The first case of Spiroxyx 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 Spiroxyx 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; Herriër et al., 2017). The nematophanaea 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 helmhinh fauna of E. orbicularis is little known and includes the trematode species Spirapalud poeliam (Ejsmont 1927) and the nematode Falcustra armenica (Massino 1924) (Ejsmont, 1927; Yamaguti, 1961; Sprent, 1980; Zalesny et al., 2009). There is also one report on the finding of the nematode Spiroxyx contortus in a pond turtle purchased in a captivity (Lukasiak, 1939).

Spiroxyx contortus, syn. Spiroptera contorta (Rudolphi 1819), Spiroxyx 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 Spiroxyx 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 testifiera (Cope, 1870), collected in the Buniirigo stream (35°01’36” S, 57°17’24” W, datum: WGS84), Buenos Aires province, Argentina, were analyzed. Seventeen Spiroxyx contortus were recovered from both turtles, six males and 11 gravid females (Palumbo et al., 2016). The female of the
of 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 Olimpus BX51 with molecular examination 18S rDNA gene was 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′-CYGCAGTTTACCTAC-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, MgCl₂, dNTPs and stabilizers at optimal concentration (TaqDNA polymerase 0.1 μ/l, MgCl₂4 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 was compared with the NCBI, DDBJ, EMBL and nematode.net databases.

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 ovjectors. 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

| Step | Temperature | Time |
|------|-------------|------|
| Initial denaturation | 95°C | 2–3 min |
| 25 - 45 cycles | 95°C | 15–30 s |
| | 60°C | 30–60 s |
| | 72°C | 15–60 s |
| Final extinction | 72°C | 7–12 min |

Table 1

| Species | Bulgaria | Poland | Romania | Slovakia | Slovenia | Spain | Turkey | References |
|---------|----------|--------|---------|----------|----------|-------|--------|------------|
| Ascarididae | | | | | | | | |
| Angusticaecum holopterum | + | | | | | | | Yamaguti (1961); Sprent (1980) |
| Camallaniidae | | + | + | + | | | | Vergles-Rataj et al. (2011) |
| Camallanus spp. | | | | | | | | Kirin (2001); Yildirimhan and Sahin (2005); Hidalgo-Vila et al. (2009) |
| Serpinema microphalus | + | | | | | | | Kirin (2001); Yildirimhan and Sahin (2005) |
| Gnathosomatidae | | + | | | + | | | Kirin (2001); Yildirimhan and Sahin (2005); Hidalgo-Vila et al. (2009) |
| Spiroxys contortus | | + | + | + | | | | Lukashik (1939); Moravec and Vojtková, 1978; Kirin (2001); Yildirimhan and Sahin (2005); Mihalca et al. (2007) |
| Kathlaniidae | | + | | | | | | Hidalgo-Vila et al. (2009) |
| Aplectana sp. | | + | | | + | | | Yamaguti (1961); Kirin (2001); Yildirimhan and Sahin (2005); Mihalca et al. (2007) |
| Falcurastra armenica | | + | | | | | | Vergles-Rataj et al. (2011) |
| Oxyuridae | Tachygenetic spp. | + | | | | | | Mihalca et al. (2007) |

* A. holopterum was detected in E. orbicularis in Europe without specifying a particular country.
The occurrence of this parasite has been widely documented in water turtles in the Nearctic and Neotropical regions, but it is even more common in European species. While the presence of this nematode has been reported in Europe, it is not yet clear how it arrived there. Some authors believe that it may have been introduced by humans, while others suggest that it may have arrived as a byproduct of the trade in exotic turtles.

Table 3

| Host          | E. orbicularis | H. tectifera |
|---------------|---------------|--------------|
| Length        | 35.6          | 31.2         |
| Width         | 0.51          | 0.48         |
| Esophagus     | 4.5           | 4.12         |
| Excretory pore | 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.

810 bp from Argentina isolate. The morphological and genetic profiles confirmed that this nematode belongs to the genus Spiroxyx and sequences of gene small subunit ribosomal RNA overlap with Spiroxyx sp. 1 MS-2018 isolate Med4 deposited in the genetic sequence database GenBank. The analyzed sample sequences show 98% similarity to the Spiroxyx 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

Spiroxyx contortus is a 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; Gonzalez 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).

Spiroxyx contortus commonly found as a parasite of several freshwater turtles in the Nearctic and Neotropics, it 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; Weiczorowski, 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 Vojtkova, 1970; Kirin, 2001; Yildirimhan and Sahin, 2005; Mihalca et al., 2007). The natural invasion of S. contortus in North America has been found in Chrysemys picta, Emydidae blandingii, Gratemys geographica, Sternotherus odoratus, Kinosternon subrubrum, Chelydra serpentina, Pseudemys taxana, 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 Vojtkova, 1970; Kirin, 2001; Yildirimhan and Sahin, 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. contortus such as Pumpkinseed sunfish (Lepomis gibbosus) and European fish lake minnow (Eupallascia perenurus) (Popiełek 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 (Sharpe, 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 (Lukasiak, 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 Spiroxyx, 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 infrorder Gna-thnostomatomorpha, 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 parasitism 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.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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