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Original Article

An investigation on the helminth parasites of Caspian turtle (*Mauremys caspica*) with a taxonomic note on recovered *Falcaustra* Lane, 1915 (Nematoda: Kathlaniidae) and *Spiroxys* Schneider, 1866 species (Nematoda: Gnathostomatidae)

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Abstract: In this study, the digestive tracts of the Caspian turtles (*Mauremys caspica*) were investigated for the presence of helminth infections. Specimens of roundworms were recovered from the large intestine and stomach of the Caspian turtles. The morphologic measures revealed the infection with nematodes of the genus *Falcaustra* and *Spiroxys*. However, some differences were found in the collected *Falcaustra* specimens compared to the previous descriptions. In parallel, samples were analyzed by sequencing of the ribosomal gene targets. The phylogenetic analysis showed that the *Falcaustra* species as members of superfamily Cosmocercoidea were grouped with some members of Ascaridoidea and Spiruroidea. Despite the significant morphologic differences, *Spiroxys* species formed a sister group with ascaroid and cosmocercoid roundworms. Therefore, our molecular findings revealed that the taxonomic position of both nematodes need be revised.

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Introduction

Nematodes of the genus *Falcaustra* Lane, 1915 and *Spiroxys* Schneider, 1866 have been reported from different species of turtles around the world. Bursey et al. (2000) listed a total of 68 *Falcaustra* species in reptilian hosts from which 29 species infect turtles. In recent two decades, a number of new species of *Falcaustra* were introduced from the digestive tract of turtles (Bursey and Kinsella, 2003; Bursey and Rivera, 2009; Bursey and Brooks, 2011) and other reptiles (Bursey et al., 2007; Bursey et al., 2009). Spirurid nematodes of the genus *Spiroxys* are also parasitic in the alimentary canal (mainly stomach) of this type of hosts (Baker, 1987) with more than 17 recorded species (Roca and García, 2008).

Most of the taxonomic knowledge on both above-mentioned genera are based on morphological features. This has been led to describing many new taxa that are often difficult to identify (Baker et al., 1986). In addition, the taxonomy of the genus *Falcaustra* is somewhat confused because of several provided descriptions of some species and the fact that the information on some species is inadequate. Although molecular-based methods have developed for the identification and taxonomy of helminths, data for molecular diagnosis of *Falcaustra* and *Spiroxys* is rare (Li et al., 2014; Rajabloo et al., 2017). Therefore, the integration of genetic markers with morphology could be important for accurate identification.

In the present study, the helminths recovered from the stomach and large intestine of the Caspian turtle (*Mauremys caspica*) (Testudines: Geoemydidae) were examined by conducting a morphological study and molecular analyses of their targeted regions in ribosomal RNA gene.

Materials and Methods

The parasite specimens and diagnosis: A total of 5 infected turtles of *M. caspica* were collected in the Sepidan (29°54'27"N 52°32'48"E) and Beyza (29°58'19"N 52°24'4"E) regions, Fars Province, southern Iran. Turtles were subjected to standard euthanasia and post mortem examination using protocols described by Mader and Garner (2006) and

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Underwood and Reymond (2020). The alimentary canal including the esophagus, stomach, small and large intestines of turtles were examined separately for the presence of helminthes. Some of the recovered specimens were preserved and fixed in 70% ethanol, cleared in lactophenol, mounted and examined for morphological examinations according to Bursey et al. (2009) and some were fixed into 96% ethanol for molecular study.

Molecular procedures: Genomic DNA was extracted from the adult nematodes using a commercial Kit (MBST, Iran) according to the manufacturer's protocol. Our investigation showed infection of the examined turtle specimens with the species of *Falcaustra* and *Spiroxys* in the large intestine and stomach, respectively. The search for the recorded *Falcaustra* in the GenBank® revealed few available items mainly ribosomal and also mitochondrial (Cytochrome Oxidase) genes. On the basis of sequences for the partial 18s rDNA, a pair of primers: F (5'-AGAAACGGCTACCACATC-3') and R (5'-TTACGGTCAGAACTAGGG-3') were designed and used for recovered *Falcaustra* sp.. The PCR condition was 94°C for 4 min (initial denaturation), followed by 35 cycles of 94°C for 1 min (denaturation), 54.6°C for 1 min and 72°C for 1 min (extension) and a final elongation at 72°C for 2 min.

For molecular diagnosis of the stomach worms i.e. *Spiroxys* sp., the partial 18s rDNA and internal transcribed spacer (ITS) regions were considered and amplified using primers: F (5'-AGAGGT GAAA TT CGTGGACC-3') and R (5'-ATATGCTTAAGTTCA GCGGGT-3') (Hasegawa et al., 2009). PCR condition was 94°C for 4 min (initial denaturation), followed by 35 cycles of 94°C for 1 min, 55.7°C for 1 min and 72°C for 1 min (extension) and a final elongation at 72°C for 25 min. The PCR reactions were adjusted in a final volume of 25 µl, including 12.5 µl of PCR premix (Ampliqon, Denmark, Cat. No. A180301) (containing Tris-HCl pH: 8.5, $(NH4)_2S04$, 3 mM $MgCl_2$, 0.2% Tween® 20, 0.4 mM of each dNTP, 0.2 units/µl Taq DNA polymerase and Inert red dye and stabilizer), 10 pmol of each primer, 6.5 µl H_2O and 4 µl of DNA extracted as template.

The presence of the expected amplicons and their size were assessed by electrophoresis on each reaction product in 1.2% (*w/v*) Tris-acetate/EDTA agarose gel and visualized under ultraviolet illumination. Products from each assay were sequenced (ABI 3730 DNA analyzer; Bioneer, Korea). Because the specimens were found for the first time in the region, no positive control was available to conduct in molecular assay. To determine the phylogenic positions of recovered parasites, the sequences were compared with previously reported other nematodes in the GenBank. Creating multiple-sequence alignment was performed using the ClustalW program embedded in the MEGA 6.0 software. Data was also used for construction of the phylogenetic trees using maximum likelihood method (Tamura et al., 2007).

Results

Morphologic measurements: The large intestines of all turtles were highly infected with round worms (*Falcaustra* sp.); however only three nematodes (one intact female and two broken) (*Spiroxys* sp.) were recovered from a gastric section. According to the morphologic characters, nematodes from the large intestine were belonged to the Family Kathlaniidae Lane, 1914, the genus *Falcaustra* (Bursey et al., 2009). Those worms have a cylindrical body tapered gently towards the tail end. At the anterior margin, the mouth opening bounded by lips, each with conspicuous pairs of large fork-like papillae (Fig. 1b). The mouth opens into the pharynx with relatively thick walls. The esophagus has subspherical isthmus and a spherical bulb at the end (Figs. 1a, c).

In males (n=18), length was 15.6±1.4 (13.2-17.2 mm) and the width (at level of esophageal-intestinal junction) was 420.7±33 (372.2-459.9 µm). The esophagus consisting of vestibule (pharynx) 74.3±6.2 (65-80 µm); corpus 1939.7±236 (1608.5-2256.3 µm); isthmus 154.1±12.8 (133.2-165 µm) and a valved bulb 222.9±27.4 (173.4-244.3 µm) in length. Nerve ring was 369.4±6.5 (363.9-376.6 µm) from the anterior end. One pseudosucker was supported by 18-20 pairs of the muscle bands terminating on the border of the structure was seen in all males (Fig. 1d). Pairs of the

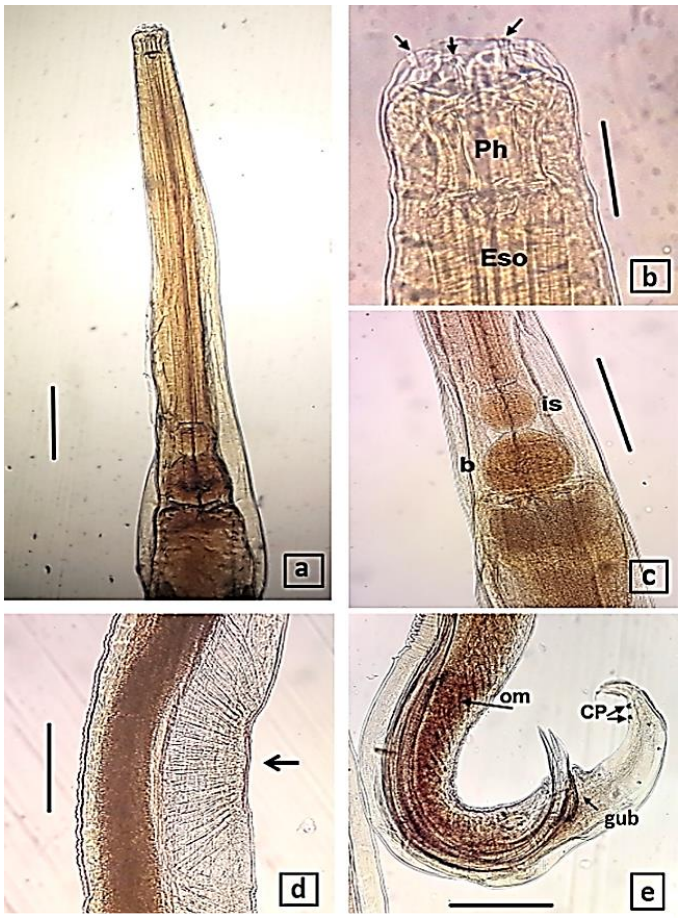

Figure 1. *Falcaustra* sp. (the Iranian isolates); (a) The anterior end (dorsal view); (b) The head region, showing large forked papillae on the head (arrows); (Ph) pharynx (vestibule) and proximal part of the esophagus (Eso); (C) Esophagus (distal part). isthmus (is) and bulbus (b) parts; (d) Male, the pseudosucker structure showing radiating muscles (arrow); (e) Caudal end of male. Oblique muscles (om); Caudal papillae (cp) spicules and gubernaculum (gub). Scale bar~300 µm.

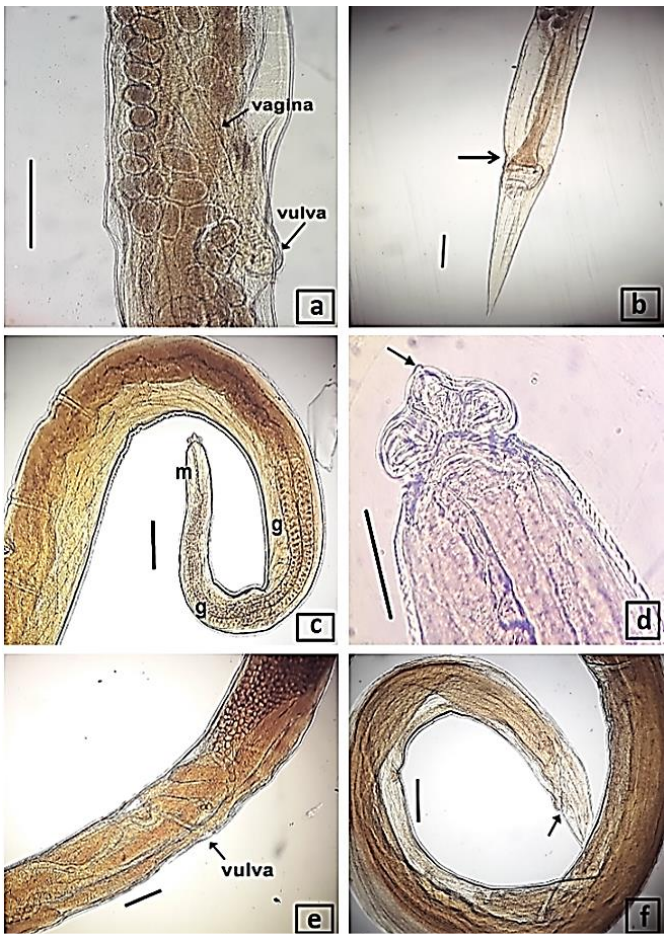

Figure 2. *Falcaustra* sp., female. (a) Distal part of reproductive system; (b) female, conical tail (arrow points to the anus); (c) *Spiroxys* sp., anterior part include the esophagus, muscular (m) and glandular (g) parts; (d) Trilobed pseudolabia forming a tooth on median part (arrow); (e) The female reproductive system; (f) Caudal end of female, arrow points to the anus. Scale bar~300 µm.

obliquely arranged muscle bands were observed beginning near posterior lip of the pseudosucker and terminating anterior to the cloaca. Five pairs of the caudal papillae, including one pair precloacal, one pair adcloacal and three pairs postcloacal (3-1+1) were observed. Spicules were similar in shape, curved, alate (which extended almost to the blunt end of the spicule), distal end pointed, 1.68±0.16 (1.48-1.9 mm) in length. Gubernaculum 178.9±17.9 (154.1-202.7 µm) in length, blunt distal tip. Tail (from the anus to the posterior end) was conical, terminated with a relatively sharp end, 574.5±68.7 (488.9-678.2 µm) in length (Fig. 1e).

Females' (n=15) length was 15.1±1.3 (13.2-17.6 mm) with width (at level of esophageal-intestinal junction) of 374.1±38.1 (319.9-455.1 µm). The esophagus consisting of vestibule (pharynx) 86.4±8.9 (70-100 µm); corpus 1689.5±163.4 (1510.1-2074.7 µm); isthmus 159.5±22.9 (138.2-212.3 µm) and the valved bulb 216.8±32.2 (174.5-288.9 µm) in length. Nerve ring was 416.8±67.8 (367.5-478.1 µm) from the anterior end. Vagina directed anterodorsally giving rise to two divergent amphidelphic uteri. Vulva, slightly prominent slit, 5.3±0.3 (5-5.4 mm) from the posterior end; eggs were oval to elliptical, thick shelled, unembryonated and 114.8±9.8 × 69.7±6 in size. Tail was conical, 1098.9±106.2 (976.5-1164.9 µm) (Figs. 2a, b).

Major morphologic measurements of *Falcaustra* species reported having one pseudosucker structure are listed in Table 1. According to described features,

Table 1. Main (selected) characterizations of some species of *Falcaustra* which are relatively closed to the specimens recovered in the present study.

Species	Host	Body length (mm)	Spicule length (mm)	Pseudo sucker	Papillae pattern	Country
Falcaustra ararath (current study)	Caspian turtle (*Mauremys caspica*)	15.3	1. 68 (1.48-1.9)	1	3-1 +1	Iran
F. ararath (Massino, 1924)	European pond turtle (*Emys orbicularis*)	15.0	1.54	1	8–6–10 +1	Armenia
F. kaveri (Karve and Naik, 1951)	cyprinid fish (*Barbus carnaticus*)	13.7–15.5	2.00–2.23	1	6–0–16 +1	India
F. armenica (Massino, 1924)	European pond turtle (*Emys orbicularis*)	7.2–8.9	1.10–1.30	1	6–2–10	Armenia
F. dubia (Yuen, 1963)	fanged river frog (*Limnonectes macrodon*)	13.5–14.0	1.54–1.69	1	6–2–12 +1	Malaysia
F. kempi (Baylis & Daubney, 1922)	Elongated tortoise (*Indotestudo elongate*)	10.9–12.8	2.90	1	10–0–8 +1	India
F. simpsoni (Johnston and Mawson, 1944)	Bell frog (*Litoria aurea*)	13.0–13.5	2.00	1	8–0–12 +1	Australia
F. chelydrae (Harwood, 1932)	Common snapping turtle (*Chelydra serpentina*)	10.0–12.5	3.4–3.9	1	6–0–14	USA

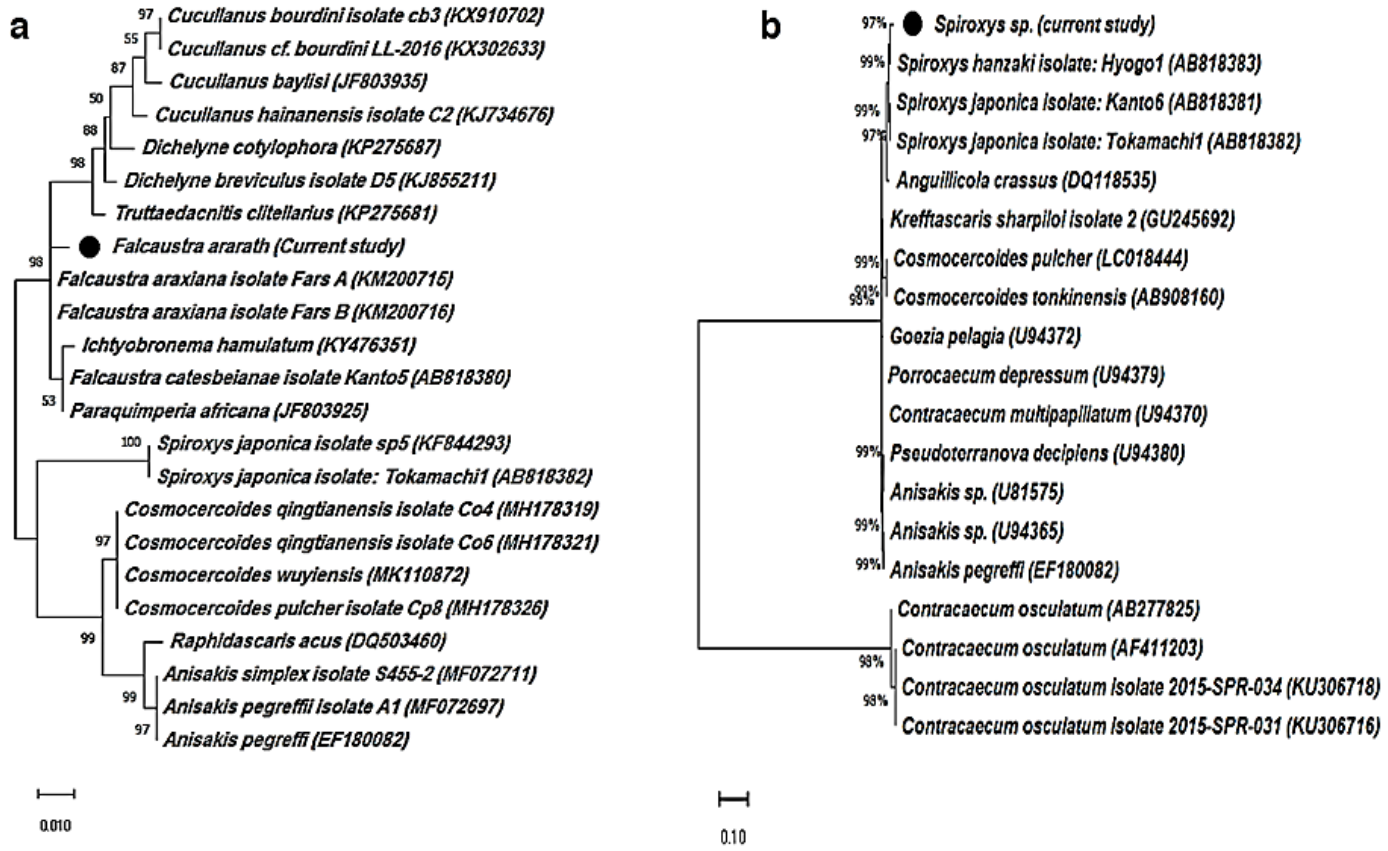

Figure 3. Phylogenetic position of the *Falcaustra* (a) and *Spiroxys* (b) isolates from Caspian turtles among nematode species in different hosts inferred from partial ribosomal gene targets using the maximum-likelihood method. Bootstrap values (2000 psuedoreplicates) represented at the nodes. The horizontal distance is proportional to evolutionary change as indicated (scale bar).

our specimens were identified as *F. ararath* Massino, 1924, described from a species of fresh water turtle, *Emys orbicularis*, in Armenia however, differences are seen in the papillae pattern and the average spicule size in males.

In this study, only one intact female nematode of

the genus *Spiroxys* was found in the stomach content and investigated. The morphology was indicative of the genus *Spiroxys* Schneider, 1866 (Figs. 2c-f). Its body length is 27.4 mm, the maximum width 676.5 µm, the oesophagus (2543.2 µm), thickened from the first third to the posterior and divided into anterior muscular, and a long posterior glandular portion (Fig. 2c). The cephalic end surrounded by the trilobed pseudolabia projectinh anteriorly in each median lobe to form a tooth (Fig. 2d). Thin-shelled sub-elliptical eggs were seen with a mean length of 56.96±6.4 × 41.04±6.6 µm (Fig. 2e). Tail was short, stout and conical, 333.18 µm in size. According to these features, our specimen was identified as *Spiroxys ankarafantsika*, described by Roca and García (2008) from Madagascan pleurodiran turtles (*Pelusios castanoides* and *Pelomedusa subrufa*).

Molecular analysis: The sequences of ribosomal gene targets were detected in samples and successfully amplified. For *Falcaustra* samples, DNA fragments of about 600bp was obtained for partial 18s rRNA gene (recorded in the GenBanks as MT160412). The comparison between our data and the three available sequences for *Falcaustra* in the GenBank revealed about 98.4 percent identity. Our samples had also a high identity with two other nematodes, *Paraquimperia Africana* (the intestinal parasite of a eel species) (98.4%) and *Ichtyobronema hamulatum* from burbot, *Lota lota* (97.9%). The phylogenetic analysis confirmed the close relationship between our samples and *F. araxiana* and *F. catesbeianae* separated from turtle and frog hosts, respectively (Fig. 3a). It was unexpected that the *Falcaustra* species as members of superfamily Cosmocercoidea were grouped with members of superfamilies Ascaridoidea (*Paraquimperia*) and Spiruroidea (Ichtyobronema); however, the other cosmocercoid nematodes were located in a separate clade. Furthermore, our specimens formed a sister group with some members of Seuratoidea (*Cucullanus*, *Dichelyne* and *Truttaedacnitis*).

The targeted ribosomal region was amplified in *Spiroxys* genetic material leading to fragments of about 1140 bp. Our specimens had nearly complete identity with *S. japonica* (AB818381 and AB818382) and *S. hanzaki (*AB818383) found in frog (*Lithobates catesbeianus*) and salamander (*Andrias japonicus*) hosts, respectively. In addition, a great identity was observed between our specimens, as members of Gnathostomidea, with *Anguillicola crassus* from superfamily Dracunculoidea. According to the phylogenetic tree, both nematodes are grouped with each other (Fig. 3b). Based on the ribosomal region, *Spiroxys* sp. formed a sister group with ascaroid and cosmocercoid nematodes, whereas there are significant differences between those taxa according to the morphologic characteristics.

Discussions

Different species of nematodes have been described occurring in the digestive tract of turtles (Bursey et al., 2009; Bursey and Brooks, 2011). Of the prevalent roundworms, the infection with the species of *Falcaustra* and *Spiroxys* were found in Caspian turtle. Previously, *F. armenica* from Caspian turtles (Youssefi et al., 2015) and *F. araxiana* from European pond turtles (*Emys orbicularis*) (Rajabloo et al., 2017) were reported in north and southwest regions of Iran, respectively. Unexpectedly, despite the similarity of the host, our samples had different morphology compared to those species. This indicates the diversity of the parasite in different localities. Having similar turtle hosts, *F. ararath*, *F. araxiana* and *F. armenica* were reported from *Emys orbicularis* in Armenia (the northwest neighbor of Iran), *F. donanaensis* from *Mauremys leprosa* in Spain (Hidalgo-Vila et al., 2006) and *F. manouriacola* from impressed tortoise (*Manouria impressa*) (Bursey et al., 2009). All the species had differences in main morphologic features, including the body size, spicule length and the number of pseudosuckers.

In this study, the structure of the esophagus, head and presence of one pseudosucker agreed with those previously described *F. ararath* Massino, 1924. In contrast, the caudal papillae pattern was not exactly unique to the previous description. In addition, regarding the average spicule length, our specimens had relatively higher measurements (1.68 comparing

to 1.54 µm). Nonetheless, these differences were not significant enough to assign our isolates into a new taxon.

Although numbers of species have explained in pervious literature for the genus *Falcaustra*, many of reports have re-described them or introduced new species during the past decades. In turtles, in addition to 29 species listed by Bursey et al. (2000), several new species have also assigned as *F. manouriacola* from *M. impressa* (Bursey and Rivera, 2009), *F. kutcheri* from *Geoemyda yuwonoi* (Bursey et al., 2000), *F. greineri* from *Orlitia borneensis* (Bursey and Kinsella 2003) and *F. donanaensis* from *M. leprosa* (Hidalgo-Vila et al., 2006). Regarding the controversial and/or overlapping morphological measurements in the past, the taxonomic investigation of those species is difficult. This was also the case for the *Spiroxys* samples. Although we did not recover male worms, the only recovered female had characteristics close to *S. ankarafantsika* separated from Madagascar freshwater turtles (Roca and García, 2008).

Many of the *Spiroxys* species have found in fresh water turtles within the suborder Cryptodira (Berry, 1985). The Caspian turtles belongs to Cryptodirans, however, *S. ankarafantsika* separated from pleurodiran turtles (belong to suborder Pleurodira) (Roca and García, 2008). According to reports on the presence of *Spiroxys* spp. in pleurodiran turtles in Australian (Berry, 1985), Madagascan (Roca et al., 2007) and Ethiopia (Berry, 1985), it was hypothesized that this nematode has been acquired from non-marine cryptodirans (Roca et al., 2007). Terefore, if we consider our samples as a type of *S. ankarafantsika*, it can be concluded that the transfer of *Spiroxys* sp. between two suborders of fresh water turtles have previously occurred.

Despite the presence of accurate morphologic characterization, molecular data on *Falcaustra* and *Spiroxys* is rare. In the GenBank, few records exist for *F. araxiana* and *F. catesbeianae* and for *S. japonica* and *S. hanzaki*. Nevertheless, on the basis of partial ribosomal gene, the cosmocercoid nematodes formed a separate clade; however, the *Falcaustra* species of turtles, as representatives of cosmocercoidea, were grouped with *Paraquimperia* (of Ascaridoidea) and *Ichtyobronema* (from Spiruroidea), both separated from kinds of fish. These groups possess significantly different morphology at least on their head region. In addition, a great identity was observed between the *Spiroxys*, as member of Gnathostomidea, with *Anguillicolaoïdes crassus* from Dracunculoidea and with ascaroid and cosmocercoid nematodes, both with a distinct structure. We found also the close relationship between *F. araxiana* and *Paraquimperia africana* showing the ambiguity on the present classification and phylogenetic relationship (Rajabloo et al., 2017). This controversial maybe because partial sequences have been considered and the number of available records is too limited to have an accurate molecular comparison.

In this study, the sequence data for the *Spiroxys* specimen was so close to *S. japonica*, but the morphologic characters were not agreed with this species well. On the other side, it was stated that the 18S rDNA and ITS regions revealed significant nucleotide differences comparing the two prevalent species, *S. japonica* and *S. hanzaki* (Li et al., 2014). Hence, it seems that the selected regions could be used for the identification at the level of species instead of the genus or higher taxa.

As a conclusion, these results criticize the morphologic data. Thus, it is believed that more molecular studies should be done to introduce a firm taxonomic framework for the prevalent nematodes of turtles. In addition, the phylogeny of both nematodes could be reconstructed according to the genetic investigations.

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