

Original papers

Revision of the species complex *Amidostomum acutum* (Lundahl, 1848) (Nematoda: Amidostomatidae) by use of molecular techniques

Katarzyna M. Kavetska[1], Daniel Polasik[2], Emil Dzierzba[1], Małgorzata Jędrzejczak[1], Elżbieta Kalisińska[3], Izabella Rząd[4]

[1]Laboratory of Biology and Ecology of Parasites, West Pomeranian University of Technology, Judyma 20, 71-795 Szczecin
[2]Department of Genetics and Animal Breeding, West Pomeranian University of Technology, Al. Piastów 45, 70-311 Szczecin
[3]Department of Biology and Medical Parasitology, Pomeranian Medical University, Powstańców Wielkopolskich 72, 70-111 Szczecin
[4]Department of Ecology and Environmental Protection, University of Szczecin, Wąska 13, 75-415 Szczecin; Poland

Corresponding author: Katarzyna Kavetska; e-mail: katarzyna.kavetska@zut.edu.pl

ABSTRACT. The aim of the work is to confirm the species differentiation of the nematodes of the Amidostomatidae family: *Amidostomoides acutum* (Lundahl, 1848) Lomakin, 1991; *Amidostomoides monodon* (Linstow, 1882) Lomakin, 1991, and *Amidostomoides petrovi* (Shakhtahtinskaya, 1956) Lomakin, 1991, which still are used in the parasitological literature as synonyms of *Amidostomum acutum* (Lundahl, 1848). The research material consisted of nematodes isolated from gizzards of dabbling ducks from the north-west of Poland. To confirm the species differentiation, DNA from the nematodes was isolated and approximately 630bp of the 28S rRNA gene were sequenced. The obtained DNA sequences were tabulated and then phylogenetic analysis were conducted using the UPGMA method. The results of the research distinctly diversify the nematodes of the genus *Amidostomoides* at the DNA level, which together with morphological and ecological differences among them (hosts from different systematic groups) enables to classify them into the separate species.

Key words: Nematoda, *Amidostomum acutum*, Amidostomatidae

Introduction

Numerous research indicate, that in ducks, both in Europe, as well as throughout whole the world, one species of nematodes, that is *Amidostomum acutum* (Lundahl, 1848) (Nematoda: Amidostomatidae), predominates. Prevalence of the parasite is large and fluctuates from 25% to 100%, having the intensity reaching even a few hundred of specimens in one infected bird [1–6].

The first description of the species, performed by Lundahl in 1848, contained very restricted information concerning morphology and hosts of the parasite [7]. Posterior mentions of different authors (Diesing 1861; Molin 1860; Seurat 1918; Cram 1927; Travassos 1937 and others) also hardly contributed, because the enumerated authors only limited themselves to quoting the earlier descriptions of the parasite.

The precise description of the species was presented by Czapliński as late as 1962. This author, relying on the morphology of nematodes coming from birds of Poland, France, and East Africa, described as many as 8 species with one tooth in a buccal cavity as *Amidostomum acutum* synonyms: *A. monodon* (Linstow, 1882); *A. chevreuxi* Seurat, 1918; *A. skrjabini* Boulenger, 1926; *A. anatinum* Sugimoto, 1928; *A fuligulae* Maplestone, 1930; *A. biziurae* Johnston et Mawson, 1947; *A. boshadis* Petrov et Fedushin, 1949, and *A. orientale* Rijikov et Pavlov, 1959. Several years later Kobuley and Ryzhikow (1968), examining again those nine

species of the „*acutum* group", accepted the validity of only three of them: *Amidostomum monodon*, *A. orientale*, and *A. cheuvreuxi*.

A work of Petrova [8] had a great importance in the classification of nematodes of the Amidostomatinae subfamily. The author analyzing morphological differences and host specificity of 110 specimens of the genus *Amidostomum* noted the need of separating it into two subgenera: for the first subgenus (with three teeth in a deep buccal cavity) she suggested leaving the name *Amidostomum,* whereas for the second one (with one tooth in a shallow buccal cavity) changing it for *Amidostomoides*. So into the subgenus *Amidostomum* (*Amidostomoides*) she included: *Amidostomum* (*Amidostomoides*) *acutum* (Lundahl, 1848), *A.*(*A.*) *quasifulicae* (Mačko, 1966), and *A.*(*A.*) *fulicae* (Rudolphi, 1819), while into the subgenus *Amidostomum* (*Amidostomum*): *Amidostomum* (*Amidostomum*) *anseris* (Zeder, 1800), *A.* (*A.*) *spatulatum* Baylis, 1932, and *A.* (*A.*) *cygni* Wehr, 1933. Basing on the suggestions of Petrova [8], Lomakin [9] proposed elevating the both subgenera *Amidostomum* and *Amidostomoides* to the genera ranks, leaving their names according to the Petrova's submissions. In his works Lomakin [9] described *Amidostomoides acutum* (Lundahl, 1848) as the type species for the genus *Amidostomoides* Petrova, 1987, enumerating as the other ones: *Amidostomoides monodon* (Linstow, 1882) nov. comb., *Amidostomoides auriculatum* (Lomakin, 1988), and *Amidostomoides petrovi* (Shakhtahtinskaya, 1956) nov. comb.

Despite significant reports of scientists from Bulgaria and Russia and present-day research of Kavetska et al. [10], using among others self-organizing neural network (Kohonen network), the two disputable species *Amidostomoides monodon* and *Amidostomoides petrovi* up to this day are recognized as synonyms of the *Amidostomum acutum*. Therefore the aim of the hereby elaboration is making an attempt of confirming a species differentiation between the three morphologically and ecologically different groups of nematodes utilizing molecular techniques, which are able to determine unambiguously their genetic kinship.

Materials and Methods

The research material consisted of nematodes isolated from gizzards of dabbling ducks wintering in the north-west of Poland in 2012–2013 (*A. acutum* from the mallard *Anas platyrhynchos*, *A. petrovi* from the tufted duck *Aythia fuligula*, *A. monodon* from the common scoter *Melanitta nigra*). The nematodes were thoroughly cleaned of remains of food and mucus, and next were subjected for deep freezing at the temperature of –20°C.

DNA from individual nematodes was isolated using a Genomic Mini kit (A&A Biotechnology) according to the attached protocol. Before isolation each sample was frozen at –80°C and subsequently heated for 10 min at 100°C. Conservative and variable regions of 28S rRNA were determined by compare *Amidostomum cygni* sequence (AM039745) with closely related species using BLAST (http://blast.ncbi.nlm.nih.gov) and Clustal Omega [11]. Following primers for PCR were designed applying [12]: AMIDOS_F 5'- TGTGAA GGAAAGTTGCAAAGAA-3', AMIDOS_R 5'-TCGGAGGGAACCAGCTACTA-3'. High genetic distance provides that these primers do not amplify the DNA of host tissues. Each PCR was performed in a reaction volume of 20µl contains: 60–80ng of DNA, 10pmol of each primer, 2xPCR Master Mix (A&A Biotechnology) and PCR grade water.

Thermal cycling was performed in Eppendorf Mastercycler gradient using the following profile: 95°C for 5min., 30 cycles of 95°C/45s, 55°C/45s, 72°C/45s and the final extension 72°C for 5min.

Presence of amplicons with expected length was confirmed by electrophoresis in 1% ethidium bromide stained agarose gels with use of GeneRuler 100 bp DNA Ladder (Fermentas). UV trans-illuminator (Vilber Lourmat) was applied to visualize electrophoretic bands.

Twenty four amplicons (8 per species) were sequenced in both directions on an ABI PRISM 3100 DNA Sequencer using ABI Prism™ BigDye Terminator Cycle Sequencing kit (Applied Biosystems) with primers AMIDOS_F and AMIDOS_R. The obtained sequences were analyzed using FinchTV 1.4.0 (Geospiza). They were subsequently aligned using MUSCLE [13] including *Amidostomum cygni* 28S rRNA gene. Phylogenetic analyses were performed by apply MEGA6 [14].

Different models were tested to find the most suitable one to infer the phylogenetic tree. Phylogenetic analysis with *Amidostomum cygni*, *Amidostomoides petrovi*, *Amidostomoides acutum and Amidostomoides monodon* was conducted using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) [15]. The evolutionary distances

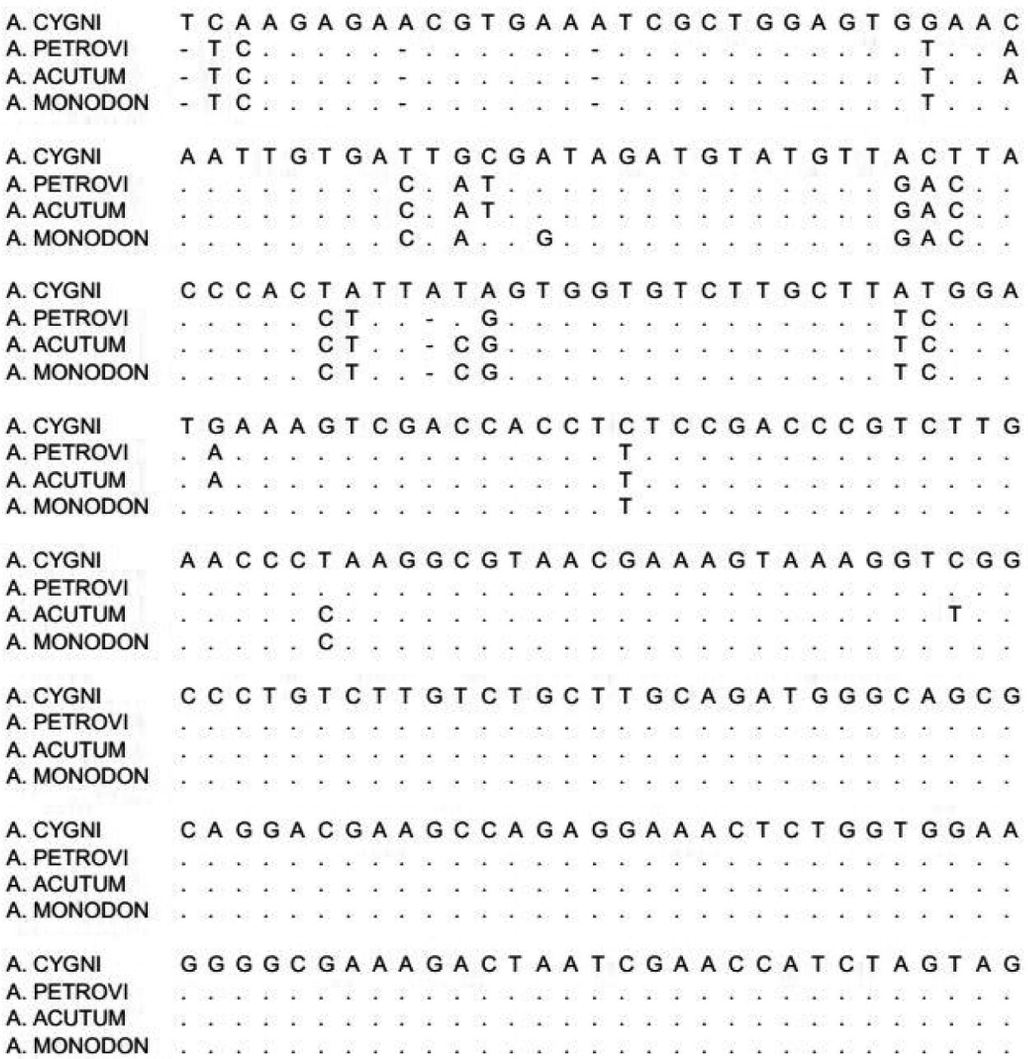

Fig. 1. Multiple alignments of the 28S rRNA sequences of the nematodes used in this study. Gaps are indicated by dashes, however nucleotides homology by dots.

were calculated using p-distance method [16]. Reliabilities for UPGMA tree was tested using 1000 bootstrap replications [17]. All positions containing gaps and missing data were eliminated.

Results

By use AMIDOS_F and AMIDOS_R primers an approximately 630bp fragments of the 28S rRNA gene were obtained. Subsequently in each of the investigated species DNA sequence was determined and deposited in GenBank under accession numbers: KJ186096 (*Amidostomoides petrovi*), KJ186097 (*Amidostomoides acutum*), and KJ186098 (*Amidostomoides monodon*). The 28S rRNA sequences of 4 taxa (including *Amidostomum cygni*) were aligned, however 19 characters (8 alignment positions of the 5' end and 11 at the 3' end of the analyzed fragment) were excluded from the phylogenetic analysis due to incomplete sequences (Fig. 1).

Maximum Likelihood (ML), Neighbor-Joining (NJ) and UPGMA methods generated trees with similar topology. UPGMA analysis of 28S rRNA nucleotide sequence (Fig. 2) showed highest homology between *Amidostomoides petrovi* and *Amidostomoides acutum* which amounted 0.91. Lower homology was found between *Amidostomoides monodon* and the above mentioned pair (approx. 0.86). The least relation regard to nematodes from *Amidostomoides* genus was noticed *Amidostomum cygni* (<0.70).

The above-mentioned results of the research distinctly diversify the nematodes of the genus

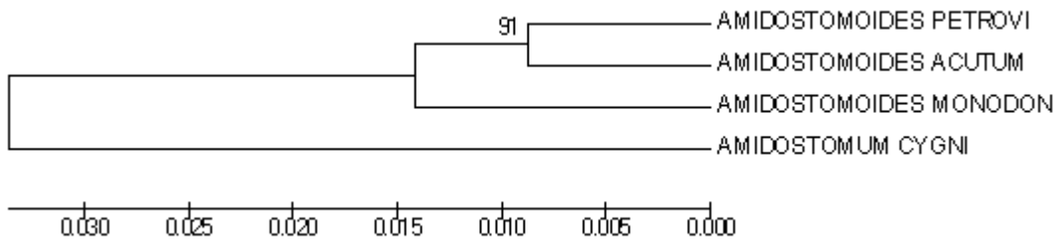

Fig. 2. Phylogenetic relationships between *Amidostomoides* and *Amidostomum* taxa as inferred by UPGMA analysis of 28S rRNA gene

Amidostomoides, which together with morphological and ecological differences among them enables to classify them into the separate species. Simultaneously more distant kinship of the analysed nematodes justifies the Lomakin's [9] proposal of excluding them from the genus *Amidostomum* and placing into the genus *Amidostomoides* Petrova, 1987.

Discussion

Although the nematodes of the genus *Amidostomum* are in Palearctic among the most often recorded parasites in birds connected with the water environment, their official systematic position still gives a lot of rise to controversy. It mainly concerns *Amidostomum acutum* (Lundahl, 1848), the parasite universally recorded both in dabbling ducks, as well as the ones living in the wild [1,18].

The crucial studies by Lomakin [1,7] have not get so far feedback from the western parasitologists community, presumably because they have been edited in Cyrillic. However, my own more than a decade conducted studies on helminths in wild Anatinae confirm the findings of the Russian researcher and indicate that *Amidostomum acutum* (Lundahl, 1848) is a collective species (*sensu lato*), and three morphologically and ecologically different species parasite on ducks: *Amidostomoides acutum* (Lundahl, 1848) Lomakin, 1991; *A. petrovi* (Shakhtahtinskaya, 1956) Lomakin, 1991, and *A. monodon* (Linstow, 1882) Lomakin, 1991. It was found that these nematodes are characterized by quite high inter-subject variability within the species, concerning mainly: the body width, the copulatory pseudobursa width, the spicule head length (among males), as well as the body width, the ovary length and the anterior uterus length (among females). The studies were confirmed with mathematical methods, including discriminant analysis. It was also found that the nematodes of this group are characterized by different ecology as well as distinct host specificity [10,19–25]. The foregoing circumstances suggest that parasites could have different breeding cycles and knowing them would be fundamental for numerous bird species protection.

Ribosomal loci represent a major tool for investigating environmental diversity and community structure through marker gene studies of eukaryotes. These loci are amenable to PCR based assays due to their pseudo-orthology and the large amount of existing data readily available in public sequence databases [26]. In this study sequences of 28S rRNA gene fragment were used to differentiate 3 nematodes from *Amidostomum* genus. The 28S rRNA gene is common used by classification of nematodes; *Calcaridorylaimus castaneae* Nedelchev et al., 2014; *Leidynema appendiculata* (Leidy, 1850) Chitwood 1932; *Mermis nigrescens* Dujardin, 1842 are the most recent examples [27–29]. There was only one species analyzed phylogenetically till now from genus *Amidostomum*, that is *A. cygni*.

Chilton et al. [30] investigated the evolutionary origins of nematodes within the order Strongylida based on 18S and/or 28S rRNA genes sequences. Among them *A. cygni* which was isolated from swan gizzard was also included. Analysis of 18S rRNA sequence showed somewhat different classification of this species to suborder Trichostrongylina than based on 28S rRNA sequence. Investigation of combined 18S and 28S rRNA gene sequences however indicated similar classification of *A. cygni* to suborder Trichostrongylina as in the case of 28S rRNA sequence.

Conclusions

The three morphologically and ecologically different groups of nematodes on dabbling ducks of the Anatinae subfamily, which should be treated as three separate species: *Amidostomoides acutum* (Lundahl, 1848) Lomakin, 1991; *A. monodon* (Linstow, 1882) Lomakin, 1991, and *A. petrovi* (Shakhtahtinskaya, 1956) Lomakin, 1991. However, *Amidostomum acutum* (Lundahl, 1848) should be recognized as a collective species.

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