary RNA structure modelling as a support for the primary molecular outcome.

Materials and Methods

Description of study area

The study area was situated on the bank of the Lesser Zab River in the northeast of Iraq in the sub-district of Altun-Kopru (Prde). It is 40 km north-west of Kirkuk City and 50 km away from Erbil City between 34° - 36° north latitude and 43° - 46° east longitudes and originates in Iran (Abdullah and Nasraddin 2015).

Fish host and parasite materials

A total of 681 fishes were collected weekly by local commercial fishermen with gill netting, from August 2016 to September 2017 and brought to the Zoology Research Laboratory, Department of Biology, College of Science, Salahaddin University-Erbil, Iraq. Containers including local river water were used for transportation and fish samples were dissected within 24-48 hours after their capture. All fishes were classified into 18 species (Family: Cyprinidae) according to Coad (2010), and the scientific names followed those provided in FishBase (Froese and Pauly 2017). Accordingly, the captured fish were *Acanthobrama marmid* (147), *Alburnus mossulensis* (36), *Arabibarbus grypus* (9), *Barbus* (*B.*) *sharpey* (18), *B. xanthopterus* (16), *Capoeta* (*C.*) *damascina* (28), *C. trutta* (12), *C. umbla* (14), *Carasobarbus luteus* (40), *Carassus* (*C.*) *auratus* (52), *C. carassus* (10), *Cyprinion macrostomum* (20), *Chondrostoma regium* (74), *Cyprinus carpio* (102), *Gara variablis* (8), *Leuciscus vorax* (78), *Luciobarbus* (*L.*) *esocinus* (10) and *L. kersin* (7).

Removed gills were placed in a Petri-dish with a small amount of water, and their filaments were tiered.

The species of *Dactylogyrus* were isolated from the gills of examined fish. After microscopic inspection, molecular analysis was carried out utilising 28S rDNA as a gene marker. At least five living Dactylogyrids (after leaving the gill filaments) were removed from the water using a small pipette and placed into an Eppendorf tube with absolute (99%) ethyl-alcohol and preserved at 4°C for further DNA extraction.

DNA extraction

Genomic DNA analysis of different isolated species was conducted using an extraction kit (BIONEER, KOREA) and pursuant to the company instructions with minor alterations (tissue lyses incubation time was prolonged to 3 hours and using absolute ethyl-alcohol for DNA precipitation as a replacement for isopropanol). The samples were soaked in Eppendorf tubes, and also the macerated tissues were then transported to a sterile tube having 200 µL lysis buffers and retained in an incubator for 3 hours. DNA concentration quantity and quality were realised using NanoDrop (ND- 1000, USA). An output of genomic DNA with more than 0.5µg amount and (A260–A320) / (A280– A320) ratio with greater than 1.7 qualities was achieved.

DNA amplification and sequencing

Universal primers were designed to amplify a partial sequence of 28S rDNA using PCR. Forward primer C1 (5′-ACCCGCTGAATTTAAGCAT-3′) at position 25, and reverse primer C3 (5′-CTCTTCAGAGTACTTTT CAAC-3′) at position 390 were selected by Mollaret et al. (2000) and predictable to be specific to flatworms. The thermal cycler PCR reaction and conditions were attained using Applied Biosystem (AB) MJ Research. The final volume of reaction mixtures was 50 µL equipped in a PCR tube containing two µL genomic DNA extract, 25 µL OnePCR™ master mixes (GENEDIREX, KOREA), one µL of each primer and 21 µL double deionised water (ddH_2O). The thermal cycling was carried out under the following conditions: 94°C for 5 min (initial denaturation), 35 cycles at 94°C for 45 sec (denaturation), 51°C for 45 sec (annealing), 72°C for 45 sec (extension), and at 72°C for 5 min as a final extension. The PCR products were analysed on 2% agarose gel electrophoresis stained with ethidium bromide and visualised under ultraviolet light. The PCR product predictable size was 365 bps. An ABI 3130X nucleotide sequence analyser (SINGAPORE) was used to find the orders of 28S rDNA nucleotides. The parasite PCR fragments as a source of DNA template were harvested from the agarose gel and submitted to sequence-specific PCR amplification.

Phylogenetic and secondary RNA structure prediction

The sequence results of 28S rDNA fragments were installed into the MEGA 7.0 software program (Kumar et al. 2016). They were aligned using ClustalW alignment for constructing the trees of evolutionary development (Hall 2013). The trees of all isolated species were constructed based on the Neighbour-Joining (NJ), Maximum Likelihood (ML), and Minimum Evolution (ME). The evolutionary distances were calculated using the p-distance model that is in the units of the number of base differences per site. Clade support was given using 1000 bootstrap replicates. The proportion of replicate trees in which the related taxa grouped in the bootstrap test was shown above the branches. The tree was drawn to scale with the length of the branches in the same units as those of the evolutionary distances used to assume the phylogenetic tree. Secondary structures for 28S rDNA fragments were predicted using minimal negative free energy state for isolated species via the online MFold package (version 3.5) (Verma et al. 2012). The study involved 12 nucleotide sequences (*Niphargus molnari* was considered as an outgroup). There were a total of 73 locations in the final data set. Bootstrap values were included to test the reliability of inferred trees, and the assessment of evolutionary divergence between sequences was calculated according to Shrivastava (2013).

Ethical approval

All examined fish, and procedures were used according to the institutional Research ethics committee office (Ref. No.: 2414/54/7(C), Date of issue: June 9, 2016).

Results

Analysis of phylogeny specified the authentication, and systematic location of eleven isolated monogeneans belonging to the Family Dactylogyridae. Figure 1A indicated the NJ phylogenetic position of the isolates.

The aforementioned methods (NJ, ML and ME) showed a similar pattern of divergence and closely comparable bootstrapped values. The trees displayed different clusters of *Dactylogyrus* species for different phylogenetic models. The first group included three species (*D. andalouensis*, *D. vistulae* and *D. squameus*) having bootstrap values ranging from 63-68%. The second group included two species (*D. omenti* and *D. malleus*) having bootstrap values between 53-61%. The third group included two species (*D. mascomai* and *D. lenkoranoides*) having bootstrap values 72-91%.

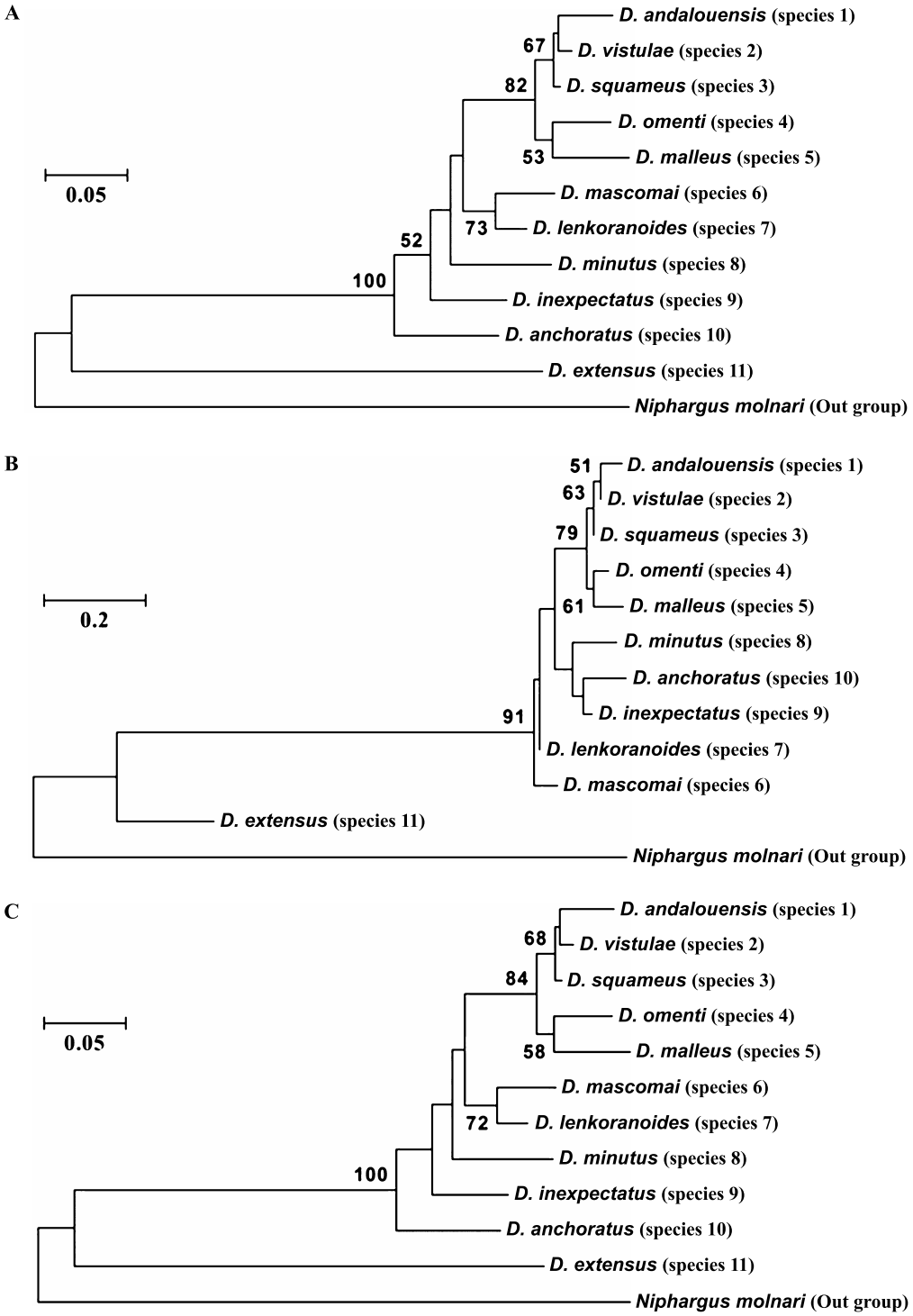

Fig. 1. Constructed phylogram of 11 *Dactylogyrus* species isolated from freshwater cyprinid fish. The trees are based on the concatenated data of partial 28S rDNA sequences. Branch lengths correspond to the expected number of substitutions per site. The trees were identical to the numbers along branches represent bootstrap values (1000 bootstraps). The species number indicates the isolated species sequenced from Iraq. *Niphargus molnari* was used as the outgroup. A- Neighbor-Joining (NJ) method B- Maximum Likelihood (ML) method C- Minimum Evolution (ME) method.

On the other hand, the combination values between the first and second bootstrap groups were 79-84%. Among the isolated species, phylogenetic relationships indicated that *D. omenti* constitutes a clade with *D. malleus*. These two mentioned species were found as a sister group to *D. andalouensis*, *D. vistulae* and *D. squameus* in the light of the three phylogenetic methods employed and they have the same position in the clades with a molecularly closely related species. *D. extensus* showed its validity as a monotypic species

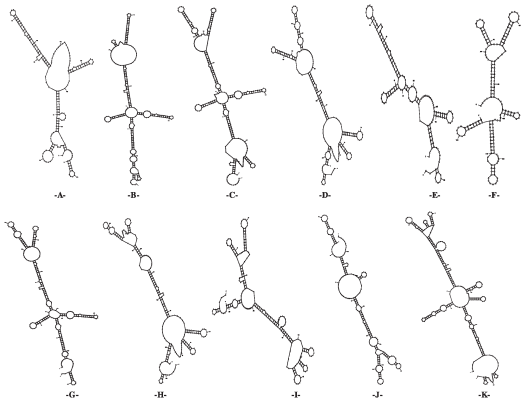

Fig. 2. Predicted secondary *28S RNA* structure orientation with the different loops types for all isolated *Dactylogyrus* (*D.*) species. Using online MFold software package based on the minimal free energy state.
(A) *D. andalouensis* **(B)** *D. vistulae* **(C)** *D. squameus* **(D)** *D. omenti* **(E)** *D. malleus* **(F)** *D. mascomai* **(G)** *D. lenkoranoides* **(H)** *D. minutus* **(I)** *D. inexpectatus* **(J)** *D. anchoratus* **(K)** *D. extensus*

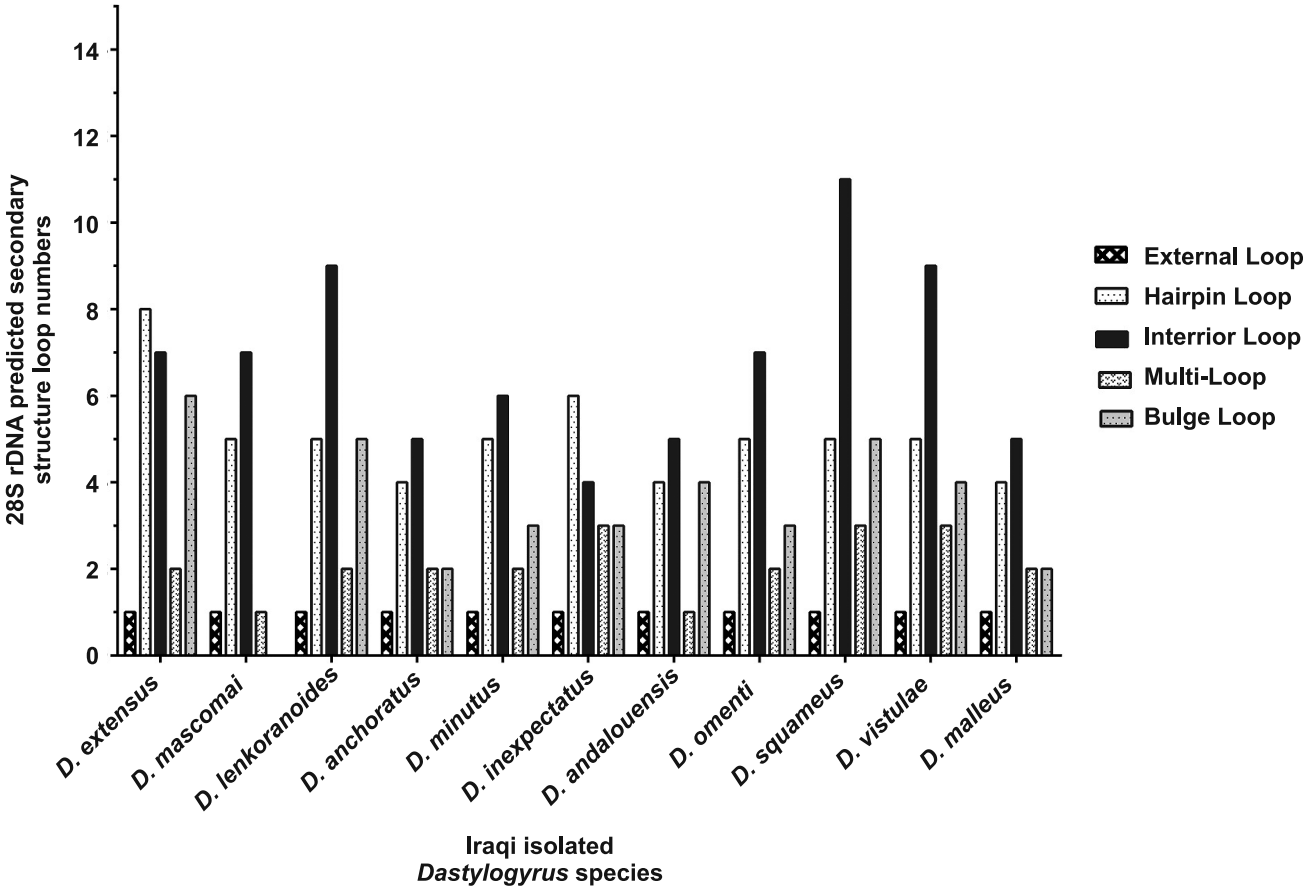

Fig. 3. Numbers and types of different loops for 28S rDNA predicted secondary structure analysis for all isolated *Dactylogyrus* species.

as compared to all isolated species and formed a separate clade. On the other hand, *D. minutus*, *D. inexpectatus* and *D. anchoratus* were constructed phylogenetically in different clades using NJ and ME. Whereas, *D. minutus* formed a sister group with both *D. inexpectatus* and *D. anchoratus* using the ML method. Also, *D. mascomai* established its location by forming a clade with its sister species (*D. lenkoranoides*), having high bootstrap values (above 70%) in NJ and ME analysis. However, for ML analysis, they were contracted in different clades.

The phylogenetic analysis using the ML-method as shown in Fig. 1B shows that the pattern of branching was significant bootstrap support for the branches. The evolutionary history was inferred using the ML method established on the Tamura-Nei model. History of the evolution was inferred using the ME method as showed in Fig. 1C, which revealed a similar topo-

Table 1. Host species, prevalence (%), free energy (ΔG) of different sequences, G+C% contents and nucleotide composition among Iraqi *Dactylogyrus* species isolates.

Species of *Dactylogyrus* (*D.*)	Host species (Family: Cyprinidae)	Prevalence (%)	Negative Free Energy (ΔG) Kcal	G+C%	T(U)	C	A	G
D. anchoratus **(Dujardin, 1845) Wagener, 1857**	***Carassius auratus*** ***Carassius carassius***	19.23 80	-73.20	50	24.6	28.2	25.8	21.4
*** *D. andalouensis*** **El Gharbi, Renaud & Lambert, 1992**	***Arabibarbus grypus***	11.11	-62.20	51	23.1	28.8	26.0	22.1
D. extensus **Mueller & Van Cleave, 1932**	***Cyprinus carpio***	14.71	-118.40	50	23.7	21.6	26.3	28.4
D. inexpectatus **Izjumova, in Gusev, 1955**	***Carassius auratus***	1.92	-78.80	53	21.8	30.3	25.2	22.7
D. lenkoranoides **El Gharbi, Renaud & Lambert, 1993**	***Carasobarbus luteus*** ***Capoeta umbla*** **	7.5 14.29	-101.10	51	24.3	28.1	24.3	23.3
D. malleus **Linstow, 1877**	***Acanthobrama marmid*** **	3.4	-44.40	51	23.8	28.0	25.0	23.2
D. mascomai **El Gharbi, Renaud et Lambert, 1993**	***Carasobarbus luteus*** **	2.5	-46.50	52	24.6	28.7	23.4	23.4
D. minutus **Kulwiec, 1927**	***Cyprinus carpio***	9.8	-71.50	52	21.1	30.1	26.8	22.0
*** *D. omenti*** **Benovics, Kicinjaova & Simkova, 2017**	***Luciobarbus xanthopterus***	12.5	-64.70	48	23.3	28.3	29.2	19.2
*** *D. squameus*** **Gusev, 1955**	***Carasobarbus luteus***	2.5	-95.30	49	26.3	28.1	25.1	20.5
D. vistulae **Prost, 1957**	***Chondrostoma regium*** **	8.22	-101.70	50	23.8	27.7	26.1	22.4
Average			-79.1	50.6	23.6	28	25.8	22.5

* New species record in Iraq
** New host record in Iraq

logy and higher bootstrap values as observed in NJ and ML trees. The isolated species were predicted for secondary structures of 28S rDNA sequences. The predicted structures of the isolates afford the necessary evidence for phylogenetic investigation with their negative free energy as shown in Figs. 2A-K. The prevalence, free energy values of all isolates and G+C% content of 28S rDNA sequences are shown in Table 1. G+C% content for all the isolated sequences ranged from 48% to 60%.

The RNA secondary structures of the 28S rDNA regions were evaluated by conserved stems and loops. The detected structural resemblances in the predicted structure are further revealed at the energy level. The maximum negative free energy was observed in the *D. extensus* (-118.40 Kcal) followed by approximately similar in both *D. vistulae* (-101.70 Kcal) and *D. lenkoranoides* (-101.10 Kcal), while *D. malleus* (-44.40 Kcal) had the least. Average length and G+C content of all isolated nucleotide sequences were 256bp and 50.6% respectively. The secondary structure analysis demonstrated the existence of external, hairpin, interior, multi-, and bulge loops. The order of preference for maintenance was first the external and multi-loops followed by the bulge and hairpin loops, and considerable differences were found in the interior loop. The external loop constantly persisted in all species (Fig. 3).

Discussion

Certain species belonging to *Dactylogyrus* described previously in Iraq are probably misidentified and have inadequate or incomplete descriptions, since the classification of this monogenean species reported in the country was based on morphological features. Therefore, applications of molecular characterizations are necessary, which will be such the first investigation in Iraq. Among isolated Dactylogyrids species three of them were considered as newly recorded in Iraq, namely *D. andalouensis*, *D. omenti*, and *D. squameus*. Alternatively, out of nine infected fish species seven of them were regarded as a new host for the isolated species. The outcomes also indicated that the 28S rDNA region is highly conserved and is therefore useful in taxonomic studies of parasitic Platyhelminthes, including monogenean parasites. The DNA sequences of *Dactylogyrus* species were put to BLAST, and then

compared with the available GenBank sequences. The BLAST results showed a similar percentage ranging between 97-100% with the available *Dactylogyrus* species sequences at the National Center for Biotechnology Information (NCBI).

PCR-based methods used in Platyhelminthes systematics have indicated that 28S rDNA sequences are dependable approach for categorizing the monogenean species and their evolutionary trees (Verma et al. 2012, Morand et al. 2015). Due to the small morphological differences in the size and shape of the attachment apparatus among the species of *Dactylogyrus*, it does not give enough information to taxonomists as compared to 28S rDNA region. This can be attributed to the natural evolving of the gene or to the fact that this genus is organized in groups with a developed taxonomic level other than previously recognised (Rana and Das 2016, Ahmadi et al. 2017). Molecular phylogenetic approaches have advantages over the morphological taxonomy. They can exploit reliable techniques which cover different well-characterised features of molecular evolution, comprising various rates of nucleotide substitution. In this regard, the aforesaid model approaches do not go through with morphological analysis. The studies performed on 28S rDNA sequences confirmed that there is a high specific similarity (Shrivastava 2013, Tamura et al. 2013, Rana and Das 2016, Ahmadi et al. 2017). The conventional bootstrap methods (NJ, ML and ME) were used for analysis of phylogeny. Accordingly, they acquired the same tree pattern and 28S rDNA regions that conserved to a high degree, which matched the findings reported by (Chaudhary and Singh 2012, Shrivastava 2013). The outcome of the trees showed different clade groups of *Dactylogyrus* species for different phylogenetic models. In relation to this and according to the requirements of phylogenetic analysis, if the value of bootstrap for a specified internal phylogenetic clade is 70% or higher, at that point the topology at the clade is deemed reliable (Soltis and Soltis 2003, Verma et al. 2012). In contrast to the above groups, *D. extensus* has formed a separate branch as compared with other species of the genus *Dactylogyrus* isolated in Iraq.

The present findings have demonstrated that the significant bootstrap values of the acquired trees were formerly documented with the same 28S rDNA marker which is available at the NCBI. Furthermore, the tree surface similarities derived from the phylogenetic analysis, carried out by Chiary et al. (2014), have revealed that *D. mascomai* and *D. lenkoranoides* are genetically closely associated sister species and a high value of bootstrap was obtained for the branch formed by these sibling species. Although the current investigation has encompassed two aspects (DNA-based sequence and morphological features), only the DNA-based was depended in this study, whereas Dactylogyrids morphology-based diversification was reported in another study (Koyee and Abdullah 2019). In this regard, certain studies have pointed out that at least three characters are required to entail 95% bootstrap support, but non-contradicted groups boosted by less than three characters will essentially receive less than 95% support. Nevertheless, two clades may each be supported at 95% and are thus not conflicting. Having said that, the problem is associated with close species that have not diverged broadly. For this reason, analysis of samples according to various phylogenetic methods has disclosed a range of traditional bootstrap values from 67 - 92% with the increasing taxa numbers (Soltis and Soltis 2003).

As regards the species divergence, it has been assumed that over the evolutionary period, the shapes and sizes of monogenean copulatory structures developed into different types, which resulted in reproductive separation within overlapping microenvironments. This was also previously known in *Dactylogyrus* species (ŠImková and Morand 2008, Benovics et al. 2018). In general, species with approximately similar haptoral shapes are grouped (Benovics et al. 2018). The best target for phylogenetic study is considered to be the rRNA gene, since it consists of an extremely well maintained region beside different domains. The ribosome comprises proteins and rRNA. In eukaryotes, it consists of small and large subunits. Furthermore, rRNA genes develop more sluggishly than protein-encoding genes and are essential for distance-based associated species. In specific, secondary RNA structure have been established almost entirely on relative sequence analysis (Patwardhan et al. 2014).

The prediction of RNA structure plays an essential part in assessing the evolutionary associations that molecularly connect all organisms (Ritz et al. 2013, Gu et al. 2014). Fundamentals of secondary RNA structure can act toward an evolving trait, and phylogenetic relations may be outlined by variations in structural character states (Knudsen and Caetano-Anolles 2008). However, this study predicted rRNA structures for isolated species and demonstrated resemblances in their structural parameters such as different types of loops and thermodynamic energy. Since the secondary structures of rRNA evolutionarily well conserved and these structures are associated with the roles of RNA molecules. The ribosomal 28S rRNA gene sequence for various metazoan major groups has developed in recent years. Also, attempts to align sequences based on the secondary structure model of 28S rRNA for the declared groups of the organism have become valuable for phylogenetic analyses. As stated by Koyee et al.

(2016) the tree of the phylogeny and secondary structure analysis can be taken as a valuable model for species identification of monogenean parasites.

Conclusions

In the light of the current results, this study is considered as the first comprehensive molecular investigation in the studied area for the monogenean parasite recognition according to the 28S rDNA sequences, which prove to be a valuable marker for identifying *Dactylogyrus* species. In addition to DNA-based identification, the secondary RNA structure analysis might be used as a respected model to discriminate new species of monogeneans since conventional morphological studies and validation of these parasites is very problematic. Hence, three isolated species were considered as newly records in Iraq.

Acknowledgements

The authors would like to thank Veterinarian and Parasitologist Dr Kareem K. Hamad from College of Science, Salahaddin University-Erbil, for his invaluable comments on an earlier draft of this manuscript.

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