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MOLECULAR CHARACTERIZATION OF THE FIRST REPORTED *NEOPLAGIOGLYPHE MEGASTOMUS* (DIGENEA, OMPHALOMETRIDAE) IN POLAND

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Molecular Characterization of the First Reported *Neoplagioglyphe megastomus* (Digenea, Omphalometridae) in Poland. Sarabeev, V., Ovcharenko, M., Ahmed, A., Sueiro, R. A., Leiro, J. M. — The diversity and taxonomy of metacercariae infecting freshwater amphipods of Poland are predominantly poorly known. During parasitological surveys of *Gammarus pulex* (Crustacea: Amphipoda) in the Pomeranian region of Poland in 2020 and 2021, some specimens of crustaceans were found to harbour metacercariae. Out of seven observed localities, metacercariae were found in the stream close to Krępa Słupska and the Lupawa River close to Smoldzino. They were morphologically studied and sequenced using universal eukaryotic primers that amplify V4–V5 regions of 18S rRNA. The BLAST analysis and phylogenetic reconstructions aid to define the affiliation of the revealed metacercariae within the Omphalometridae Looss, 1899. The further differential analysis based on morphologic information led to the conclusion that the metacercarial form studied herein represents *Neoplagioglyphe megastomus* (Baer, 1943). The degree of morphometric variations of taxonomic important features in populations from different geographic regions was followed up. The reported here form was characterized by a larger body size that was affected by the processing methods used in the present and previous studies. To our knowledge, this is the first report of *Neoplagioglyphe megastomus* in Poland and is the first molecular characterization of the worm.
Key words: 18S rRNA, *Gammarus pulex,* Pomeranian region, metacercaria.

Introduction

Digeneans are a group of phylogenetically diverse parasitic flatworms that have complex life cycles with two or three hosts, where vertebrates are definitive hosts and molluscs are first intermediate hosts. The infective metacercarial stage may pass through a variety of invertebrate and vertebrate hosts serving as second intermediate hosts in which they undergo extensive organogenesis (Saville and Irwin, 1991). As crustaceans are highly abundant in the aquatic environment and play a key role as an important source of nutrition, digeneans are known to frequently include pelagic and benthic crustaceans in their life cycle to use the trophic interactions in food webs for facilitating the transmission of parasites to the successive hosts (Busch et al., 2012). Metacercariae diversity in amphipods was recently evaluated by Bojko and Ovcharenko (2019), who counted 14 and over 30 species of digeneans from marine and freshwater amphipods, respectively.

Since Digenea commonly includes species, genera and families with few morphological distinctions, their accurate identification is complicated (Gibson, Jones and Bray, 2002; Goswami et al., 2013). The metacercarial stages often lack reliable distinguishing morphological characters, especially for structures associated with the reproductive system, and thus their identification to the species level is complicated or even impossible. However morphological data remain the cornerstone of trematode systematics (Blasco-Costa et al., 2016). Over recent decades there has been an increased integration of genetic data to overcome problems in establishing accurate species limits and higher taxonomic groups of digeneans (Pérez-Ponce de León and Hernández-Mena, 2019). The current taxonomy and classification of digeneans is based on molecular data predominantly derived from phylogenetic assessments of two nuclear rRNA genes, 18S and 28S (Olson et al., 2003; Blasco-Costa et al., 2016; Pérez-Ponce de León and Hernández-Mena, 2019).

During parasitological surveys of *Gammarus pulex* (L.) (Crustacea: Amphipoda) in the Pomeranian region of Poland in 2020 and 2021, some specimens of crustaceans were found to harbour metacercariae. The metacercariae were studied alive, microphotographed and measured with subsequent fixation and total genomic DNA extraction. The obtained sequences were analysed using the BLAST Sequence Analysis Tool and phylogenetic reconstruction of the closest taxa found in GenBank. The final species identification was performed using diagnostic morphological features.

Material and methods

Material collection and processing

G. pulex were collected from streams and rivers in the Pomeranian region of Poland. In total 233 individuals from seven localities were surveyed for parasites (table 1). Amphipods were measured under a stereomicroscope (SMZ-161 with digital camera Moticam BTU) and dissected on the object-glass. Parasites were counted, excysted with syringe needles and transferred for further inspection under a compound microscope. Cysts and metacercariae were microphotographed and measured alive (table 2) using a digital camera Optikam B3 and microscope Delta Optical Evolution 300. Selected specimens were preserved in absolute ethanol for molecular analysis. The line drawing was prepared from a set of microphotographs obtained from alive worms with magnifications 100x and 400x. Similarly to previous studies (Blasco-Costa et al., 2006, 2010), ratios were counted to compare literature descriptions with specimens of digeneans collected here. Parasites were identified to the species level based on morphology after defining their family affinity with aid of molecular analysis.

DNA extraction, amplification, sequencing and analysis

Genomic DNA was extracted from a single metacercaria using a quick alkaline lysis protocol (Klimyuk et al., 1993; Stanton, McNicol and Steele, 1998). Individual metacercariae were transferred to 10 µL 0.25 M NaOH in 0.2 mL tube and sonicated for several seconds. The sample was incubated in NaOH for 3 min at 95 °C and subsequently neutralized by addition of 9 µL 0.25 M HCl and 8 µL 1 M Tris-HCl (pH 8.5), 1 µL 2 % Triton X-100 was also added as detergent. The mixture was again incubated for 3 min at 95 °C. The PCR was performed using universal eukaryotic primers F-566:5'-CAG CAG CCG CGG TAA TTC C-3' and R-1200:5'-CCC GTG TTG AGT CAA ATT AAG C-3' to amplify V4 and V5 variable regions of 18S rRNA gene as those with high taxonomic information (Hadziavdic et al., 2014).

The PCR mixtures (25 µL) contained reaction buffer, 0.2 mM of each deoxynucleoside triphosphate (dNTPs, Nzytech, Portugal), 0.4 µM of each primer; 0.4 units of high fidelity NZYProof DNA polymerase (Nzytech) and 50 ng of genomic DNA. The reactions were run in an automatic thermocycler (T100™ Thermal Cycler, BioRad, USA) as follows: initial denaturing at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, annealing at 57 °C for 45 s, and 72 °C for 1 min; and finally, a 7 min extension phase at 72 °C. The PCR products were confirmed by 1.5 % agarose gel in Tris-acetate ethylenediaminetetraacetic acid buffer containing Sybr Green at 1× concentration, to verify the presence of bands of the correct size under a variable-intensity 312 nm ultraviolet (UV) transilluminator (Spectroline, USA). The PCR product was purified by a PCR purifica-

Table 1. Results of *Gammarus pulex* samplings from streams and rivers in the Pomeranian region of Poland with information on the date, locality, number of studied crustaceans and infection parameters, prevalence (P) and mean abundance (A) with an intensity range in parentheses

Sample ID	Date	Locality name	Geographic coordinates	n	P, %	A
GPO	27/09/2020	Orzechowa	54.598841, 16.918841	43	0	0
GPLD	10/10/2020	Lesny Dwor	54.358391, 17.155713	28	0	0
GPSL	31/01/2021	Smoldzino Lupawa	54.662085, 17.212266	31	10	0.23 (1–4)
GPDP	20/02/2021	Debki Piasnicy	54.832288, 18.061855	30	0	0
GPSS	28/02/2021	Slupsk Stadion	54.475260, 17.042841	30	0	0
GPKS	05/03/2021	Krępa Słupska	54.403371, 17.047010	41	38	0.8 (1–8)
GPSW	25/03/2021	Wodnica Slupia	54.556568, 16.875233	30	0	0

Table 2. Comparative metrical data for metacercariae of *Neoplagioglyphe megastomus* from *Gammarus pulex* and adults from water shrews

Metrical data	Metacercariae				Adult	
	Gammarus pulex				*Neomys fodiens*	*Neomys anomalus*
	Mean	Range	n	Range	Range	Range
Body length	930.5	866–995	2	500–600	375–600	672–800
Maximum body width	230.5	220–241	2	120	200–300	208–210
Forebody length	415	352–478	2	–	–	–
Oral sucker length	150.3	137–164	3	110–122	126–150	128–147
Oral sucker width	132.6	113–150	5	90–108	115–126	128
Prepharynx length	32.5	22–43	2	–	–	–
Pharynx length	45.3	41–53	6	–	40–50	38–57
Pharynx width	50.8	35–65	4	–	–	38–54
Ventral sucker length	66.5	56–77	6	47–58	65–73	67–96
Ventral sucker width	65	51–90	6	–	–	60–96
Cirrus sac length	211	186–236	2	–	216–240	–
Cirrus sac width	36	32–40	2	–	36–40	–
Cirrus length	83	61–105	2	–	–	–
Cirrus width	9	7–11	2	–	–	–
Prostatic bulb length	22	–	1	–	–	–
Prostatic bulb width	15	–	1	–	–	–
Genital atrium length	19.5	17–22	2	–	–	–
Genital atrium width	15.5	15–16	2	–	–	–
Proximal portion of seminal vesicle length	23	–	1	–	–	–
Proximal portion of seminal vesicle width	11	–	1	–	–	–
Distal portion of seminal vesicle length	49	–	1	–	–	–
Distal portion of seminal vesicle width	12	–	1	–	–	–
Metraterm length	86.5	80–93	2	–	–	–
Metraterm width	18.5	15–22	2	–	–	–
Anterior testis length	90.5	78–109	4	–	–	108–118
Anterior testis width	78.5	62–102	4	–	–	92–93
Posterior testis length	94.8	71–124	4	–	–	96–115
Posterior testis width	76.8	60–100	4	–	–	89–115
Ovary length	53.8	42–62	4	–	–	76–83
Ovary width	57.8	50–62	4	–	–	51–67
Laurer's canal length	105		1	–	–	–
Laurer's canal width	13		1	–	–	–
Mehlis' gland length	99		1	–	–	–
Mehlis' gland width	84		1	–	–	–
Excretory bladder length	219		1	–	–	–
Post-caecal field length	77.5	67–88	2	–	–	–
Post-testicular field length	127	126–128	2	–	–	–
Ratios*				–	–	–
Maximum body width as a percentage of body length	25	22–28	2	–	18–36	24–25
Length of the forebody as a percentage of body length	44	40–48	2	–	37–46	42–46
Sucker length ratio	2.0	1.9–2.1	3	–	2.1–2.6	1.4–1.5
Sucker width ratio	2.0	1.7–2.5	5	–	2.0–2.2	1.3–1.4
Cirrus sac length to ventral sucker length	2.8	2.6–3.1	2	–	2.4–4.1	1.9–2.1
Post-testicular field length as a percentage of body length	13.7	13–15	2	–	14–20	15.1–15.3
Post-caecal field length as a percentage of body length	8.4	7-10	2	–	3–8	5.0–5.5
Cysta length	315.3	273–352	4	520	–	–
Cysta width	249.3	205–280	4	360	–	–
References	The present study			Baer (1943)	Baer (1943)	Matskási (1971)

* For literary material the ratios were evaluated from published line drawings.

tion kit (PureLink™, Invitrogen, USA) and was sequenced in complementary directions using Sanger sequencing service (Eurofins Genomics, Germany).

Obtained forward and reverse sequences were assembled and visualised using MEGA-11 (Tamura, Stecher and Kumar, 2021). The obtained sequences were compared with GenBank entries by using BLAST tool (Altschul, 1997) to define the taxonomic position of the metacercaria. According to the Blast search, 20 ad-

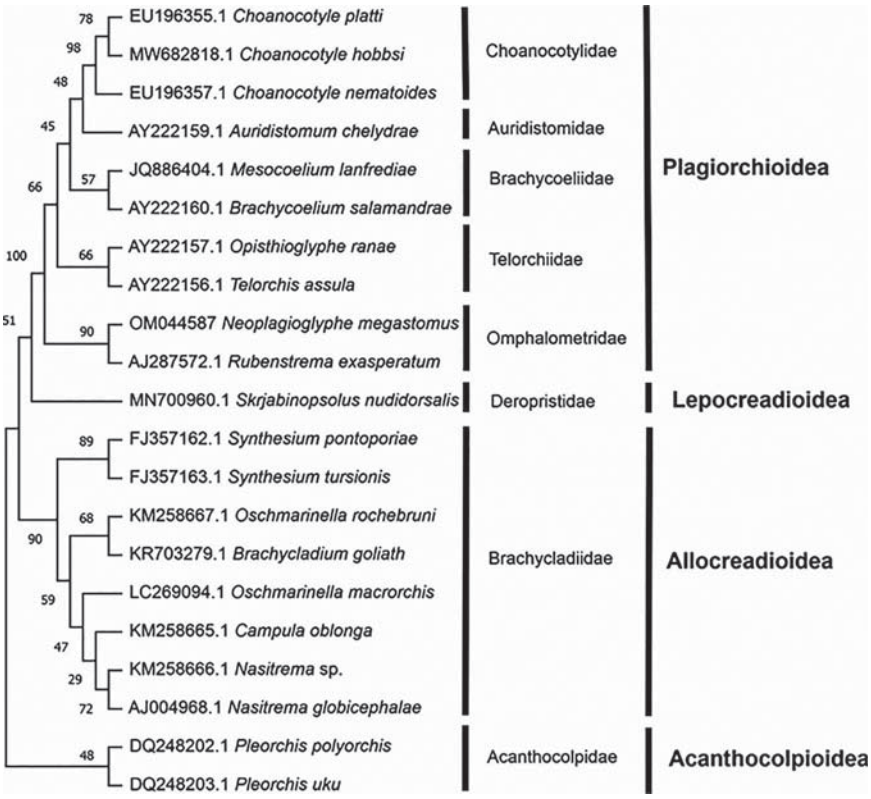

Fig. 1. Phylogenetic trees based on 18S rRNA sequences using the ML method with 500 bootstrap replicates. The tree with the highest log likelihood (-1959,87) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

Table 3. Results of BLAST analysis performed on 18S rRNA sequence obtained from the *Gammarus pulex* metacercaria. Best hits (with highest similarity scores) are shown as retrieved in October 2021 from GenBank

Species	Family	Superfamily	Coverage, %	Similarity, %	GenBank Accession number
Rubenstrema exasperatum	Omphalometridae	Plagiorchioidea	100	98.20	AJ287572.1
Mesocoelium lanfrediae	Brachycoeliidae	Plagiorchioidea	100	96.82	JQ886404.1
Brachycoelium salamandrae	Brachycoeliidae	Plagiorchioidea	100	96.54	AY222160.1
Opisthioglyphe ranae	Telorchiidae	Plagiorchioidea	100	96.54	AY222157.1
Choanocotyle nematoides	Choanocotylidae	Plagiorchioidea	100	96.40	EU196357.1
Auridistomum chelydrae	Auridistomidae	Plagiorchioidea	100	96.13	AY222159.1
Choanocotyle hobbsi	Choanocotylidae	Plagiorchioidea	100	95.99	MW682818.1
Choanocotyle platti	Choanocotylidae	Plagiorchioidea	100	95.99	EU196355.1
Telorchis assula	Telorchiidae	Plagiorchioidea	100	95.84	AY222156.1
Oschmarinella macrorchis	Brachycladiidae	Allocreadioidea	100	95.59	LC269094.1
Campula oblonga	Brachycladiidae	Allocreadioidea	100	95.59	KM258665.1
Pleorchis uku	Acanthocolpidae	Acanthocolpioidea	100	95.59	DQ248203.1
Skrjabinopsolus nudidorsalis	Deropristidae	Lepocreadioidea	100	95.59	MN700960.1
Pleorchis polyorchis	Acanthocolpidae	Acanthocolpioidea	100	95.45	DQ248202.1
Oschmarinella rochebruni	Brachycladiidae	Allocreadioidea	100	95.45	KM258667.1
Nasitrema sp.	Brachycladiidae	Allocreadioidea	100	95.45	KM258666.1
Brachycladium goliath	Brachycladiidae	Allocreadioidea	100	95.32	KR703279.1
Synthesium tursionis	Brachycladiidae	Allocreadioidea	100	95.31	FJ357163.1
Synthesium pontoporiae	Brachycladiidae	Allocreadioidea	100	95.31	FJ357162.1
Nasitrema globicephalae	Brachycladiidae	Allocreadioidea	100	95.31	AJ004968.1

ditional species revealed as the closest relatives were chosen for the phylogenetic analysis (table 3). The most distant species from the Acanthocolpidae were used to root obtained trees. Nucleotide sequences were aligned using the Clustal W option of MEGA-11. Trees were obtained using maximum likelihood (ML) with Tamura-Nei model, neighbour joining (NJ) and minimum evolution (ME) methods as applied in MEGA-11. Clade support was assessed by bootstrap resampling with 500 replicates.

Results

Molecular identification

18S rRNA sequence obtained from the metacercaria studied here is deposited in GenBank under accession numbers OM044587. BLAST analyses on this sequence showed the closest similarity with trematodes belonging to the superfamily Plagiorchioidea (Bray, 2008) (Trematoda: Digena: Plagiorchiida) (table 3). Phylogenetic analysis further confirms that the studied here metacercariae belong to this superfamily (strongly supported by a high bootstrap value, 100 %) and is in the sister relationship with *Rubenstrema exasperatum* (Rudolphi, 1819) of the Omphalometridae Looss, 1899 (highly supported by a bootstrap, 90 %) (fig. 1). Phylogenetic trees built with NJ and ME algorithms showed the same topology, but the bootstrap support for clade formed by *R. exasperatum* and the metacercaria sequenced in the present study was slightly higher (94 %).

Differential analysis within the Omphalometridae

The last revision of Omphalometridae by Tkach (2008) defined 5 genera within the family: *Omphalometra* Looss, 1899, *Rutshurutrema* Baer, 1959, *Rubenstrema* Dollfus, 1949, *Neoglyphe* Shaldybin, 1953 and *Neoplagioglyphe* Tkach, 2008. Specimens of metacercariae found in the present study are morphologically similar to *Neoplagioglyphe*. They are distinguished from *Omphalometra*, *Rutshurutrema*, *Rubenstrema* and *Neoglyphe* by the following combination of characters: i) body small, slender, elongate, narrows at posterior end; ii) oral sucker much larger than ventral; iii) testes entire, rounded or spherical, contiguous, tandem, in posterior half of body; iv) cirrus sac claviform, curved, in hindbody, extends posteriorly beyond posterior margin of ventral sucker, contains bipartite seminal vesicle, prostatic complex and ejaculatory duct, cirrus unspined; v) ovary posterolateral or lateral to ventral sucker. Whereas those features are following: i) body very elongate, ii) suckers relatively small, either equal in size or oral sucker slightly larger than ventral sucker, iii) testes deeply lobed, iv) ovary median, in mid-region of body in *Omphalometra*; i) body oval or elongate, ii) suckers approximately equal in size, iii) testes lobed, iv) ovary median in *Rutshurutrema*; i) body relatively large, very muscular and thick, ii) ventral sucker larger than oral sucker, iii) testes entire or lobed, iv) cirrus sac entirely in forebody in *Rubenstrema*; i) body pear-shaped or oval, extremely flattened dorsoventrally, ii) suckers approximately similar in size or oral sucker slightly larger than ventral, iii) testes irregularly shaped, frequently lobed, strongly transversely elongate, iv) cirrus covered with very small spines in *Neoglyphe*.

Morphological description of the studied metcercariae (table 2, fig. 2)

Excysted metacercariae obovoid, tapered at posterior end, small, slender, elongate, maximum width in mid-body; oral sucker subterminal and round much larger than ventral one; ventral sucker at mid-body; prepharynx very short; pharynx ovoid broadly, well developed; oesophagus indistinct; intestinal bifurcation occurs at one-fourth of body length, distant from ventral sucker; ventral sucker small, at one-third to one-half of the total length according to the condition of contraction; caeca terminate close to posterior extremity; testes mature, entire, rounded or spherical, contiguous, tandem, in posterior third of body; cirrus sac claviform, curved, reaches well into hindbody to left of ovary, contains bipartite seminal vesicle, prostatic complex and ejaculatory duct; genital pore in forebody, submedian; ovary posterolateral or lateral to ventral sucker; vitellarium comprises

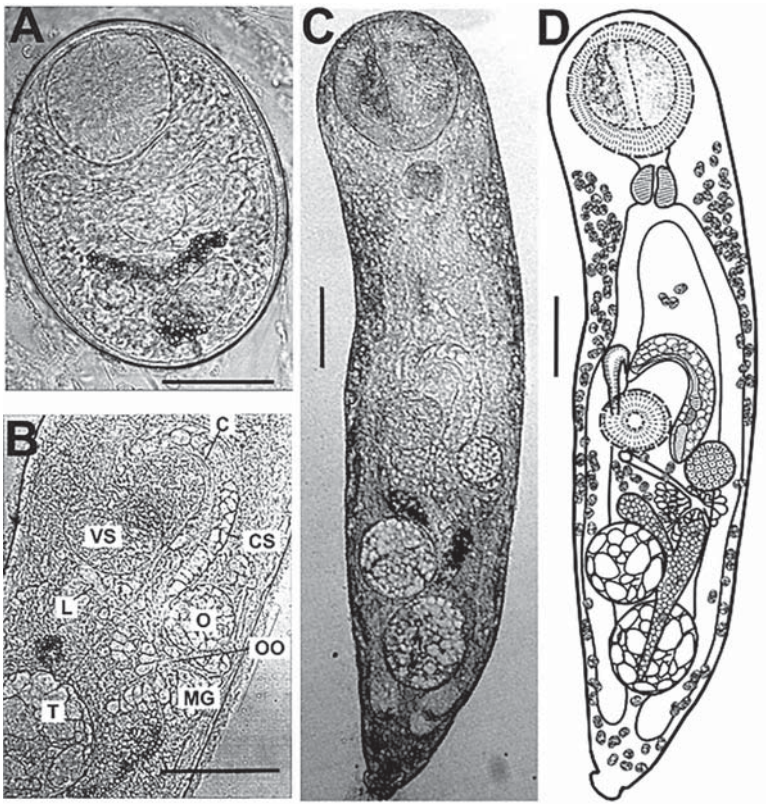

Fig. 2. Microphotographs and line drawing of live metacercariae of *Neoplagioglyphe megastomus* from *Gammarus pulex*. A. Encysted metacercaria. B. Dorsal view of ovarian complex and male terminal genitalia. C. and D. dorsal view of metacercaria removed from cyst. Abbreviations: C, cirrus; CS, cirrus sac; L, Laurer's canal; MG, Mehlis' gland cells; O, ovary; OO, ootype; T, anterior testis; VS, ventral sucker.

numerous follicles forming lateral fields confluent posterior to testes and extends anteriorly to level of pharynx; excretory vesicle Y-shaped. Seminal receptacle and uterus were not observed.

Discussion

Metacercariae reported here are identified as *Neoplagioglyphe megastomus* (Baer, 1943) based on their morphological and biological features. The dimensions of metacercariae and the condition of the genital glands indicate that those were mature and quite similar morphologically to the adult form. Our finding corroborates the results of Baer (1943), who reported metacercariae with well developed genital systems and measurements similar to or even larger than in the adults. The individuals revealed here were about one-third larger in the body size than those reported by Baer (1943), including both adult and larval stages (table 2). The larger dimensions in the body size can be explained by the fact that different processing techniques were used when manipulating metacercariae. We measured alive worms, while Baer (1943) used ethanol fixed and stained material. The influence of storage and examination methods on the size of metacercariae was investigated by Lepitzki, et al. (1994), who showed that variation between measurements of ethanol fixed and alive worms may be substantial, reaching as high as 40 % to 50 %. Another reason that may explain metrical distinctions is the presence of two geographically isolated forms. In the body shape and measurements, specimens examined here are more similar to the form described by Matskási (1971) (e. g. figs 11 and 12). The ratios used to compare metacercariae and adults, drawn from published figures for the latter ones (Baer, 1943; Matskási, 1971), showed

that those are in the range or overlapped broadly (table 2), thus further supporting our identification of these worms as *N. megastomus*.

The Omphalometridae is a small group of plagiorchioid digenean taxa, which combines *Omphalometra*, *Rutshurutrema*, *Rubenstrema*, *Neoglyphe* and *Neoplagioglyphe* (Tkach, 2008). Adults are parasites of the stomach, intestine and gallbladder of shrews, moles and desmans. The family is characterized by the uterus, which never entered the post-testicular region of the body and usually not beyond the anterior testis (Tkach, 2008). Although the uterus as a diagnostic feature was not observed in the present study, the family affinity of the metacercariae was determined based on molecular information. *Neoplagioglyphe megastomus* was in the sister relationship with *Rubenstrema* comprising together the most basal clade of the monophyletic Plagiorchioidea clade. The BLAST search revealed 98.2 % identity for sequence of *R. exasperatum* with that obtained here. The species identity threshold for V4 and V5 regions of 18S rRNA is usually assigned at the level of 97–98 % (Aguilar et al., 2016; Choi and Park, 2020; Sarabeev et al., 2020). This finding further supports the morphological observation that specified taxonomic distinctions between *N. megastomus* and *R. exasperatum*.

Adults of *N. megastomus* were described from Eurasian water shrew, *Neomys fodiens* (Pennant, 1771), in streams around Neuchatel, Switzerland. Encysted metacercariae of this species were found from *Gammarus pulex* (L.) in the same waters in which the shrew was caught. The full life cycle was elucidated by Vaucher (1971), who obtained cercariae from *Radix peregra* (Müller, 1774) sampled in the Areuse River near Neuchatel and infected individuals of *Gammarus* in the experimental study. The literature review of geographic records of *N. megastomus* indicates that the distribution of this species is related to freshwater ecosystems of Europe. In addition to Neuchatel, *N. megastomus* was also reported from the type host in the Giessen Area, Germany (Brendow, 1970), the High Tatras, Slovakia (Prokopic, 1957); from *Neomys anomalus* Cabrera, 1907 in Németbány, Hungary (Matskási, 1971); from *N. anomalus* and *Sorex araneus* L. in Sopron, Hungary (Matskási, 1971; Gubányi et al., 2002). Out of 7 observed localities in the Pomeranian region of Poland *N. megastomus* was found in the stream close to Krępa Słupska and the Lupawa River close to Smoldzino (table 1). The intensity of infection reached 4 and 8 individuals per host with a prevalence 10 % and 38 % in Smoldzino and Krępa Słupska, respectively. Cystes were localized in the body cavity along the intestinal tract. Similarly, a low prevalence of infection in amphipods was reported by Baer (1943) in Neuchatel.

Given the evident similarity in morphological and biological characteristics, supplemented by molecular data, it may be concluded that the metacercariae studied herein represent *N. megastomus*. To our knowledge, this is the first report of *N. megastomus* in Poland and is the first molecular characterization of the worm. Further molecular data is needed to elucidate relationships between geographically isolated forms that differ in dimensions.

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