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The first case of *Spiroxys contortus* in European pond turtle (*Emys orbicularis)* in the wild in Poland

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ABSTRACT

During standard parasitological dissection of 4 individuals of European pond turtle (*E. orbicularis*) (Linneaus, 1758) one nematode was found. In a morphological examination the parasite was identified as *Spiroxys contortus* (Rudolphi, 1819) and confirmed by DNA analysis based on sequencing of the small subunit ribosomal RNA gene. The partial 18S rDNA gene was deposited to NCBI GenBank with the accession number MN629917. This is the first molecular evidence of *S. contortus* in *E. orbicularis* from wild from Poland. The analyzed sample genotype sequence shows 100% similarity to the reference specimen from Argentina.

1. Introduction

The European pond turtle is a freshwater turtle reported from northeastern Africa, across most of Europe to Asia Minor and the Caspian and Aral Seas (Fritz, 2003). Until the 19th century it was the most widely distributed turtle in Europe, but at present populations are gradually disappearing from most of its range. Therefore, it was registered as an endangered species in the Bern Convention (Appendix II) (Cadi and Joly, 2004; Iglesias et al., 2015; Héritier et al., 2017). The nematophauna of *E. orbicularis* living in Europe is presented in Table 1. The European pond turtle is the only representative of the order Testudines in Poland. In this country the helminth fauna of *E. orbicularis* is little known and includes the trematode species *Spirhapalum polesianum* (Ejsmont 1927) and the nematode *Falcaustra armenica* (Massino 1924) (Ejsmont, 1927; Yamaguti, 1961; Sprent, 1980; Zaleśny et al., 2009). There is also one report on the finding of the nematode *Spiroxys contortus* in a pond turtle purchased in a captivity (Łukasiak, 1939).

Spiroxys contortus, syn. *Spiroptera contorta* (Rudolphi 1819), *Spiroxys contorta* (Schneider 1866) belongs to the family Gnathostomatidae and it is the nematode mainly found in freshwater turtles (Hedrick, 1935; Mascarenhas and Muller, 2015). Formerly there was no documented case of invasion of *S. contortus* in *E. orbicularis* in the wild in Poland. In addition no gene sequence of this species has been published.

The aim of this work is confirmation of possibility of invasion of adult nematode from the genus *Spiroxys* in *E. orbicularis* in the wild in Poland.

2. Material and methods

2.1. Sampling

In present study we have examined 4 individuals of European pond turtle (three female and one male) which were found dead in November 2016 in the Western Polesie region, in the nearby Polesie National Park (South-Eastern Poland), obtained from Department of Animal and Environmental Hygiene, University of Life Sciences in Lublin, Poland. The turtles died of unknown causes. The consent for the use of biological material for research purposes from animals of species subject to close and partial protection WPN.6401.45.2015.MPR March 25, 2015. In addition, the stomach contents of 25 *Hydromedusa tectifera* (Cope, 1870), collected in the Buñirigo stream (35°01′36″ S, 57°17′24″ W, datum: WGS84), Buenos Aires province, Argentina, were analyzed. Seventeen *Spiroxys contortus* were recovered from both turtles, six males and 11 gravid females (Palumbo et al., 2016). The female of the

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S. contortus individuals from Argentina was sent to Poland and used as a reference individual for the species.

2.2. Dissection and microscopical examination

The animals were dissected the day after they were found. In order to detect helminths organs were separated, and their contents and mucous membranes were examined. The stomach wall was tested with compression method between two glass plates. The helminth found during dissection was identified by microscopical examination and diagnosed by morphological features according to the previous reports (Hedrick, 1935; Moravec and Vargas-Vázquez, 1998; Palumbo et al., 2016). The sample was fixed in 70% ethanol for molecular examination. The dimensions of helminth were measured using light microscope Olimpus BX51 with differential interference contrast (DIC) and integrated software Cell by Olympus Life Science. In order to compare helminth obtained from *E. orbicularis* from Poland and a reference individual of *S. contortus* originating from *Hydromedusa tectifera* (Cope, 1870) from Buenos Aires, Argentina (Centro de estiudios parasitológicos y de vectores CEPAVE-UNLP), we examined the differences in the dimension of the recovered worms and provided the molecular examination.

2.3. Molecular examination

For molecular examination 18S rDNA gene the analysis of one female of *S. contortus* from Poland and one female from Argentina was conducted. The partial 18S (SSU) and internal transcribed spacers and gene (ITS-1, ITS-2, 5.8S) of ribosomal DNA were amplified using the polymerase chain reaction. Genomic DNA from both individual worms was extracted using versatile kit for genomic DNA purification from various sources Genomic Mini (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. The procedure of amplification the purified DNA was conducted with forward EK-82F (5′-GAAACTGC-GAATGGCTC-3′) and reverse EK-1520R (5′-CYGCAGGTTCACCTAC-3′) primers. PCR reaction was performed with PCR Mix Plus (A&A Biotechnology, Gdynia, Poland) an optimized and ready to use high specificity PCR mixture containing Taq DNA polymerase, PCR buffer, $MgCl_2$, dNTPs and stabilizers at optimal concentration (TaqDNA polymerase 0.1 U/µl, $MgCl_2$4 mM, dNTPs 0.5 mM of each dNTP, stabilizers: red dye and loading buffer). These additives enable direct loading of PCR products on agarose gel upon completing the PCR. The reaction was performed under conditions optimized by the manufacturer of the kit (Table 2). The post-PCR samples were load directly on a 2% agarose gel for electrophoresis. DNA sequencing was done by Genomed Joint-Stock Company (Genomed S. A., Warszawa, Poland). The obtained sequence DNA was compared with the NCBI, DDBJ, EMBL and nematode.net databases.

Table 2
PCR reaction condition.

step	temperature	time
initial denaturation	95 °C	2–3 min
25 - 45 cycles	95 °C	15–30 s
	50–68 °C	30–60 s
	72 °C	15–60 s
final extinction	72 °C	7–12 min

3. Results

3.1. Morphological analyses

From 4 examined individuals of European pond turtle only one female harbored a parasite. The nematode was identified as female of *Spiroxys contortus*. The adult slender, colorless worm was found in the stomach. The oral opening of the nematode was surrounded by two lateral trilobed lips with a rather thick cuticular lining projecting anteriorly in each median lobe to form a blunt tooth (Fig. 1A, a). Each lip beared two submedian and one small lateral papillae (Fig. 1A and b). Two prominent cervical spines were occurred on each side of anterior margin of the collar (Fig. 1A, c). Small cervical papillae were located posterior from the excretory pore. Tail rather short ending in an abrupt, sharp, conical tip (Fig. 1B), with pair of distinct dorsolateral phasmids. Two cuticular prominences guarded the opening of the vulva, which was just posterior to the middle of the body (Fig. 1C). The vagina was directed anteriorly, thick-walled, muscular, and annulated. No ovijectors. The morphological features of the specimen were compared with the available literature and fully correspond to the previous descriptions of the species *S. contortus* (Hedrick, 1935; Moravec and Vargas-Vázquez, 1998; Palumbo et al., 2016). A detailed morphological description of individuals from Argentina has been included in the original article (Palumbo et al., 2016). The morphometric data of both Polish and Argentinian individuals are compiled in Table 3. The measurement results vary, but they are similar and remain within the measuring range of this species (Hedrick, 1935; Moravec and Vargas-Vázquez, 1998; Palumbo et al., 2016).

3.2. Molecular analyses

The following product lengths were obtained from the PCR reaction of small subunit ribosomal RNA gene: 845 bp from Poland isolate and

Table 1
Species of nematodes reported from *Emys orbicularis* in Europe.

Species	Bulgaria	Poland	Romania	Slovakia	Slovenia	Spain	Turkey	References
Ascarididae								
Angusticaecum holopterum[a]								Yamaguti (1961); Sprent (1980)
Camallanidae								
Camallanus spp.					+			Vergles-Rataj et al. (2011)
Serpinema microcephalus	+					+	+	Kirin (2001); Yildirimhan and Şahin (2005); Hidalgo-Vila et al. (2009)
Gnahthosomatidae								
Spiroxys contortus	+	+	+	+			+	Łukasiak (1939); Moravec and Vojtková, 1970; Kirin (2001); Yildirimhan and Şahin (2005); Mihalca et al. (2007)
Kathlaniidae								
Aplectana sp.						+		Hidalgo-Vila et al. (2009)
Falcaustra armenica	+	+	+				+	Yamaguti (1961); Kirin (2001); Yildirimhan and Şahin (2005); Mihalca et al. (2007)
Oxyuridae								
Tachygonetria spp.					+			Vergles-Rataj et al. (2011)

[a] *A. holopterum* was detected in *E. orbicularis* in Europe without specifying a particular country.

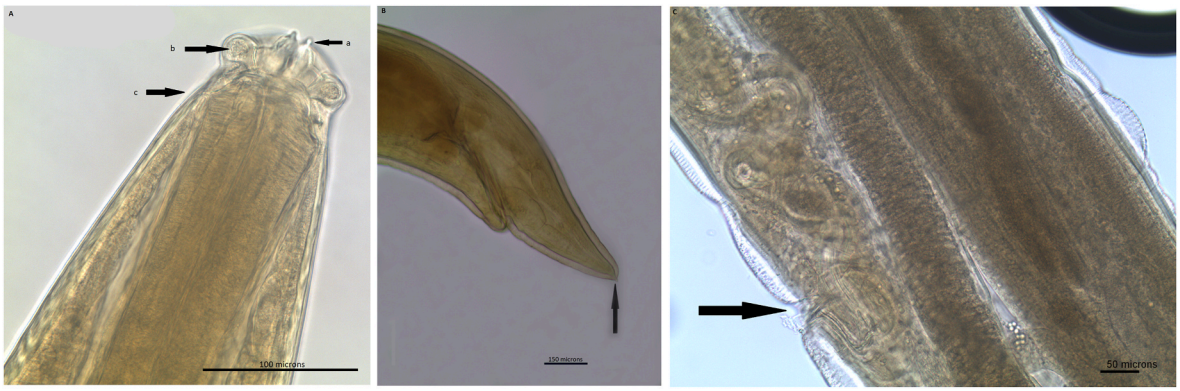

Fig. 1. A–C Female of *Spiroxys contortus* (*Gnathostomatidae*) parasite of wild *Emys orbicularis* (European pond turtle) in Poland. A. Anterior region (a) median lobe with a tooth; (b) the lip with submedian papilla; (c) cuticular spine on the margin of the collar; B. Posterior region with a conical tip (arrow) C. Vulvar opening (arrow).

Table 3
Comparative features on individuals *S. contortus* in *E. orbicularis* and *H. tectifera*.

Host	*E. orbicularis*	*H. tectifera*
Length	35,6	31,2
Width	0,51	0,48
Esophagus	4,5	4,12
Excretory pore[a]	9,51	9,02
Eggs	73-77,2 x 45,5-48,6	79-80 x 50

Comparative features on individuals *S. contortus* in *E. orbicularis* and *H. tectifera*.
[a] From anterior extremity.

810 bp from Argentina isolate. The morphological and genetic profiles confirmed that this nematode belongs to genus *Spiroxys* and sequences of gene small subunit rybosomal 18S rRNA overlap with *Spiroxys* sp. 1 MS-2018 isolate Med4 deposited in the genetic sequence database Genbank. The analyzed sample sequences shows 98% similarity to the *Spiroxys* genus. In addition, the alignment of the sequence generated for both samples (from Poland and Argentina) indicates 100% similarity between the samples. The received sequences were deposited to NCBI GenBank with the accession numbers MN629917 (from Poland) and MN629933 (from Argentina).

4. Discussion

Spiroxys contortus is nematode living threaded in the stomach mucosa of turtles. It is also detected in the esophagus, small and large intestines (Mascarenhas et al., 2013). According to Hedrick (1935), in life cycle of *S. contortus* there are two intermediate hosts, firstly - cyclops and secondly - tadpoles or fish (Hedrick, 1935). Other authors mention here other amphibians, mollusks, and also dragonfly nymphs (Moravec et al., 1995; Anderson, 2000; González and Hamann, 2010; Santos et al., 2019). However, due to the possibility of the development of an invasive larval stage in body cavities of cyclops, as well as effective infection of the final host with them, it gives the conclusion that it is most likely that nematode requires only a cyclop to close the life cycle. This theory is indirectly confirmed by other authors who classify fish and other hosts as paratenic hosts of this species (Moravec et al., 1995; Pilecka-Rapacz and Sobecka, 2008). The effects of parasitism of *S. contortus* are inflammatory and hypertrophic changes in mesothelium and in heavy infections deep ulcers and the destruction of tissues involving the submucosa, mucosa, and the circular muscle layer (Hedrick, 1935; Miclaus et al., 2013).

Spiroxys contortus commonly found as a parasite of several freshwater turtles in the Nearctics and Neotropics, is even more common in Palearctic region. While the occurrence of this parasite has been widely described in turtles from North and South America (Hedrick, 1935; Wieczorowski, 1939; Everhart, 1957; Yamaguti, 1961; Rosen and Marquardt, 1978, 1986; Moravec and Vargas-Vázquez, 1998; Platt, 2000; Bolek, 2001; Mascarenhas and Muller, 2015; Palumbo et al., 2016) only a few articles come from Europe (Moravec and Vojtková, 1970; Kirin, 2001; Yildirimhan and Şahin, 2005; Mihalca et al., 2007). The natural invasion of *S. contortus* in North America has been found in *Chrysemys picta, Emydoidea blandingii, Graptemys geographica, Sternotherus odoratus, Kinosternon subrubrum, Chelydra serpentina, Pseudemys texana, Trachemys scripta* (Hedrick, 1935) and *Trachemys dorbigni, Acanthochelys spixii, Hydromedusa tectifera Phrynops hilarii* in South America (Mascarenhas et al., 2013; Mascarenhas and Muller, 2015; Palumbo et al., 2016). So far, in Europe a natural invasion of this adult nematode in wild in *E. orbicularis* was reported in Bulgaria, Romania, Czechoslovakia and Turkey (Moravec and Vojtková, 1970; Kirin, 2001; Yildirimhan and Şahin, 2005; Mihalca et al., 2007). However, these cases were not molecularly confirmed, and to this day it is not known which genotype of this nematode population is present in Europe. What is surprising the genotype of an individual isolated in Poland is identical to the genotype of an individual originated from South America (Argentina) as reference specimen. The question arises whether the genotype of this species is so little variable, ubiquitous, or whether the diversity concerns different sequence from the studied one. The turtle described in this article was found in the near Polesie National Park, the area with the largest native population of *E. orbicularis* in Poland. Individuals of turtles inhabiting this area feed on water insects and fish, which can carry larvae of *S. controtus* such as Pumpkinseed sunfish (*Lepomis gibbosus*) and European fish lake minnow (*Eupallasella perenurus*) (Popiołek et al., 2005; Pilecka-Rapacz and Sobecka, 2008). Each potential host could introduce eggs and larvae of *S. contortus* into the environment just like infected alien and native species of freshwater turtles occupied lakes and rivers of countries bordering Poland e.g. Russia, Ukraine and Hungary (Sharpilo, 1976). However, turtles not only migrate via natural routes, but are also imported by humans, only between 1994 and 1997, 448 000 american freshwater turtles were officially imported to Poland (Najbar, 2001). It has led to a frequent release of these terrapins into natural habitats, which also applies to the Polesie National Park (Marini, 2017; Kolenda et al., 2019). The presence of introduced animals in new habitats may increase the risk of co-introducing new parasites, which in turn may be a threat to indigenous European turtles. So far, the only one case of *S. contortus* invasion in a final host in Poland is reported and it concerns an animal, which was kept in a pet shop (Łukasiak, 1939). This turtle had contact with imported turtle species sold in this store, so this case was probably an instance of the host-switching in captivity.

Our findings have confirmed that comparing the genome sequences of two samples belonging to the genus *Spiroxys*, nematode from Poland and from Argentina, indicates 100% similarity between them. Results of the morphological comparison confirm the same species affiliation.

However the obtained partial 18S rDNA gene was also compared to literature data and similarities with gene sequence of *Pseudoterranova decipiens* were found (Mafra et al., 2015). Both species of nematodes belong to the suborder Spirurina, but *S. contortus* to infraorder Gnathostomatomorpha, and *P. terranova* to Ascaridomorpha and they differ in morphological features. The reason for this similarity may be the analysis of a short segment of genetic material. It is possible that examining a longer sequence would show discrepancies between these species.

5. Conclusions

So far there has been no available molecular data on *S. contortus* parasitizing freshwater turtles, which makes it difficult to identify the source of this parasite individual. The lack of data can be explained by a small interest in this nematode in America, but in Europe the issue has become important because it concerns the epidemiological threat to *E. orbicularis*, which is here endangered species. For this reason, research on the parasitofauna of these reptiles is limited, which results in a small number of animals tested. Information on the *E. orbicularis* parasites in this area is still incomplete, therefore research on it should be conducted in order to prevent native fauna. Assessing the genetic variability of *S. contortus* from fast evolving genes within populations sampled from distinct biogeographical areas should also be continued.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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