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Community of Monogenea in populations of *Cichla monoculus* from two tributaries of the Amazon River in the Northern Brazil

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Summary

This study compared the monogeneans community in *C. monoculus* from the Tapajós River (state of Pará) and Jari River (state of Amapá), northern Brazil. A total of 2188 monogeneans belonging to eight taxa were collected from the gills of fish: *Gussevia arilla*, *Gussevia longihaptor*, *Gussevia tucunarense*, *Gussevia undulata*, *Sciadicleithrum ergensi*, *Sciadicleithrum umbilicum*, *Sciadicleithrum uncinatum* and *Tucunarella cichlae*. *Gussevia arilla* was the dominant species for *C. monoculus* from the Tapajós River basin, while *S. umbilicum* predominated among the hosts from the Jari River basin. For the two populations of *C. monoculus*, the prevalence, mean intensity and mean abundance of monogeneans were different and the of parasites community had a high qualitative similarity (87.5 %). The monogeneans community of *C. monoculus* was characterized by high species richness, with infection values varying from low to moderate. The geographic distance and differences in environmental characteristics arising from the same did not influence the richness of species of monogeneans infesting *C. monoculus* in the Tapajós and Jari rivers, but appear to have been determinants in the differences observed in the structure of the monogenean communities in each region.
Keywords: Ectoparasites; Jari River; Monogenean; Peacock bass; Tapajós River; Tucunaré

Introduction

The Tapajós River basin is formed from the confluence of the Teles Pires River and the Juruena River in the state of Mato Grosso, and flows into the middle Amazon River, in the region of Santarém, in the state of Pará (Umetsu *et al*., 2007). It has transparent waters and due to its distance from the sea (around 650 km) suffers little influence from the tide of the Amazon River. The Jari River basin, meanwhile, is formed in the Tumucumaque Mountains National Park, on the border between Brazil and Suriname, and flows into the lower Amazon River in the south of the state of Amapá (Amapá, 2012). Its mouth is around 270 km of the Atlantic Ocean, and suffers strong influence of tides of the Amazon River. For this reason, the Jari River it has white waters downstream and black waters upstream, varying the amount of organic matter in suspension (Abreu & Cunha, 2015).

The genus *Cichla* Block & Schneider, 1801 (Cichlidae) comprise 15 fish species that are popularly known as peacock bass. They are endemic to the Amazon River system and, due to the excellence of their meat are important in extractive fishery and fish farming (Batista & Petrere Júnior, 2003; Kullander & Ferreira, 2006; Santos *et al*., 2012). *Cichla monoculus* is widely distributed in the

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Amazon region and can be found from Peru to French Guiana (Kullander & Ferreira, 2006). Due to the importance in the sport fishing, some species of *Cichla* have been introduced in other Brazilian river basins (Agostinho & Júlio Júnior, 1999; Chellappa *et al*., 2003; Gomiero & Braga, 2004).

Studies on parasites of wild fish populations, in addition to increasing knowledge of diversity, can generate information about the parasite-host-environment relationship (Oliveira *et al*., 2016; Oliveira & Tavares-Dias, 2016). In addition, some studies have tried to elucidate the main factors that influence the parasite composition of host population (Poulin, 1995; Poulin *et al*., 1999; Marcogliese *et al*., 2006; Braicovich & Timi, 2008; Francová & Ondračková, 2011; Santana-Pineros, *et al*., 2012; Lagrue & Poulin, 2015; Marcogliese, *et al*., 2016). Monogenea Van Beneden, 1858, which belongs to the Platyhelminthes Gegenbaur, 1859 phylum, is the most diverse group of parasites, with around 835 species described parasitizing fish from South America (Luque *et al*., 2017). While these are mainly ectoparasites of fish, and are usually found on gills, body surface and nasal cavities, but some species are endoparasites inhabiting the intestine, stomach and urinary bladder of hosts (Bilong-Bilong, *et al*., 1996; Guidelli *et al*., 2003a; Boeger & Viana, 2006). They exhibit high host specificity in comparison with

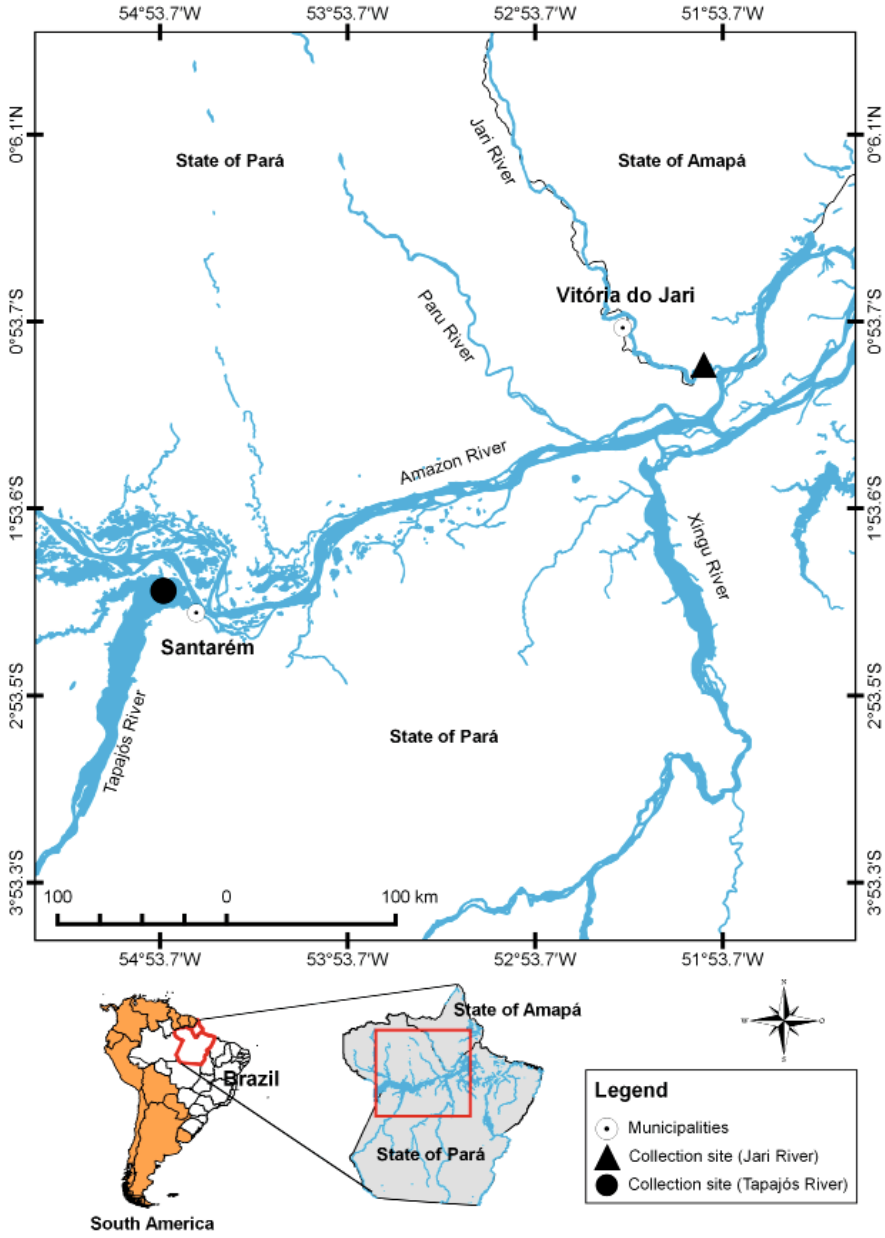

Fig. 1. Sampling sites of *Cichla monoculus* in the tributaries from the Amazon River, in eastern Amazon, Northern Brazil.

other helminths taxa (Boeger & Viana, 2006; Braga *et al*., 2014).
In Brazil, most of the monogenean species described from freshwater fish belong to the Dactylogyridae and Gyrodactylidae families, with the dominance of dactylogyrids (Cohen *et al*., 2013; Luque *et al*., 2017). Among fish from the Cichlidae family, infestations were recorded mainly by dactylogyrids species (Cohen *et al*., 2013; Ferreira-Sobrinho & Tavares-Dias, 2016). For the *Cichla* genus, eight species of monogeneans are known (Cohen *et al*., 2013), but only *Gussevia longihaptor*, *Tucunarella cichlae* (Mendoza-Franco, *et al*., 2010) and *Gussevia undulata* (Mendoza-Franco *et al*., 2010) have been recorded parasitizing *C. monoculus*, and such studies has been restricted to the Peruvian Amazon. Thus, this study compared the community of monogeneans in gills of *C. monoculus* from the Tapajós and the Jari rivers, both tributaries of the Amazon River system in the northern Brazil.

Materials and Methods

Fish collection
The specimens of *C. monoculus* were captured in March 2015 in the Jari River, near the community of Jarilândia, in the municipal of Vitória do Jari, in the state of Amapá (1°7'39.48"S - 51°59'43.94"W) and in the Tapajós River, near the community of Jari do Socorro, in the municipal of Santarém, in the state of Pará, Brazil (2°20'2.58" S 54°52'34.08" W) (Fig. 1).
These two locations are approximately 320 km apart in a straight line. Gillnets measuring 30 m in length and 2.5 m in height, and with a mesh size of 30, 35 and 40 mm between knots were used to capture the fish, along with artificial bait. The fish were identified in accordance with Kullander & Ferreira (2006). Voucher specimens were deposited at the Platyhelminthes of the Zoology Museum (ZUEC) from the Universidade Estadual de Campinas (Campinas Federal University – Brazil), under accession number 94 – 99, 101, 104 – 109, 111 – 113, 115, 121 – 135.

Collection, fixation and identification of parasites
Following collection, the fish were euthanized by spinal cord transection. Their standard length and weight (g) were then measured, and they were necropsied for the removal of the gills, which were transferred to flasks containing heated water (60-70°C) and shaken vigorously for detachment of the parasites (Kritsky & Stockwell, 2005). The collected monogenea were fixed in formalin 5 % for 24h and preserved in alcohol 70 %. The methodology used to quantify the parasites was that recommended by Eiras *et al*. (2006), and identification was in accordance with Kritsky *et al*. (1986), Kritsky *et al*. (1989) and Mendoza-Franco *et al*. (2010).

Data analysis procedures
The prevalence, mean intensity, mean abundancy (Bush *et al*., 1997) and frequency of dominance, i.e. percentage of infracommunities in which a given species of parasite is numerically dominant were determined (Rohde *et al*., 1995). The differences in prevalence for each species of monogenean from the host populations were evaluated using the Williams' *G*-test with Yates's correction; and the differences in abundance and intensity were compared using the Mann-Whitney test (*U*) (Zar, 2010).
To test the differences between the monogenean communities of *C. monoculus*, Tapajós River and Jari River, the (ANOSIM) test was used with 999 permutations using the similarity index Jaccard (J) (qualitative), and dissimilarity index of Bray-Curtis (B) (quantitative). Principal Component Analysis (PCA) was carried out to compare the monogenean communities in the gills of fish from both hydrographic basins. These analyzes were carried out using the Past-Paleontological Statistics software package (Hammer *et al*., 2001).

Ethical Approval and/or Informed Consent

The fish capture was authorized by the Ministry of the Environment (SISBIO nº 44268-4) and the methodology of the present study was approved by the Ethics Research Committee of the Universidade Federal de São Paulo (São Paulo Federal University) (CEUA No 92090802140) in accordance with Brazilian legislation (Federal Law 11794, dated October 8, 2008).

Results

The 19 specimens of *C. monoculus* from the Tapajós River measured 37.4 ± 2.6 cm and weighed 657.5 ± 142.5 g, and the 20 specimens from the Jari River measured 29.9 ± 3.7 cm and weighed 737.0 ± 240.1 g. All the fish examined were parasitized by species of monogeneans one or more species. A total of 561 monogeneans were collected from *C. monoculus* from the Tapajós River and 1627 from the Jari River, totaling 2188 parasites. These parasites were distributed into the following taxa: *Gussevia arilla* Kritsky, Thatcher & Boeger, 1986; *Gussevia longihaptor* Kritsky, Thatcher & Boeger, 1986; *Gussevia tucunarense* Kritsky, Thatcher & Boeger, 1986; *Gussevia undulata* Kritsky, Thatcher & Boeger, 1986; *Sciadicleithrum ergensi* Kritsky, Thatcher & Boeger, 1989; *Sciadicleithrum umbilicum* Kritsky, Thatcher & Boeger, 1989; *Sciadicleithrum uncinatum* Kritsky, Thatcher & Boeger, 1989 and *Tucunarella cichlae* Mendoza-Franco, Scholz & Rozkošná, 2010. *Gussevia arilla* was the dominant species in the *C. monoculus* population from the Tapajós River, and *S. umbilicum* was the dominant species in hosts from the Jari River. Of these eight species of monogeneans found, seven species were commons for hosts of both basins, but *T. cichlae* occurred only in hosts from the Tapajós River. The infestation levels of monogenean species varied among themselves and between the regions studied. In hosts from the Tapajós River, the highest values of infestation were caused by *G. arilla* and *S. umbilicum*, and in the Jari River by *S. umbilicum* and *S. ergensi*. The lowest values of infestation in fish from the Tapajós River were caused by *G. undulata* and in the Jari River by *T. cichlae* of (Table 1).

Table 1. Infestation by monogenean species in the gills of *Cichla monoculus* from the Tapajós River and the Jari River in the eastern Amazon, Northern Brazil. P: Prevalence; MI: Mean intensity; MA: Mean abundance; FD: Frequency of dominance; TNP: Total number of parasites. SD: Standard deviation.

Parasites	Tapajós River (N = 19)					Jari River (N = 20)				
	P (%)	MI	MA ± SD	FD (%)	TNP	P (%)	MI	MA ± SD	FD (%)	TNP
Gussevia arilla	100	10.8	10.8 ± 7.3	0.363	205	80	11.1	8.9 ± 16.2	0.109	178
Gussevia longihaptor	42.1	2.4	1.0 ± 1.6	0.034	19	80	6.8	5.4 ± 9.2	0.066	108
Gussevia tucunarense	100	4.1	4.1 ± 4.1	0.138	78	55	4.3	2.3 ± 4.6	0.029	47
Gussevia undulata	36.8	2.7	1.0 ± 1.5	0.034	19	60	8.0	4.8 ± 6.4	0.059	96
Sciadicleithrum ergensi	94.7	4.7	4.5 ± 3.7	0.150	85	100	12.5	12.5 ± 9.1	0.154	250
Sciadicleithrum umbilicum	100	5.9	5.9 ± 3.7	0.200	113	100	41.8	41.8 ± 36.5	0.514	836
Sciadicleithrum uncinatum	68.4	3.2	2.2 ± 2.7	0.074	42	90	5.7	5.15 ± 5.4	0.063	103
Tucunarella cichlae	0	0	0	-	-	35	1.3	0.45 ± 0.7	0.006	9

Table 2. Williams' *G*-test (G), and Mann-Whitney (*U*) test, considering ($p \leq 0.05$), for levels of monogenean infestation in the gills of *Cichla monoculus* from the Tapajós River and the Jari River in the eastern Amazon region, Northern Brazil. P: prevalence; MI: Mean intensity; MA: mean abundance.

Parasites	P (%)		MI		MA	
	G	p	*U*	p	*U*	p
Gussevia arilla	5.17	0.10	93.50	0.03	93.50	0.003
Gussevia longihaptor	6.09	0.01	46.50	0.14	100.50	0.006
Gussevia tucunarense	14.61	0.0001	92.50	0.30	92.50	0.003
Gussevia undulata	2.11	0.15	21.00	0.03	125.00	0.03
Sciadicleithrum ergensi	1.47	0.23	78.50	0.001	78.50	0.0009
Sciadicleithrum umbilicum	-	-	24.00	0.0001	24.00	0.0001
Sciadicleithrum uncinatum	2.87	0.10	79.50	0.07	111.50	0.01

There were significant differences in the prevalence, mean intensity and mean abundance of monogeneans for both host populations (Table 2). The monogeneans community of *C. monoculus* of the Tapajós River and Jari River presented homogeneity according to the qualitative index Jaccard (J = 0.875) (R = 0.370, p = 0.001) and quantitative index Bray-Curtis (B = 0.459) (R = 0.643, p = 0.001).

A positive correlation was observed between the abundance of monogeneans in *C. monoculus* (Table 3). In fish from the Tapajós River, were predominant hosts with 5 – 6 species of parasites, while in fish from the Jari River there was a predominance of hosts with 4 – 8 species of monogeneans (Fig. 2). Multivariate analysis of the monogenean communities of *C. monoculus* from the Tapajós and Jari rivers revealed small differences between the host populations, caused by *G. arilla* and *S. umbilicum* (Fig. 3).

Discussion

Fish have an important role in the life cycle of various species of monoxenic parasites (Hoffman, 1999; Thatcher, 2006; Oliveira *et al*., 2016), including monogenean species. These associations are highly complex and dynamic, resulting from the interaction of evolutionary systems and ecological processes acting simultaneously (Alarcos & Timi, 2012). Therefore, phylogenetically proximal fish populations, living in the same environment, can exhibit major similarity in the community and richness of parasite species (Alarcos & Timi, 2012; Hoshino & Tavares-Dias, 2016; Oliveira *et al*., 2016). In contrast, when great distances separate such host populations, these similarities tend to diminish (Poulin *et al*., 1995; Poulin & Morand, 1999; Lagoue & Poulin, 2015). The community of monogeneans of *C. monoculus* from the Tapajós River and Jari

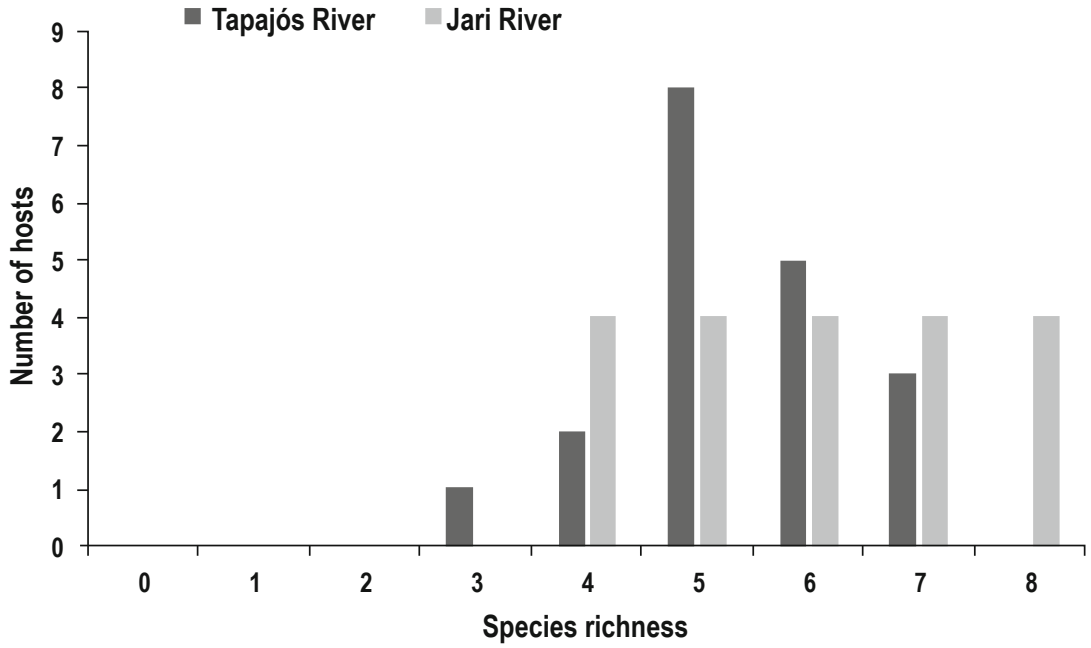

Fig. 2. Species richness of monogeneans in the gills of *Cichla monoculus* from the Tapajós River and the Jari River in the eastern Amazon, Northern Brazil.

Table 3. Coefficient of Spearman's correlation (*rs*), considering ($p \leq 0.05$), between abundance of infracommunities of monogeneans in the gills of *Cichla monoculus* from the Tapajós River and the Jari River in the eastern Amazon, Northern Brazil.

	Gussevia longihaptor		*Gussevia tucunarense*		*Gussevia undulata*		*Sciadicleithrum ergensi*		*Sciadicleithrum umbilicum*		*Sciadicleithrum uncinatum*		*Tucunarella cichlae*	
Tapajós River	*rs*	p	*rs*	p	*rs*	P	*rs*	p	*rs*	p	*rs*	p	*rs*	p
Gussevia arilla	0.47	0.04	0.69	0.001	0.47	0.04	0.55	0.01	0.25	0.29	0.56	0.01	-	-
Gussevia longihaptor			0.37	0.12	0.14	0.57	0.24	0.31	0.28	0.25	0.49	0.03	-	-
Gussevia tucunarense					0.31	0.19	0.25	0.30	-0.17	0.48	0.61	0.005	-	-
Gussevia undulata							0.35	0.15	0.25	0.29	0.28	0.33	-	-
Sciadicleithrum ergensi									0.41	0.08	0.62	0.004	-	-
Sciadicleithrum umbilicum											0.13	0.59	-	-
Jari River														
Gussevia arilla	0.55	0.01	0.60	0.005	0.21	0.37	0.36	0.12	0.04	0.84	0.38	0.10	0.36	0.11
Gussevia longihaptor			0.61	0.004	0.52	0.01	0.30	0.19	0.35	0.13	0.07	0.76	-0.009	0.97
Gussevia tucunarense					0.51	0.02	0.39	0.09	0.47	0.03	0.41	0.07	0.07	0.75
Gussevia undulata							0.31	0.18	-0.05	0.86	0.17	0.48	0.15	0.52
Sciadicleithrum ergensi									0.69	0.004	0.29	0.21	0.05	0.82
Sciadicleithrum umbilicum											0.43	0.06	0.34	0.13
Sciadicleithrum uncinatum													0.15	0.52

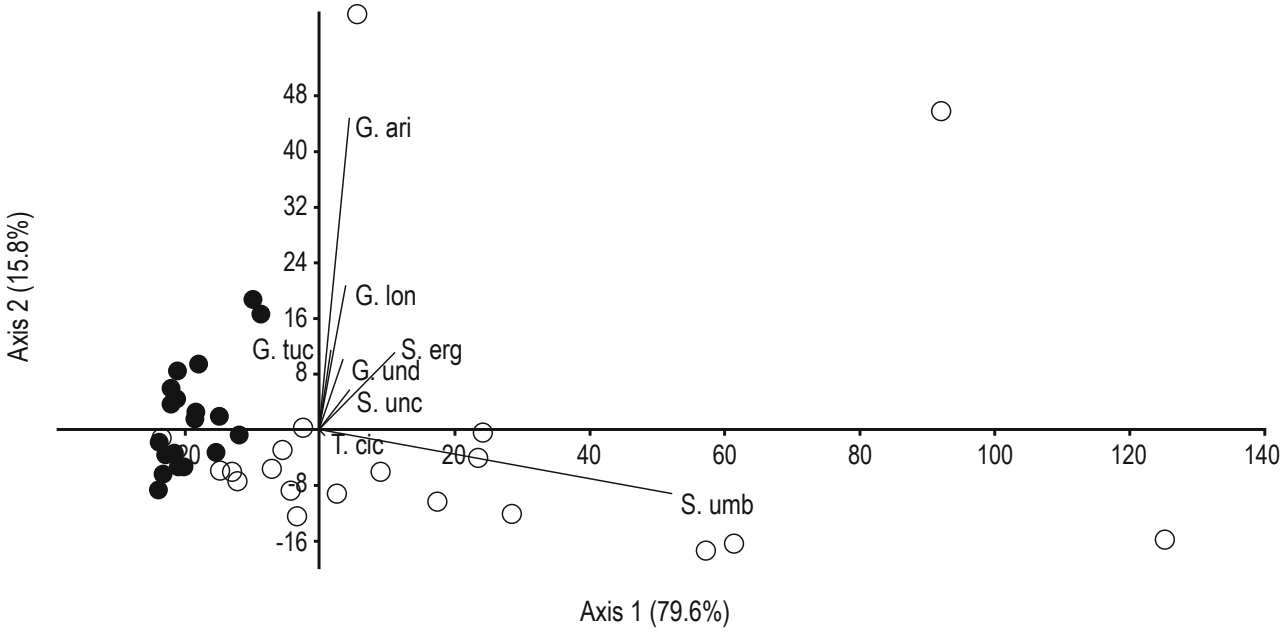

Fig. 3. Scatter plot of principal component analysis (PCA) of infracommunities of monogenea from the gills of *Cichla monoculus* from the Tapajós River (O) and the Jari River (●) in the eastern Amazon, Northern Brazil. G. ari: *Gussevia arilla*; G. lon: *Gussevia longihaptor*; G. tuc: *Gussevia tucunarense*; G. und: *Gussevia undulata*; S. erg: *Sciadicleithrum ergensi*; S. umb: *Sciadicleithrum umbilicum*; S. unc: *Sciadicleithrum uncinatum*; T. cic: *Tucunarella cichlae*.

River had a similarity of 87.5 %, and this similar composition can be expected for a same species of similar environment (Oliveira *et al*., 2017).

The *Cichla* species includes typically sedentary fish species, whose movements are restricted to a few kilometers (Hoeinghaus *et al*., 2003). The composition and structure of the monogenean communities of *C. monoculus*, a widely-distributed fish in the Amazon region (Kullander & Ferreira, 2006; Willis *et al*., 2007), from two rivers 320 km apart, was compared. Cohen *et al*. (2013) listed eight species of monogeneans described for *Cichla* species, and we found these same species in *C. monoculus* from the Jari River, and only seven in hosts from the Tapajós River, which not had *T. cichlae*. These absence of *T. cichlae* of *C. monoculus* from the Tapajós River may be due to the low sampling of fish. In comparison with other studies, the species richness of monogeneans found here was greater than that registered for *Cichla kelberi* Kullander & Ferreira, 2006 from the Paraná River basin (Takemoto *et al*., 2009), Rosana Reservoir (Yamada & Takemoto, 2013) and Laje Reservoir (Yamada & Takemoto, 2011), as well as for *Cichla piquiti* Kullander & Ferreira, 2006 from the Itaipu Reservoir (Yamada & Takemoto, 2011). However, all these studies were carried out with species of *Cichla* introduced into such watersheds, a condition that certainly influenced the diversity of the monogeneans of these hosts, because the parasites loss can occur after introduction into a new environment (Lacerda *et al*., 2013).

The present study, besides extending the knowledge of the geographic distribution of monogeneans for species of *Cichla*, also provides comparative data on species richness, prevalence, mean intensity and mean abundance, which are of great importance in the understanding in parasite-host-environment interaction. The Tapajós and Jari rivers are large tributaries of the Amazon River system and have their own environmental characteristics, due to the location of their mouths, and thus suffer differing influences from the waters of the Amazon River, due to the occurrence of daily tides in the Jari River (Abreu & Cunha, 2015). Daily tides of Amazon River can directly affect the local biota of its tributaries (Junk, 2013). The similar species richness and structure of the monogenean communities from the two populations of *C. monoculus* suggest the wide distribution of parasites of this host throughout its area of occurrence in the Amazonian biome. However, the community structure of these parasites revealed different levels of prevalence and abundance, as well as the dominance of *G. arilla* in *C. monoculus* from the Tapajós River and dominance of *S. umbilicum* in hosts from the Jari River. Similarly, differences in the structure of the parasite communities related to the characteristics of each environment have been reported for different host species (Paulin & Morand, 1999; Francová & Ondračková, 2011; Santana-Pineros *et al*., 2012; Marcogliese *et al*., 2016).

Levels of parasitism for the same host can vary spatially, with higher values at sites where hosts are more abundant and environmental conditions are more suitable for development, transmission and survival during the free-living and infectious stages of the parasites (Lagrue & Poulin, 2015). Possibly, differences in environmental characteristics influenced the levels of parasitism in *C. monoculus*, because the prevalence of *G. arilla* and *G. tucunarense* was greater in hosts from the Tapajós River, while the

prevalence of *G. longihaptor* was greater in hosts from the Jari River. The mean intensity of *G. arilla*, *G. tucunarense*, *G. undulata*, *S. ergensi* and *S. umbilicum* was greater in fish from the Jari River, as was the abundance of *G. longihaptor, G. undulata*, *S. ergensi*, *S. umbilicum* and *S. uncinatum*.

In *C. monoculus* from the Tapajós River there was a predominance of hosts infested by five to six species of monogeneans, while in fish from the Jari River there was a predominance of hosts with four to eight species. Such differences can be related to the low abundance and mean intensity of some species of monogeneans in the gills of hosts from the Tapajós River, resulting in greater micro-habitat availability for the establishment of different monogenean infracommunities. Competition among species of parasites can be verified by the negative correlation between the abundance of the same (Šimková *et al*., 2000). However, only significant positive correlations were found between the abundance of the monogenean species from the Tapajós and Jari rivers, suggesting that there no competition among species, which can facilitate the coexistence of these parasites in the gills (Desdevises *et al*., 2000). Oliveira & Tavares-Dias (2016) also reported similar results for *Piaractus brachypomus* parasitized by *Anacanthorus spathulatus*, *Mymarothecium viatorum* and *Notozothecium janauachensis*.

In summary, this study increased the knowledge on the diversity of monogeneans in *C. monoculus* from the Amazon basin, and showed a moderate parasitism in the host population, which had an aggregate dispersion of parasites. The *C. monoculus* population of both localities shared the mostly of the species of monogeneans, as expected. However, were found differences in the levels of infection by monogeneas in both host populations, influenced by geographic distance and the differences in the environmental characteristics, which is peculiar to each basin investigated. Further studies should focus on the environmental characteristics and seasonality of monogeneans in *C. monoculus* from these two Amazonian basins.

Conflict of Interest

Authors state no conflict of interest.

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Structure and morphometrics of *Ancyrocephalus paradoxus* (Monogenea: Ancyrocephalidae) from *Sander lucioperca* (Percidae) in Czechia

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Article info

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Summary

New morphometric data, including details of the copulatory system and attachment structures, as well as inner organs are provided for *Ancyrocephalus paradoxus* Creplin, 1839. Scanning electron microscopy reveals new information of the body shape, position of the cephalic organs' openings, and structure of anchors, as well as differences in the in anchors' structure in adults and sub-adults of *A. paradoxus.* Energy dispersive analysis for X-ray was conducted for the first time for anchors in Monogenea and revealed structural differences between different parts of the anchors in two age groups.
Keywords: *Ancyrocephalus paradoxus*; morphometrics; SEM; EDXA

Introduction

Ancyrocephalus paradoxus Creplin, 1839, the type species of genus *Ancyrocephalus*, is an euryhaline monogenean, known from Baltic to Aral Seas, Black and Azov seas, the Caspian, and the Mediterranean. Gusev (1985) reported that *A. paradoxus* is distributed worldwide together with its host. It has been reported from Europe and Asia. In the Ukrainian territory of Europe, this species was recorded from many localities including Danube, Dniester, Tisza, Prypyat and Dnieper rivers and its reservoirs, and the Black and Azov Sea basins (Kulakivska, 1954, 1973, 1974; Komarova, 1964, 1972; Pashkevichute, 1971; Iskov & Koval, 1973; Koval, 1978; Solonchenko, 1982; Rubtsova, 2003; 2015). In Poland *A. paradoxus* was reported from Lakes Jamno, Lebsko, Dargin, Gulf of Gdansk, Vistula Lagoon and from the Pomeranian Bay of the Baltic Sea (Wierzbicki, 1970; Rolbiecki & Rokicki, 1996; Rolbiecki, 2003; Zaostrovtseva, 2009; Bielat *et al*, 2015). It was registered in Great Britain (Brewster, 2016); Hungary (Molnar *et al*., 2016); Czechia (Mendoza-Palmero *et al*., 2015; Acosta *et al*.; 2017); Romania (Cojocary, 2009); Azerbaijan (Ibragimov & Shakaralieva, 2014); Russia (Izyimova 1958; Gusev, 1985; Zharikova *et al*., 2002; Rumyantsev, 2004); Turkey (Ozturk & Ozer, 2014), Iran (Pazooki & Masoumian, 2012). Chubb (1977) studied the occurrence of *A. paradoxus* in different climate zones. Starovoitov (1989, 1999) studied different ecological aspects and relationships in the host-parasite system. Molnar *et al*. (2016) provided histological investigations of *A. paradoxus.*

In spite of these extensive reports, the description of *A. paradoxus* was very brief and a few redescriptions and records were lacking some basic morphometric data and accuracies (Ergens, 1966; Bykhovsky & Nagibina, 1970; Lom & Ergens, 1970 and Gusev, 1985). Metal analysis have never been performed for *A. paradoxus*. Though it was successfully used for differentiation of two age stages in Polystomatidae (Rubtsova & Heckmann, 2017), it was never carried out for different gallium cuts of the anchors in Monogenea, accomplished with SEM. In the present study, we provide metric parameters according to Gusev (1985) that now are widely used in studies of Monogenea (Šimková *et al*., 2013; Acosta *et al*.,

* – corresponding author

2018). For instance, “complete anchor length” in Ergens (1966) is what was later accepted as ventroapical length (for four-anchored monogeneans) (Gusev, 1985). Thus, the addition and expansion of the morphometric data, the results of metal analysis of the anchors, as well as the results of gallium cuts of different parts of anchors of *A. paradoxus* in two age groups prompted this study and represents its major contributions.

Material and Methods

Sample collection

Fish was collected with gill-netting – a 22 mm mesh benthic gillnet (15 m length, 1.5 m height) was installed across the pond (5 m from the bank) for 3 h during the day and controlled every 0.5 h. Sampling time: 11:00−12:00 h. All fish were transported alive in aerated barrels to the laboratory of the Institute of Vertebrate Biology, Czech Academy of Sciences (Brno), where they were transferred to a 1 m^3 outdoor holding basin (separate basin for each sampling method). Before dissection, the standard length (SL) of each fish was determined and gills were examined for monogeneans. All fish were dissected within 48 h of sampling (Kvach *et al*., 2016). Thirty-two mature specimens of *Sander lucioperca* age 3+, SL 26.9 (21.4 – 30.5 cm) were studied for the presence of *Ancyrocephalus paradoxus* at Cezarka pond, Vodnany, Czechia (49°08’47.0”N 14°11’28.7”E) on 17 – 18 October 2017. Twenty specimens of *A. paradoxus* were used for morphometric studies, and 8 specimens were used for SEM and metal analysis.

Light microscopy

Worms were stained in Mayer’s acetic carmine, destained in 4 % hydrochloric acid in 70 % ethanol, dehydrated in ascending concentrations of ethanol (12 hr. each), cleared in 100 % xylene and then in 50 % Canada balsam and 50 % xylene (12 hr. each). Whole worms were then whole mounted in Canada balsam. Measurements of sclerotized parts in the present study were made using the scheme shown at Fig. 1. The range is followed by the mean values between parentheses.

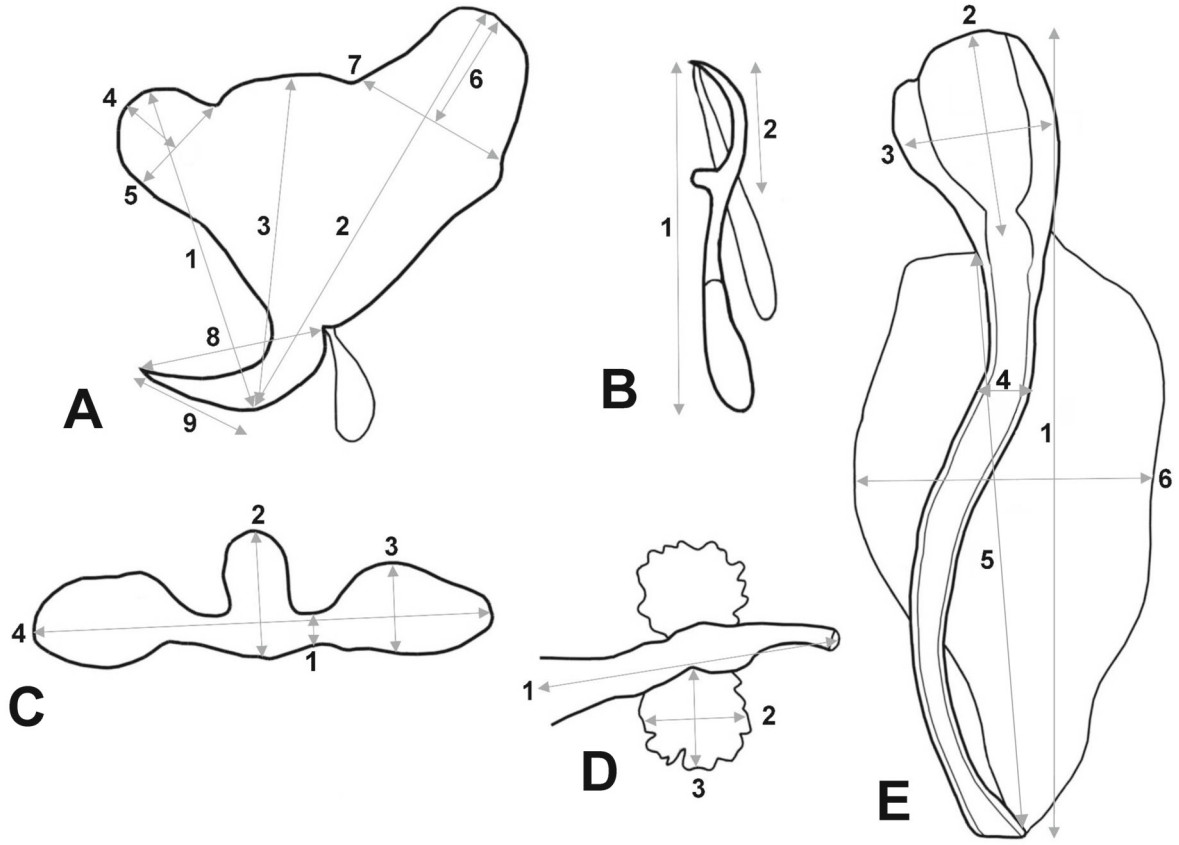


Fig. 1. Scheme of measurements of sclerotized parts of *Ancyrocephalus paradoxus.*
A – ventral/dorsal anchor (1 – dorsoapical length, 2 – ventroapical length, 3 – base part length, 4 – inner root length, 5 – inner root width, 6 – outer root length, 7 – outer root width, 8 – blade length, 9 – point length); B – marginal hooklet (1 – marginal hooklet length, 2 – marginal hooklet blade length); C – ventral/dorsal bar (1 – length in the narrowest part, 2 – length of the middle extension, 3 - length of the lateral extensions); D – vagina (1 – vagina length, 2 – comb-like structures length, 3 - comb-like structures width); E – copulatory organ (1 – copulatory organ total length, 2 – copulatory organ wide part length, 3 – copulatory organ wide part width, 4 – copulatory organ tube diameter, 5 – accessory piece of copulatory organ length, 6 – accessory piece of copulatory organ width)

Scanning Electron Microscopy (SEM)

Samples of parasites fixed and stored in 70 % ethanol were processed following standard methods [Lee, 1992] which included critical point drying (CPD) in sample baskets and mounted on SEM sample mounts (stubs) using conductive double-sided carbon tape. Samples were coated with gold and palladium for 3 minutes using a Polaron #3500 sputter coater (Quorum [Q150 TES] www.qurumtech.com) establishing an approximate thickness of 20 nm. Samples were placed and observed in an FEI Helios Dual Beam Nanolab 600 (FEI, Hillsboro, Oregon) Scanning Electron Microscope with digital images obtained in the Nanolab software system (GEI, Hillsboro, Oregon) and then transferred to a USB for future reference. Samples were received under low vacuum conditions using 10 KV, spot size 2, 0.7 Torr using a GSE detector.

Energy Dispersive X-ray Analysis (EDXA)

Standard methods were used for preparation similar to the SEM procedure. Specimens were examined and positioned with the above SEM instrument, which was equipped with a Phoenix energy-dispersive x-ray analyzer (FEI, Hillsboro, Oregon). X-ray spot analysis and live scan analysis were performed at 16Kv with a spot size of five and results were recorded on charts and stored with digital imaging software attached to a computer. The TEAM (Texture and Elemental Analytical Microscopy), a modification of the EDAX (Energy Dispersive analysis for X-ray) system, software system (FEI, Hillsboro, Oregon) was used. The data included weight percent and atom percent of the detected elements following correction factors.

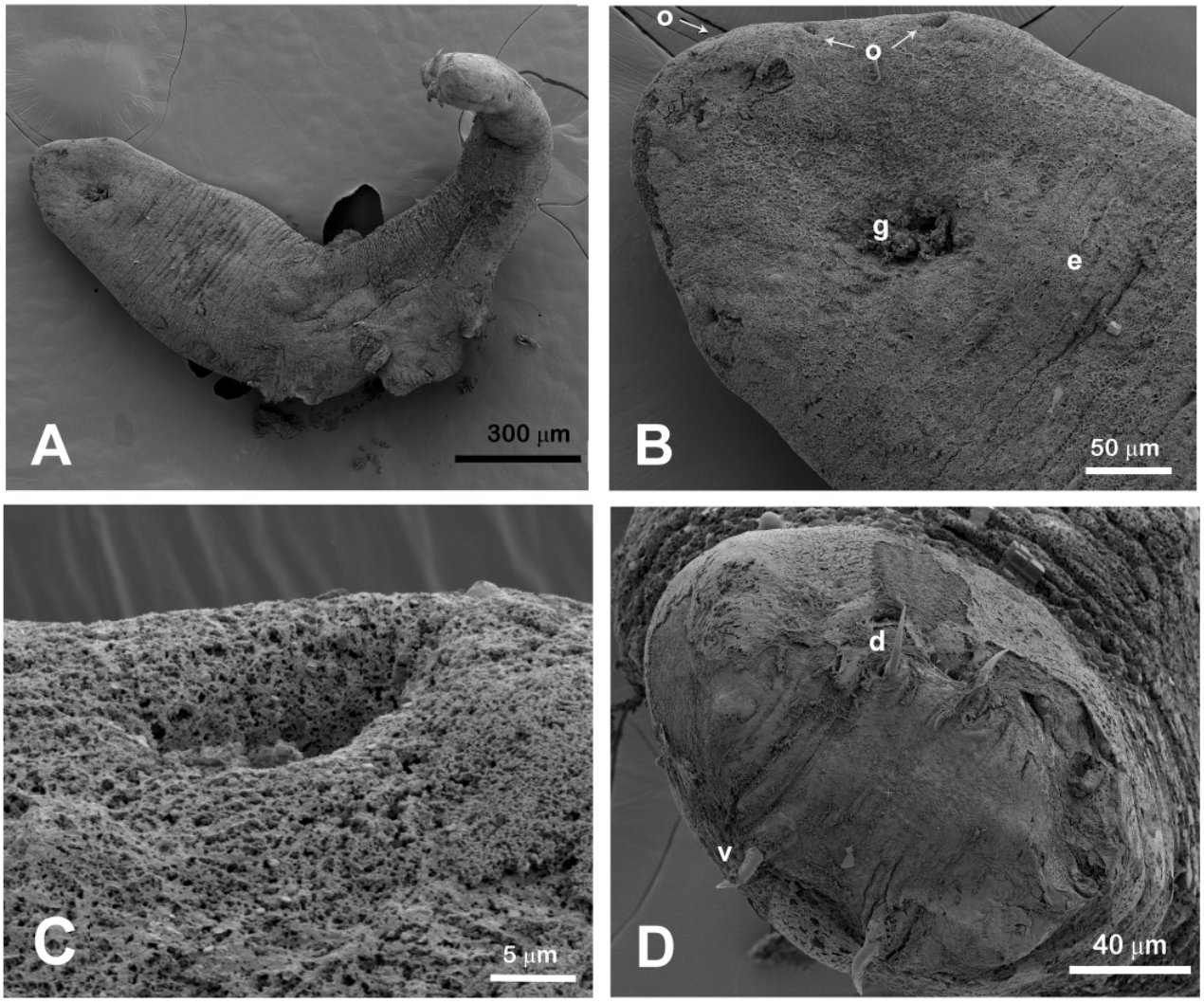


Fig. 2. SEM of an adult of *Ancyrocephalus paradoxus*.
A – whole body, ventral view; B – prohaptor of *A. paradoxus* (o – cephalic gland openings; g – mouth groove, e – an elevation in the area of protrusion of the copulatory organ); C – one of the six cephalic gland openings of the prohaptor; D – opisthohaptor of *A. paradoxus*, an end view (v – ventral anchor; d – dorsal anchor)

Ethical Approval and/or Informed Consent

The research related to animals has been complied with all relevant national regulations and institutional policies for the care and use of animals.

Results

Infection levels

All hosts were highly infected with *A. paradoxus* with intensity of 138 (101 – 188) parasites per fish.

Measurements of A. paradoxus

In the present study, we provide detailed measurements of hard parts and soft inner organs of mature specimens of *A. paradoxus* (Table 1), namely providing the metrical information on haptor, peduncle, detailed measurements of dorsal and ventral anchors, that include ventro- and dorsoapical lengths, roots parameters, base, blade and point lengths. We also provide marginal hooklet metric parameters, as well as copulatory system parameters, that include details of copulatory organ tube, accessory piece, vaginal tube and its accessory parts, as well as pharynx, ovary and testis parameters.

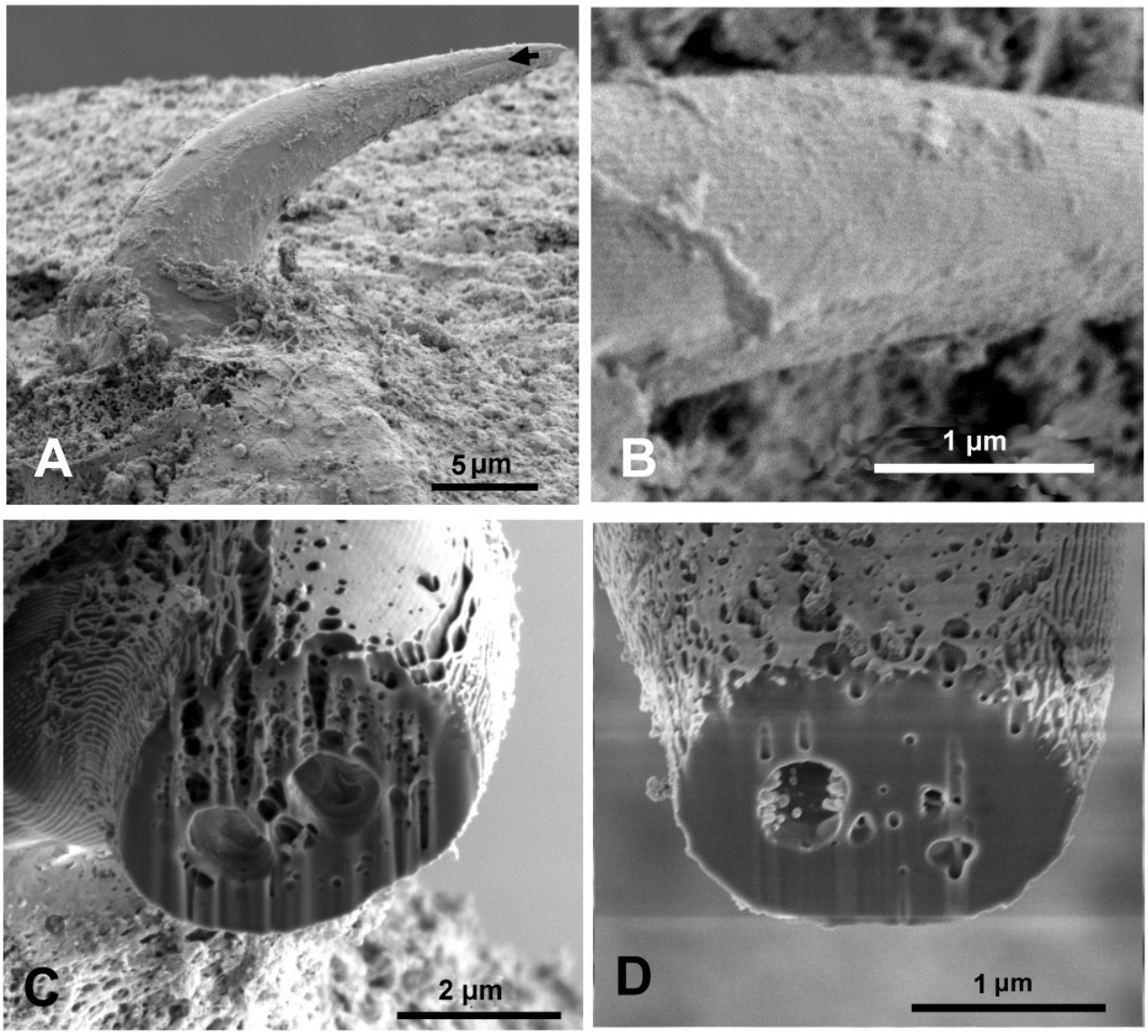


Fig. 3 SEM of an anchor of an adult of *Ancyrocephalus paradoxus*.
A – note longitudinal depression (arrow) in the distal part of the blade; B – an approximate view of the anchor surface. Note the longitudinal stiffeners; C – a gallium cut of the anchor, close to the base. Note the inner porous structure; D – a gallium cut of the anchor, close to its tip. Note dense, homogeneous, calcified tissue of the anchor on the cut

Table 1. Comparative metric characteristics of *Ancyrocephalus paradoxus* from *Sander lucioperca*, in mm.

	Ergens, 1966	Bykhovsky & Nagibina, 1970	Gusev, 1985	Present study
Body length	2.34 – 4.68	1.50 – 3.50	4.7	1.965 (1.750 – 2.125)
Body width	0.39 – 0.78	0.50 – 0.70	0.8	0.505 (0.375 – 0.750)
Haptor length	0.15 – 0.39	–	–	0.034 (0.028 – 0.045)
Haptor width	0.15 – 0.49	–	–	0.048 (0.040 – 0.063)
Peduncle length	– ***	–	–	0.096 (0.075 – 0.138)
Peduncle width	–	–	–	0.048 (0.030 – 0.063)
Ventral anchor:				
dorsoapical length *	–	–	–	0.042 (0.038 – 0.051)
ventroapical length	0.056 – 0.063	0.054 – 0.057	0.050 – 0.063	0.057 (0.053 – 0.063)
base length	–	–	–	0.039 (0.035 – 0.043)
inner root length	d.**	–	–	0.008 (0.006 – 0.011)
inner root width	–	–	–	0.014 (0.013 – 0.015)
outer root length	d.	–	–	0.008 (0.001 – 0.011)
outer root width	–	–	–	0.017 (0.015 – 0.018)
blade length	0.023 – 0.026	–	–	0.022 (0.018 – 0.025)
point length	–	–	–	0.009 (0.007 – 0.013)
Ventral bar length 1	0.003 – 0.005	–	–	0.005 (0.003 – 0.008)
Ventral bar length 2	0.007 – 0.016	–	0.009 – 0.014	0.010 (0.006 – 0.013)
Ventral bar length 3	0.011 – 0.014	–	–	0.012 (0.010 – 0.013)
Ventral bar width	0.037 – 0.060	0.045 – 0.050	0.045 – 0.060	0.047 (0.038 – 0.060)
Dorsal anchor:				
dorsoapical length	–	–	–	0.041 (0.038 – 0.043)
ventroapical length	0.050 – 0.060	0.052 – 0.060	–	0.057 (0.055 – 0.060)
base length	–	–	–	0.033 (0.028 – 0.038)
inner root length	d.	–	–	0.010 (0.008 – 0.013)
inner root width	–	–	–	0.015 (0.014 – 0.015)
outer root length	d.	–	–	0.008 (0.002 – 0.015)
outer root width	–	–	–	0.020 (0.015 – 0.023)
blade length	0.023 – 0.028	–	–	0.027 (0.025 – 0.028)
point length		–	–	0.013 (0.010 – 0.015)
Dorsal bar length 1	0.002 – 0.004	–	–	0.005 (0.004 – 0.008)
Dorsal bar length 2	0.007 – 0.008	–	0.008 – 0.012	0.009 (0.007 – 0.015)
Dorsal bar length 3	0.007 – 0.011	–	–	0.011 (0.009 – 0.013)
Dorsal bar width	0.049 – 0.060	0.060 – 0.064	0.060 – 0.070	0.050 (0.043 – 0.055)
Marginal hooklet length		0.018 – 0.020	0.017 – 0.020	0.021 (0.015 – 0.023)
Marginal hooklet blade length	–	–	–	0.007 (0.005 – 0.009)
Copulatory organ total length	–	–	–	0.148 (0.133 – 0.163)
Copulatory organ wide part length	–	–	–	0.036 (0.028 – 0.043)
Copulatory organ wide part width	–	–	–	0.026 (0.023 – 0.030)
Copulatory organ tube diameter	–	0.008 – 0.010	0.006 – 0.010	0.007 (0.005 – 0.009)
Copulatory organ tube length	–	0.10	0.13 – 0.16	–
Accessory piece length	–	–	–	0.088 (0.063 – 0.100)
Accessory piece width	–	–	0.070	0.060 (0.055 – 0.065)
Vaginal tube length	–	–	0.040 – 0.050	0.050 (0.038 – 0.058)
Vaginal tube diameter	–	–	0.010	0.010 (0.008 – 0.011)
Comb-like growths if vaginal tube length	–	–	–	0.015 (0.015 – 0.015)
Comb-like growths if vaginal tube width	–	–	0.020	0.019 (0.018 – 0.020)
Pharynx length	–	0.14 – 0.16	–	0.138 (0.130 – 0.146)
Pharynx width	–	–	–	0.109 (0.104 – 0.114)
Ovary length	–	0.16 – 0.20	–	0.139 (0.125 – 0.156)
Ovary width	–	–	–	0.166 (0.125 – 0.208)
Testis length	–	0.16 – 0.18	–	0.140 (0.125 – 0.156)
Testis width	–	–	–	0.177 (0.166 – 0.187)

* – see Fig. 1 for the scheme of measurements and abbreviations

** d. – data provided in literature are doubtful (see Discussion part)

*** data not available

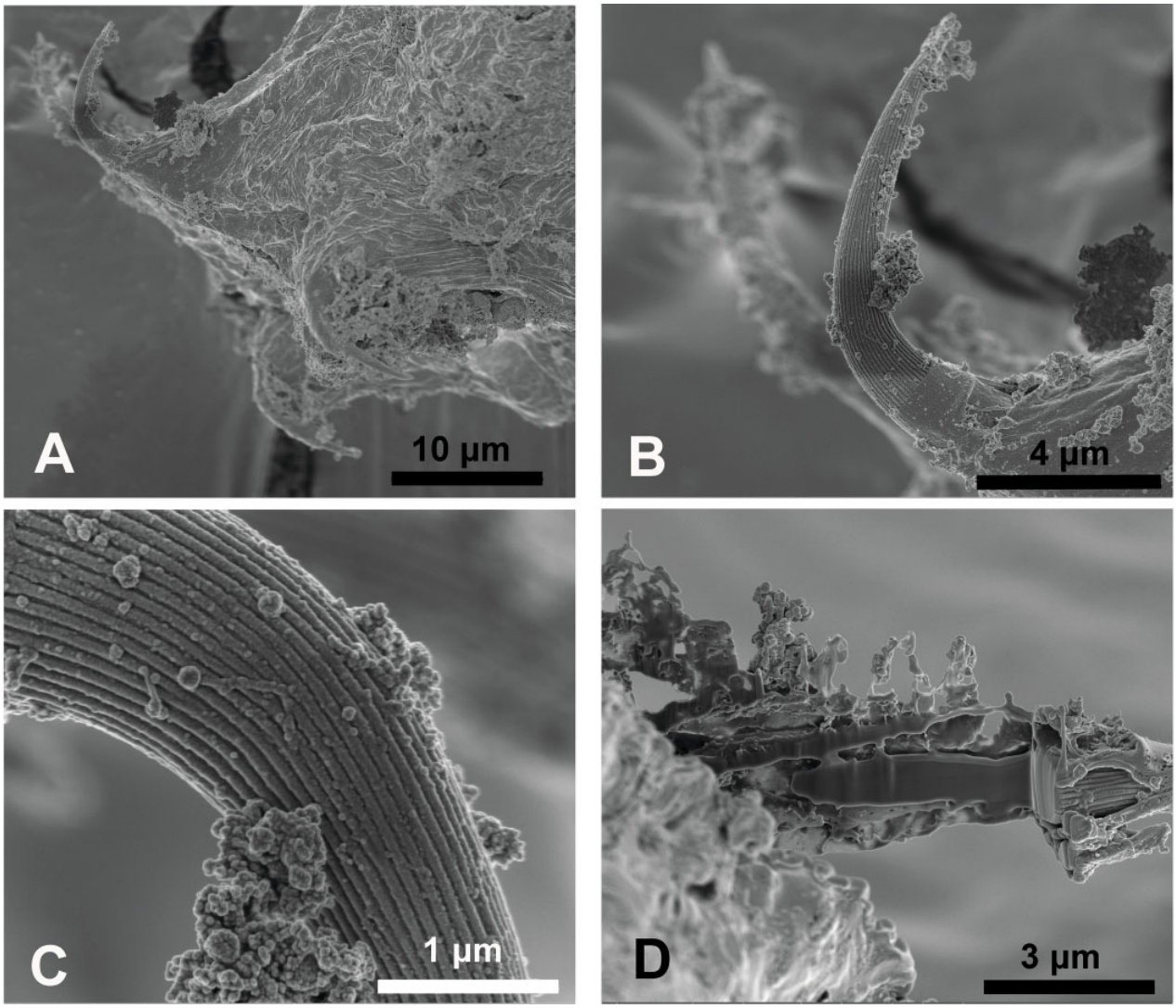


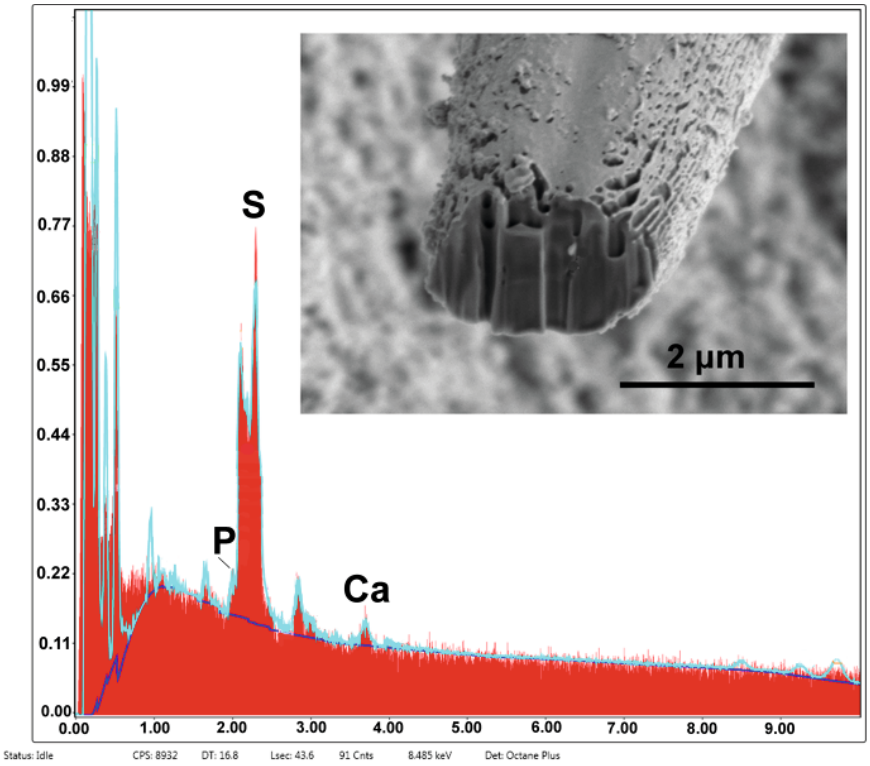
Fig. 4 SEM of a sub-adult of *Ancyrocephalus paradoxus*.
A – opisthohaptor of *A. paradoxus*; B – an anchor of *A. paradoxus.* Note the longitudinal stiffeners; C – an anchor of *A. paradoxus* (enlarged). Note the longitudinal stiffeners; D – frontal plane gallium cut of an anchor base of *A. paradoxus*

Results of scanning electron microscopy (SEM)

SEM reveals the following features in the anatomy of *A. paradoxus*. The forebody has a characteristic broad rhomboid-shaped prohaptor (Fig. 2A). A close SEM microphotograph of a prohaptor (Fig. 2) shows the outer structure of three pairs of cephalic gland openings, mouth groove and an elevation in the area of protrusion of the copulatory organ (Figs. 2B, 2C). The haptor of sub-adult (Fig. 4A) is compared to its juvenile shape with outstanding anchors and almost square end of a haptor, opposite to the haptor of an adult, with rectangular shape and relative sizes of anchors being twice as small as the haptor itself (Fig. 2D).

The cross-section of the point of the anchor also differs in two different age stages. In the adult worm, it has an oval shape; the sub-adult has a triangular shape with rounded edges or near circular (Figs. 4, 5). In spite of the opinion of Ergens (1966) that the ventral and dorsal anchors are similar, they are clearly different in their thickness; see the distal end view of the haptor (Fig. 2D) and compare ventral and dorsal ones. The anchors themselves differ by their surface structure. In adults and sub-adults, they possess longitudinal ribs (compare Fig. 3B, Fig. 4 B and C). In adults, the ribs are more numerous and not so pronounced.

The blade of anchors has a characteristic longitudinal depression (Fig. 3, A). A close SEM microphotograph of the surface of the anchor (Fig. 3B) demonstrates that its entire surface is covered with uniform longitudinal ribs. Figs. 3 C and 3 D show gallium cuts in two different parts of the anchor – a thick part that is closer to the base (Fig. 3 C) and a narrow part at the distal point of the anchor (Fig. 3D). The central part of the blade close to the base



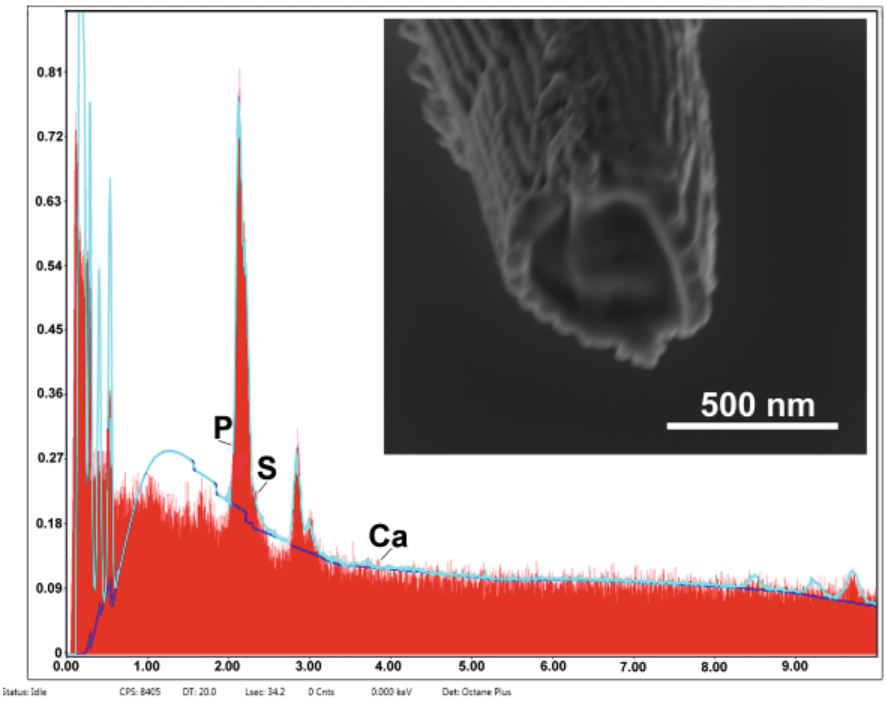
Element	Weight %	Atomic %	Error %	Net Int.	K Ratio
C K	24.92	38.67	8.89	228.25	0.121
N K	20.5	27.28	15.22	108.93	0.0637
O K	20.43	23.79	10	229.93	0.0661
Ga L	1.18	0.31	17.25	15.69	0.0084
Mg K	0.22	0.17	62.14	7.4	0.0015
P K	1.11	0.67	18.58	33.08	0.009
Au M	15.37	1.45	9.09	189.73	0.1134
S K	10.72	6.23	5.03	299.54	0.0855
Pd L	3.95	0.69	29.15	44.59	0.026
Ca K	1.6	0.74	32.15	25.05	0.0127

Fig. 5. An X-ray elemental analysis of the distal part of anchor of an adult of *A. paradoxus*.

has cavities and pores. Two bigger central cavities, apparently extend along the length of the anchor. A multitude of smaller pores of different sizes are located randomly, but mostly at the external curvature of the anchor's shaft. On the other hand, the distal part of the anchor (Fig. 3D) has a dense structure devoid of pores demonstrating the strength of the hook in this section. Scanning electron micrographs of the studied areas are shown on Figs 4 and 5 as well. The general shape of the sub-adult anchor's cut demonstrates its softness (Fig. 6), while in the mature specimen the structure is solid (Fig. 5). Visually and by chemical analysis, drastic chemical and morphological changes in the attachment structures are well demonstrated. The characteristic longitudinal depression of the blade of anchors (Fig. 3, A) resembles a trough on the blade of hunting knives, and, apparently, providing an additional elasticity and hardness to the anchor Anchors of both ages also have longitudinal ribs that apparently, give the anchor additional strength.

Results of the EDXA study

Other novel studies used in the present work is Energy Dispersive X-ray Analysis of different parts of the anchors, never performed before for anchors in Monogenea. An X-ray elemental analysis of the middle part and distal part of anchors of *A. paradoxus* in two different life stages are compared, see Table 2 for % weight of Mg, P, S and Ca. Common elements (C, H, O) that are present in all protoplasm and processing elements (Ga, Pd, Au) are omitted.



Element	Weight %	Atomic %	Error %	Net Int.	K Ratio
C K	23.29	36.13	9.12	224.2	0.1197
N K	21.26	28.28	14.69	117.38	0.0691
O K	22.53	26.23	9.8	254.92	0.0737
Ga L	1.17	0.31	15.59	15.4	0.0083
Mg K	0.21	0.16	65.16	6.93	0.0014
P K	0.91	0.55	22.34	26.89	0.0073
Au M	15.22	1.44	8.83	185.87	0.1119
S K	9.1	5.29	5.61	252.4	0.0726
Cl K	0.09	0.05	99.99	2.16	0.0007
Pd L	4.6	0.81	26.74	51.77	0.0304
Ca K	1.61	0.75	31.5	25.1	0.0129

Fig. 6. An X-ray elemental analysis of the distal part of anchor of a sub-adult of *A. paradoxus*.

The uncut anchor demonstrated dominating high level of sulfur. We provide a comparison of chemical elements of gallium cut anchors of a mature adult specimen of *A. paradoxus* and immature sub-adults for the first time in Monogenea. There is a clear tendency for increased phosphorus weight percentage in an immature (sub-adult) specimen compared to mature specimens where the sulfur is about 10 times higher. Calcium is the prevalent element in the distal part of anchor's blade of sub-adult and dominates in the distal part of anchor in sub-adult in comparison to the middle part, but in adult calcium is prevalent in the middle part in comparison with the distal part of the anchor (Table 2). Figures 4 and 5 show results of the spectrum analysis of the gallium cuts of the distal parts (tips) of anchors.

Discussion

Measurements of A. paradoxus

By providing detailed measurements of hard parts and soft inner organs of mature specimens of *A. paradoxus* (Table 1), we are filling a large gap in a very scant information in the redescriptions by Ergens (1966), Gusev, Kulemina (1971) and Gusev (1985).

Influence of the method of preservation and fixation on measurements

Parameters of soft body structures, especially body length and width in Monogenea can change depending on methods of preservation and fixation. As a rule, hard parts of monogeneans remain

Table 2. X-ray scans of Ga cuts of anchors of *Ancyrocephalus paradoxus*.

	Sub-adult		Adult		Adult
Element	Distal part of anchor's blade	Middle part of anchor's blade	Distal part of anchor's blade	Middle part of anchor's blade	Uncut anchor
Magnesium (Mg)	0*	0.25	0.22	0.2	0.09
Phosphorus (P)	0.21	1.81	1.11	1.62	0.21
Sulfur (S)	0.16	0.31	10.72	14.6	5.49
Calcium (Ca)	0.43	0.29	1.6	2.74	0.04

* – see spectrum figures (Figs. 4 and 5); numbers represent % wt

the same in different types of preservation and fixation. The worm keeps its "3D" body shape when preserved in alcohol for SEM or in acetic carmine for staining before mounting in Canada balsam (see SEM, Fig. 2A). When fixed in ammonia picrate or glycerin jelly (that allows better vision of sclerotized structures), the body and inner soft organs appear flattened which influences measurements. Describing the shape of haptor in the present study in non-pressed state, its width appears almost equal to the length, while in glycerin jelly whole mounts, it has clear rectangular shape with the width appearing noticeably larger than length, as it was drawn in Bykhovsky & Nagibina (1970). For taxonomic purposes, we consider glycerin jelly fixation as an optimal procedure for small monogeneans, without strong pressure, just under natural weight of the coverslip.

Ergens (1966) redescribes *A. paradoxus* from fishes in Central Europe (Danube, Elbe and Oder rivers) and provides drawings and limited measurements (see Table 1), that lacked details which we clarify in the present study. In his redescription, Ergens (1966) did not describe the inner soft anatomy of *A. paradoxus*, and focused only on the measurements and morphology of sclerotized parts of copulatory system and haptor. Ventral and dorsal pairs of anchors, named "first pair" and "second pair", did not provide an understanding of the position of each pair of anchors in the haptor in his study (Ergens, 1966). Measurements of anchors provided in his paper were not complete. Moreover, he used untraditional metric parameters. For instance, he provided measurement of the length of inner and outer roots that did not, however, correspond to the length of root itself, but the root and a base of the anchor (see Fig. 1 A in Ergens, 1966). In the present study we are providing this information, according to the scheme of measurements (see Fig 7 (5) in Gusev, 1985). This parameter is critical for species definitions in Monogenea, because some parts of the anchor keep growing during the lifespan of the worm (Gusev & Kulemina, 1971). The length of the blade named "point" in Ergens (1966) is practically a different anchor parameter, according to Gusev (1985) (see Fig. 1). In his redescription Ergens (1966) did not distinguish dorsal and ventral bars, calling them first pair and second pair. From his drawings, we assume that he considered the ventral set of anchors as the first pair and its bar (the ventral bar) and the second pair as the dorsal anchors pair and its bar (the dorsal bar).

Bykhovsky & Nagibina (1970) provided a redescription of *A. paradoxus* with some information on inner anatomy of the worm that included diameters of pharynx, ovary and testis (Table 1) together with few measurements of sclerotized parts (copulatory organ tube length and diameter). For both ventral and dorsal anchors, they provide a single measurement, a "ventroapical length". Information on the sizes of ventral and dorsal bars was given for the bar's width only (mistakenly called length).

Gusev (1985) in his "Keys to freshwater fish parasites of USSR" (1985) gave brief information on the main metric parameters of *A. paradoxus*, that included body width and length, a single metric parameter (dorsoapical length) for both ventral and dorsal anchors, considering them to have similar morphology, measurements of bars, hooklets and copulatory system. In spite of giving only one parameter for anchors, Gusev (1985) gave a comprehensive set of measurements of *A. paradoxus* – type anchors [see Fig. 7.5 in Gusev (1985)], that included proper measurements for inner and outer roots, blade dorsoapical and ventroapical lengths, that we are currently using as a base in the present study (Fig. 1).

Cephalic organs

We provide SEM photographs of cephalic organs' openings in *A. paradoxus*. Bakke *et al*. (2004) reported a high number of sensilla distributed ventrally around the oral pore and the region of the penis, that probably indicates that the sensilla serve to orient the gyrodactylid during feeding and copulation. Bakke *et al*. (2004) claimed these sensilla might have a different function to those sensilla distributed around the cephalic lobes, which must play a crucial role when transferring between hosts and moving over the host's epidermis (Bakke *et al*, 2004).

EDXA of different parts of anchors from two different age groups of A. paradoxus

The comparison between gallium cuts from medial and distal parts of the anchor show that at the middle part, this structure is more flexible, while it is the hardest and calcified terminally. The amount of calcium (Ca), phosphorus (P), and sulfur (S) (Fig. 4) is emphasized because they metabolize into hardened structures as found in mammalian teeth. Same tendencies were recently reported in the attachment structures of acanthocephalans (Heckmann *et al*.,

2012). The calcium and phosphorus form a rigid phosphate apatite similar to the enamel of mammalian teeth with disulfide bonds (cysteine) enhancing the strength of the structure. The enamel of mammalian teeth is over 95 % inorganic matter representing the hardest tissue in the body (Heckmann *et al*., 2012). The levels of structural minerals especially calcium and phosphorus at the central part of the anchor are too low to have any structural/attachment utility. These unique characters may be novel because they were simply not seen or reported by earlier researchers.

Conflict of Interest

Authors state no conflict of interest.

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New host and locality record of *Parapharyngodon japonicus* (Nematoda: Oxyuroidea) from the Egyptian changeable lizard *Agama mutabilis* (Agamidae): A light and scanning electron microscopy

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Summary

Parapharyngodon (Oxyurida) is a lizard gastrointestinal nematode parasite with a life cycle including lizards as main hosts. However, some species are known to parasitize anurans. In the present study, *P. japonicus* isolated from the large intestine of the Egyptian changeable lizard, *Agama mutabilis* was described and illustrated. Forty five specimens of these animals were collected from south Sinai desert, Egypt during the period from May to September 2017. After necropsy, the body was opened by a longitudinal incision from vent to throat, and the gastrointestinal tract was removed. The esophagus, stomach, small and large intestines were examined separately for helminthes. The recovered nematodes were examined by light and scanning electron microscopy. Thirty six specimens (80.0 %) were found to be naturally infected. The parasite was robust with prominent cuticular transverse annulations. Mouth surrounded by three bilobed lips, each with tiny labial papillae. Three pairs of caudal papillae were observed in male worms; 1 pair precloacal, 1 pair sublateral in cloacal opening line, 1 pair in proximal region of caudal appendage on its narrowed point. The posterior extremity beard dorsally directed caudal appendages. Females were with a conical posterior end terminated at a terminal spike. Ovaries reached esophageal isthmus but not wrapped around corpus. The parasite recorded was compared morphologically and morphometrically with the most similar species, it was found that it was most similar to *P. japonicus* with new host and locality records.

Keywords: *Parapharyngodon japonicas*; Nematoda; *Agama mutabilis*; Agamidae; light and scanning electron microscopic study

Introduction

The taxonomic status and validity of the genus *Parapharyngodon* have been questioned almost since its proposal by Chatterji (1933). Baylis (1936) considered it to be a synonym of the genus *Thelandros* Wedl (1861) (see Adamson, 1981; Adamson & Nasher, 1984; Bursey & Goldberg 1999, 2005). Later on, Freitas (1957) reinstated the genus *Parapharyngodon* which was accepted by several authors (Skrjabin *et al*., 1960; Baru & Coy-Otero, 1969; Baru, 1973; Sharpilo, 1976; Baker, 1987; Castazo-Fernandez *et al*., 1987; Ashour *et al*., 1994; Bursey & Goldberg, 2007a). Adamson (1981) re-established *Parapharyngodon* based on dietary habits of the host, morphology of the male genital cone and female tail and eggs. Males of *Parapharyngodon* spp. lack conical-shaped genital area and accessory piece. They have mammilliform papillae surrounding the more-or-less terminal anus and sub-terminal dorsally directed tail. Whereas males of *Thelandros* have a genital cone with pendulant papillae outside this cone, an accessory piece as well as terminal posteriorly directed tail is present in some species (Bursey *et al*., 2013; Pereira *et al*.,

* – corresponding author

2017). Females of *Parapharyngodon* spp. possess a conical tail terminated in a short stout spike, eggs with sub-terminal operculum in the early stages of cleavage when released (Bursey *et al*., 2004; Anjum *et al*., 2013). Females of *Thelandros* have various tail morphologies, eggs with a terminal operculum larvated when released (Bursey *et al*., 2013; Pereira *et al*., 2017). More than 40 well described species are assigned to the genus *Parapharyngodon* according to Bursey and Goldberg (2007a,b) and Gupta *et al*. (2009). The Egyptian changeable lizard, *Agama mutabilis* Merrem (1820) is widespread across northern Africa, occurring from Western Sahara, Mauritania and Morocco east to Egypt and Sudan. This is an active predator exhibits a diurnal behavior and insectivorous including beetles, caterpillars and ants in diets and in some instances it has been known to eat large migratory locusts as they pass through its habitat. The present study reports the finding of *P. japonicus* in a new host, *Agama mutabilis* and locality, Egypt, including detailed morphological and morphometric characterization of this species using light and scanning electron microscopy (SEM).

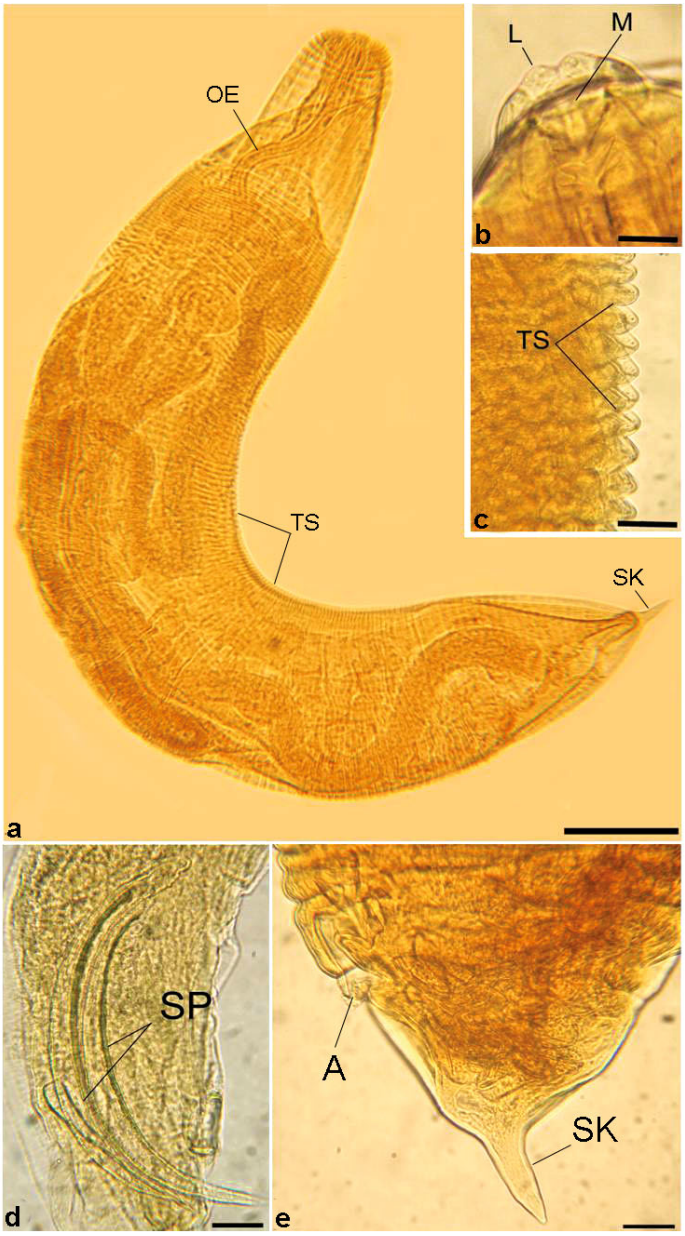

Fig. 1. **(A – E):** Photomicrographs of *P. japonicus* showing: **A** – Females, lateral view with terminal spike (SK), TS transverse cuticle striations. **B** – Details of the cephalic end, mouth opening (M) surrounded by three bi-lobed lips. **C** – Transverse cuticle striations (TS). **D** – Posterior end of male with two spicules (SP). **E** – Posterior end of female terminated at a caudal spike (SK). Scale bars: A = 300 µm; B, C = 10 µm; D = 100 µm; E = 50 µm.

Materials and Methods

Forty-five specimens of the mean snout-vents *Agama mutabilis* (length up to 9.4 cm) were collected by hand or noose from South Sinai desert, Egypt during the period from May to September 2017. Animals were subjected to euthanasia using 20 % benzocaine gel (Anbesol, Pfizer, Inc., New York). Each specimen was subsequently necropsied and all organs were examined for helminthes using a ZEISS Compact Greenough stereomicroscope (Model Stemi 305). All animal procedures were carried out according to the regulatory laws regarding experimental Animal Ethics Committee. Nematode worms were isolated from host intestines, heat fixed in 10 % neutral buffered formalin for 15 min and then preserved in 70 % ethanol in 5 % glycerol solution to avoid sudden drying. Finally, samples were transferred to lactophenol for clearance. The prepared samples were examined using differential interference contrast (DIC) light microscopy with digital image analysis system (analysis auto 5.0). Drawings were made with the aid of a camera lucida. Measurements were in micrometer unless otherwise stated. For SEM, samples were fixed in 4 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), washed in the same buffer, and dehydrated in a graded alcohol series. Samples were then processed in a critical point drier "Bomer–900" with freon 13, sputter–coated with gold–palladium in a Technics Hummer V, and finally examined with a Jeol scanning electron microscope (Model JSM7610F).

Ethical Approval and/or Informed Consent

All animal procedures were carried out according to the regulatory laws regarding experimental Animal Ethics and Use Committee.

Results

Thirty six out of 45 (80.0 %) specimens of the Egyptian changeable lizard, *A. mutabilis* were infected with nematode parasites isolated from their intestines. Worms were examined morphologically by light and SEM.

Parapharyngodon japonicus Houttuyn (1782)
Description based on 13 specimens (Figs. 1 – 3): Robust cylindrical nematodes with prominent cuticle annulations from beginning of the esophagus to the anal opening. Oral opening was triangular, surrounded by three bilobed lips. Each lobe beard tiny labial papillae. Buccal capsule absent. Sexual dimorphism evident, females larger and more robust than males. Lateral alae present in males, but absent in females. Males without caudal alae, caudal filament subterminal and directed dorsally. Females with conical tail terminated in a short stout spike.
Male: Small fusiform nematodes measured 1735 – 2986 (2280 ± 10) µm long, 385 – 490 (438 ± 11) µm wide at the level of the excretory pore. Lateral alae began at the level of esophageal isthmus. Total esophagus length 290 – 460 µm (388 ± 7). Bulb was

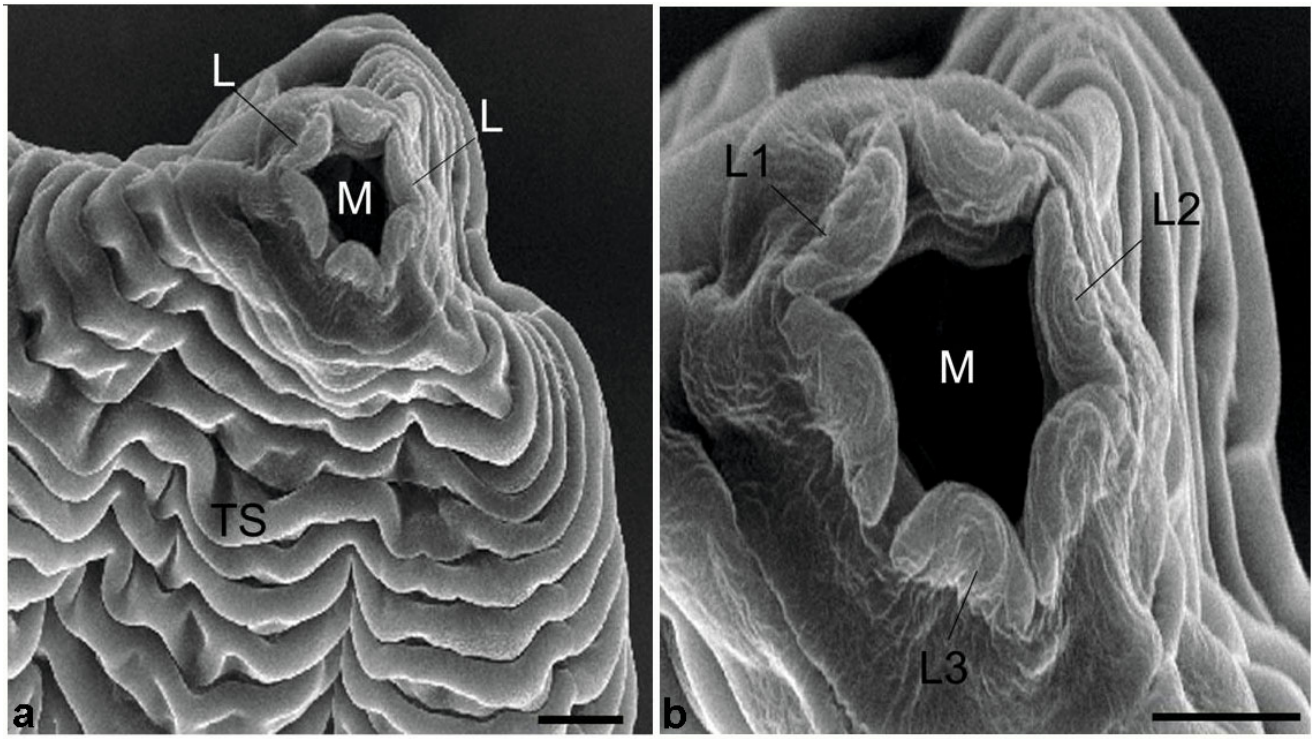

Fig. 2. **(A, B)**: Scanning electron micrographs showing apical views of the cephalic end, L symbolized for the three bilobed lips (L1, L2, L3) surrounding mouth (M); TS for transverse cuticle striations. Scale bar = 10 µm.

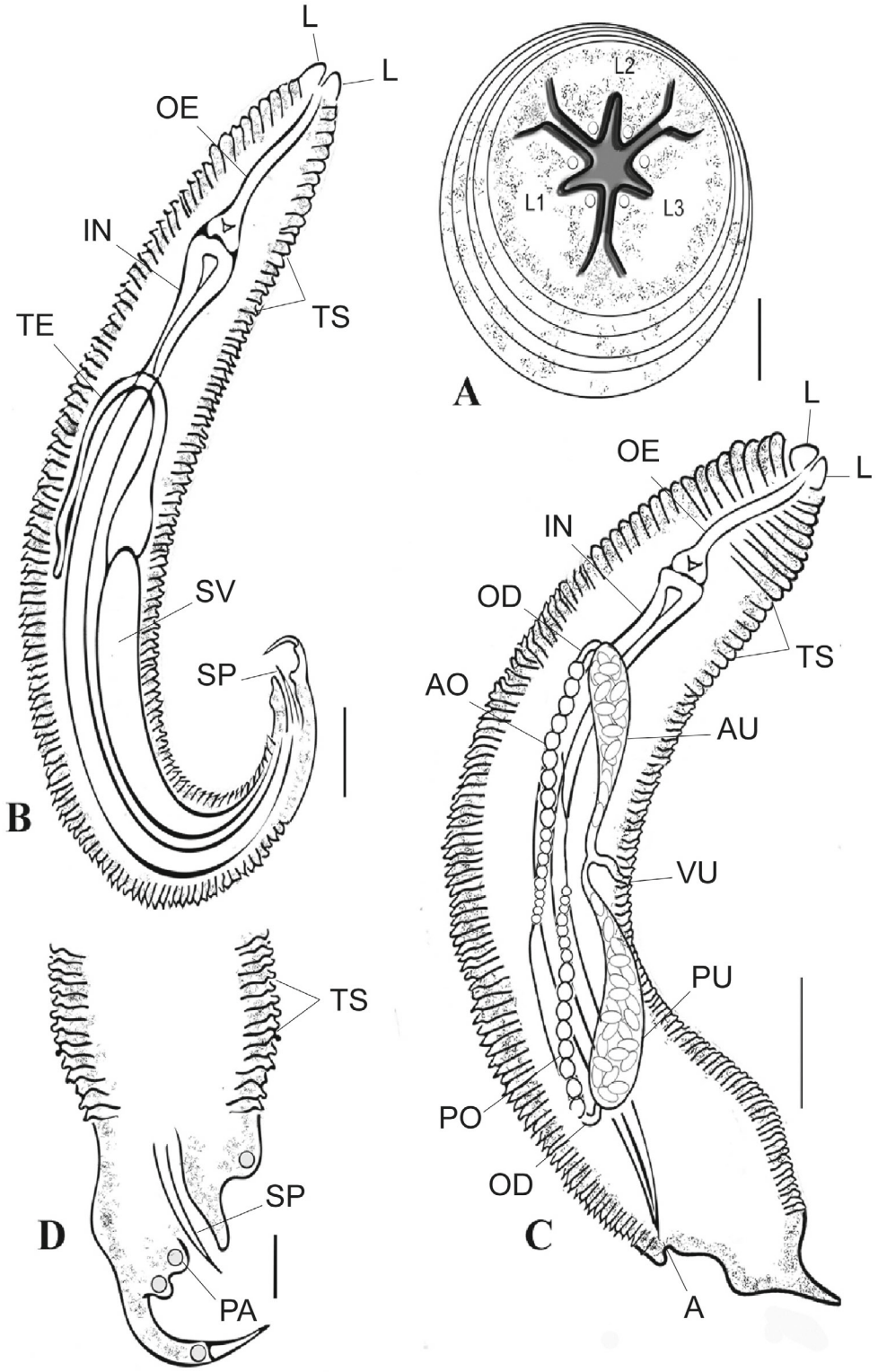

Fig. 3. Line drawings of *P. japonicus.* **A,** cephalic end of paratype female, apical view. **B,** Holotype male, lateral view. **C,** paratype female, lateral view. **D,** Posterior end of male, lateral view. L lips; OE oesophagus; IN intestine; TS transverse striations; TE testis; SV seminal vesicle; SP spicule; AO anterior ovary; PO posterior ovary; OD oviduct; AU anterior uterus; PU posterior uterus; A anus; PA caudal papillae. Scale bars: A = 10 µm; B = 200 µm; C = 300 µm; D = 100 µm.

Table 1. Comparative measurements between the present species and members of family Pharyngodonidae previously reported from lizards in Egypt, measurements in mm, otherwise stated.

Species	Host	locality	length	width	Spicule (mm)	Cloacal lip	Ovary	Egg size	Reference
T. alatus (Wedle, 1862)	*Agama stellio*	El Quseima, North Sinai	5.94	0.44	–	–	–	14X(72 – 84) µm	Edward A. Belle, 1954
T. kasauli (Chatterji, 1936)	*Agama stellio*	El Quseima, North Sinai	Female: 5.23 Male: 3	Female: 0.35 Male:0.31	100 µm	–	–	128X68 µm	Edward A. Belle, 1954
*T. bulbosus*v. (Linstow, 1899)	*Agama – Scincu*s	Berg el Arab (*Agama*), Wadi Faran, S.Sinai (*Scincus*)	Female 3.8 – 4.61 Male: 2.6	–	74 – 79 µm	–	|Prebulbar	97X57 µm	Edward A. Belle, 1954
T. micipsae (Seurat, 1917)	*Chalcides sepoides*	Zawiet, Abu Musa1lam, Giza	Female : 6.90 – 7.0 Male: 2.30	Female: 8.0 Male: 0.17	74 µm	–	Postbulbar	50X90 mm	Edward A. Belle, 1954
T. cameroni Edward A. Belle 1954	*Chaleides sepoides – Scincus*	Berg el Arab, W. Desert, Kom. Aushdm, Faiyam Province	Female:3.28 Male: 2.34	Female: 0.31 Male:0.20	72 µm	–	Postbulbar	79 – 95 X 52 – 54 µm	Edward A. Belle 1954
T. kuntz (Edward A. Belle 1954)	*Agama*	Wadi Faran, S. Sinai	Female:3.20 – 4.20 Male:2.23 – 2.65	Female:0.39 Male:0.20	50 µm	–	–	100X58 µm	Edward A. Belle, 1954
Thelandros sp.	*Chalcides ocellatus Chalcides sepsoides*	S.Sinai	Female: 2.65 – 3.85 Male:1.85 – 3.02	Female: 0.36 – 0.46 Male:0.17 – 0.25	–	–	Prebulbar	78 – 84 x 51 – 68 µm	Rabie *et al*, 2012
Pharyngodon hindlei (Thapar, 1925)	*Eumeces schneiderii*	Berg El Arab, W. Desert.	Female:4 – 5 Male:2.5 – 0.3	Female:0.4 – 0.47 Male:0.16	0.045 – 0.054	–	–	140X42 – 51	Edward A. Belle, 1954
Pharyngodon extenuates (Rudolphi, 1819)	*Acanthodactylus*	Baltim, Fouadiya Province,	Female:5 – 6.7	Female:0.36	–	–		144X33 – 36	Edward A. Belle, 1954
Pharyngodon inermicauda (Baylis, 1923)	*Tarentola mauritanica*	Abu Rawash	Femal:3.60 – 3.81 Male:1.46 – 1.84	Female:0.326 – 0.340 Male:0.095 – 0.163	absent	–	In the middle third	0.150 – 0.165 X 0.042 – 0.51	Moravec *et al* ., 1987
Pharyngodon mamillatus (Linstow, 1897)	*Chalcides ocellatus*	Abu Rawash	Female:3.26 – 3.63 Male:1.07 – 2.23	Female: 0.340 – 0.394 Male:0.109 – 0.204	0.033 – 0.045	–	In the middle third	0.135 – 0.144 X 0.036 – 0.042	Moravec *et al* ., 1987
P. bulbosus (Linstow, 1899)	*Chalcides ocellatus*	Abu Rawash	Female:2.86 – 4.28 Male:2.14 – 2.46	Female:0.340 – 0.449 Male:0.231 – 0.236	0.051 – 0.063	smooth	Postbulbar	90 – 99X54 – 57	Moravec *et al* ., 1987
P. micipsae (Seurat, 1917)	*Scincus scincus*	Abu Rawash	Female:4.46 – 0.6.77 Male:1.71	Female:0.503 – 0.830 Male:0.095mm	88 µm	echinate	prebulbar	91 X 50	Moravec *et al* ., 1987
P. Japonicus (Present study)	*Agama mutabilis*	*S. sinai*	Female: 2150 – 3690 µm Male:1735 – 2986 µm	Female: 386 – 630 µm Male:385 – 490 µm	381 – 590 µm	smooth	Postbulbar	76 – 120 µm	

96 – 161 (140 ± 6) µm long and 65 – 105 (86 ± 5) µm wide. Nerve ring and excretory pore were 60 – 118 (70 ± 9) µm and 40 – 76 (46 ± 6) µm from the anterior end respectively. Testis reflexed posteriorly behind esophagus and the vas deferens separated from testis by a narrow tube. Three pairs of caudal papillae; 1 pair precloacal, 1 sublateral pair in cloacal opening line, 1 pair in proximal region of caudal appendage on its narrowed point. Spicules were 381 – 590 (550 ± 11) µm long. Posterior extremity of the body beard dorsally directed caudal appendages, terminated at a thin tip, 18 – 23 µm (20 ± 5) long.

Female: Body length 2150 – 3690 (2450 ± 17) µm long x 386 – 630 µm (510 ± 12) wide at the level of vulva. Esophagus length was 282 – 460 (375 ± 8) µm long; Bulb length 89 – 170 (153 ± 7) µm; bulb width 85 – 120 (96± 10) µm. Nerve ring and the excretory pore were at 64 – 110 (81 ± 10) µm and 42 – 86 (56 ± 7) µm from the anterior end respectively. Uteri were Amphidelphic and divergent; the anterior uterus directed anteriorly while the posterior uterus was posteriorly directed and joined at the mid body. Ovaries reached esophageal isthmus but not wrapped around corpus. Eggs were oval, slightly flattened, thin-shelled and with subterminal operculum. Asymmetrical eggs extracted from ovijector in the early stages of cleavage. The posterior end of female was conical with terminal stout spike 76 – 120 (90 ± 13) µm long.

Taxonomic Summary

Species: *Parapharyngodon japonicus* Houttuyn (1782)

Host: *Agama mutabilis* (Family: Agamidae)

Infection Site: Small intestine.

Locality: South Sinai, Egypt.

Prevalence: 36 out of 45 (80.0 %) specimens were infected

Deposition: Permanent slides of paratype female and holotype male were deposited at the Parasitology Division, Zoology Department Museum, Faculty of Science, Cairo University, Egypt.

Discussion

The nematode fauna of Egyptian reptiles has received little attention in recent years. The only studies include those of Seurat (1917), Baylis (1923) and Moravec *et al*. (1987); they described several species of nematodes from African reptiles, mostly of the genera *Agama* and *Scincus* (Table 1). The recorded species in the present study was assigned to the genus *Parapharyngodon* according to the key published by Bursey and Goldberg (1999) where species of *Parapharyngodon* are distinguished on the basis of the pattern of caudal papillae, morphology of the anterior cloacal lip, the location of the ovary, and geographical distribution. Worldwide, there are currently 83 nominal species have been assigned to *Parapharyngodon*, with sufficient morphological features for both male and female nematodes (Bursey & Goldberg, 2015; Ramallo *et al*., 2016). The parasite recorded herein can be differentiated from *Thelandros* sp. based on egg development during posture and the posterior end morphology in both sexes (Bursey & Goldberg, 2005). *Parapharyngodon* sp. males do not have a conical-shaped genital area, or an accessory piece. They have mammilliform papillae and a dorsal subterminal tail. Males of *Thelandros* sp. have a conical-shaped genital area with papillae disposed outside this cone (Bursey & Goldberg, 1999; Bursey *et al*., 2013). Females of *Parapharyngodon* sp. generally have a cone shaped tail with a thick pointed end, like a spike, eggs with a subterminal operculum that are un-cleaved, or in early stages of cleavage when released. In contrast, *Thelandros* sp. females have diverse tail morphology, eggs with terminal operculum, larvae are fully developed when they are released (Ramallo *et al*., 2016; Bursey *et al*., 2013; Velarde-Aguilar *et al*., 2015). *Parapharyngodon japonicus* recovered from *Onychodactylus japonicus* by Bursey and Goldberg (1999) in Japan is most similar to the present nematode isolated. Where both share the presence of postbulbar ovarian coils, the tail of female terminated at a small spike, eggs are thin-walled and the anterior cloacal lip is smooth. They resemble *P. tyche* in the presence of smooth anterior cloacal lip, ovary is postbulbar, and the eggs are thin-walled and oval in outline, the female spike is small and uterus is thick-walled. They differ from each other in that the spicules in *P. japonicus* male is half the length of those in *P. tyche,* and the lateral alae of *P. japonicus* end abruptly about 80 µm anterior to the cloaca, whereas in *P. tyche,* the lateral alae continue to the end of the body. All of the nematode species of family Pharyngodonidae reported from lizards in Egypt were compared (Table 1); two of them are from the same genus, *P. bulbosus* (Linstow, 1899) by Moravec *et al*. (1987) from *Chalcides ocellatus* in Egypt and *P. micipsae*. *P. bulbosus* differ from the present species in that tails of females which is conical without distinct caudal spike and ovarian coils not reaching anterior level of esophagus; while *P. micipsae* differ by the presence of postbulbar ovaries in females and their anterior ends forming prominent coils around the base of esophagus while their males have 4 pairs of caudal papillae and echinate anterior cloacal lip. Moravec *et al*. (1987) isolated a nematode *P. aegyptiacus* which further has since been transferred to *Skrjabinodon inglis* by Moravec and Barus (1990).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Effect of coffee silver skin and brewers' spent grain in the control of root-knot nematodes

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Article info

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Summary

Plant parasitic nematodes (PPN) are important pests of numerous agricultural crops especially vegetables, able to cause remarkable yield losses correlated to soil nematode population densities at sowing or transplant. The concern on environmental risks, stemming from the use of chemical pesticides acting as nematicides, compels to their replacement with more sustainable pest control strategies. To verify the effect of aqueous extracts of the agro-industry waste coffee silverskin (CS) and brewers' spent grain (BSG) on the widespread root-knot nematode *Meloidogyne incognita*, and on the physiology of tomato plants, a pot experiment was carried out in a glasshouse at 25 ± 2 °C. The possible phytotoxicity of CS and BSG extracts was assessed on garden cress seeds. Tomato plants (landrace of Apulia Region) were transplanted in an artificial nematode infested soil with an initial population density of 3.17 eggs and juveniles/mL soil. CS and BSG were applied at rates of 50 and 100 % (1L/pot). Untreated and Fenamiphos EC 240 (nematicide) (0.01 µL a.i./mL soil) treated plants were used as controls. Reactive oxygen species (ROS) and chlorophyll content of tomato plants were estimated during the experiment. CS extract, at both doses, significantly reduced nematode population in comparison to the untreated control, although it was less effective than Fenamiphos. BSG extract did not reduce final nematode population compared to the control. Ten days after the first treatment, CS 100 %, BSG 50 % and BSG 100% elicited the highest ROS values, which considerably affected the growth of tomato plants in comparison to the untreated plants. The control of these pests is meeting with difficulties because of the current national and international regulations in force, which are limiting the use of synthetic nematicides. Therefore, CS extracts could assume economic relevance, as alternative products to be used in sustainable strategies for nematode management.

Keywords: *Meloidogyne incognita*; phytochemicals; sustainable nematode control; tomato; by-products valorization

Introduction

Plant parasitic nematodes (PPN) are important pests of numerous agricultural crops especially vegetables, able to cause remarkable yield losses correlated to soil nematode population densities at sowing or transplant (Sasanelli, 1994; Perry & Moens, 2011). They can also cause indirect damages by opening penetration ways to soil pathogens (*Fusarium* spp., *Verticillium* spp., *Pyreno-*

* – corresponding author

chaeta lycopersici etc.) and/or to viruses (Brown *et al*., 1988) because of the mechanical action of their stylet on the root surface (Ciccarese *et al*., 2008; Sasanelli *et al*., 2008).
In particular, the widespread root-knot nematodes (*Meloidogyne* spp.) are of remarkable importance due to their polyphagy. Some of these species are included in the quarantine pest list either of the European Union (EU, Directive 2000/29) and of the European and Mediterranean Plant Protection Organization (EPPO) (Wesemael *et al*., 2010). Concerns for the environmental risks, stemming from the use of chemical pesticides acting as nematicides, recently ended in restrictions provided by the European legislation (EU Reg. 396/2005, 1095/2007, 33/2008, 299/2008, 1107/2009, 459/2010 and 293/2013), that impel their replacement with more sustainable pest control strategies (Renčo, 2013; Abdel-Daym *et al*., 2014). The use of eco-friendly agro-industrial by-products in pest control is nowadays regarded with increasing interest (Abdel-Dayem *et al*., 2012; Luque & Clark, 2013). In particular, those with high polyphenols content seems to be particularly effective in controlling plant parasitic nematodes (Chitwood, 2002; Oka, 2010). From this point of view, coffee silverskin (CS) and brewer's spent grain (BSG) are among the most interesting readily available, high volume and low cost agro-industry by products with high polyphenols content. These by-products, rich in polyphenols content (Regazzoni *et al*., 2016; Santi Stefanello *et al*., 2018), are produced in large amount throughout the year (Mussatto & Teixeira, 2010; Lynch *et al*., 2016).
Coffee silverskin, the only by-product generated during the coffee roasting process (dos Santos Polidoro *et al*., 2017), is a thin tegument of the outer layer of coffee beans and represents about 4.2 % (w/w) of the entire seed weight (Janissen & Huynh, 2018). The average basic chemical composition of CS is 16 – 18 % of proteins, 2 % of lipids and 4 – 7 % of ash (Borrelli *et al*., 2004; Carneiro *et al*., 2009). This by-product is also rich in specific bioactive compounds such as chlorogenic acids (1 – 6 %), caffeine (0.8 – 1.3 %), and melanoidins (17 – 23 %) (Mesías *et al*., 2014; Behrouzian *et al*., 2016). CS is used as biofuel (Woldesenbet *et al*., 2016), fertilizer (Hachicha *et al*., 2012) and as mushroom cultivation substrate (Fan *et al*., 2003).
Brewer's spent grain is the by-product of the beer fermentation process and consists of the husk-pericarp-seed coat layers covering the barley grain. The husk contains considerable amounts of silica and polyphenolic components of the barley grain (Macleod, 1979). The chemical composition of BSG varies according to barley variety, harvest time, malting and mashing conditions (Huige, 1994; Santos *et al*., 2003). In general, BSG is considered as a lignocellulosic material rich in protein and fibers, containing 15 – 24 % of proteins, 10 % lipids and 2 – 4 % of ash (Kanauchi *et al*. 2001; Mussatto & Roberto, 2005) and remarkable quantity of bioactive phytochemicals, such as phenolic compounds (Connolly *et al*., 2015). Among its different uses, it is employed to increase the protein and dietary fibre content of food, in animal feeding (Öztürk *et al*., 2002) and in industrial processes (Tsaousi *et al*., 2011; Aggelopoulos *et al*., 2013).
This work was aimed at studying, in a tomato plant pot experiment, the effect of the aqueous extract of CS and BSG on the root-knot nematode *Meloidogyne incognita* (Kofoid and White) Chitw. and on the physiology of tomato plants.

Materials and Methods

The pot experiment was carried out at the Institute of Sustainable Plant Protection (IPSP) of the Italian National Research Council (CNR) in Bari (Italy) (40°16'22"N, 16°88'16"East Greenwhich) in a glasshouse, the temperature of which was set at 25 ± 2 °C.

Extracts preparation and characterization
CS and BSG were crushed and suspended in deionized water (1:10 w/vol) in a blender at 8,000 rpm for 5 min, shaken for 1 hour and filtered using a Whatman n.1 filter. The pH of extracts was measured using the pHmeter Basic 20 Crison and the electrical conductivity (EC) by a Sension+ EC7 (Hach) conductivity meter. Total nitrogen and total polyphenols were determined according to Bremner (1996) and Waterhouse's (2002) methods, respectively. UHPLC Dionex Ultimate 3000 RS system coupled by the HESI–II probe and TSQ Quantum Access Max triple quad mass spectrometer (Thermo Fischer Scientific) was used for the qualitative assessment of polyphenols in CS and BSG aqueous extracts. The separation of compounds was performed at 30 °C on Hipersyl Gold C18 column, 3 µm particle size, i.d. 2.1 mm, 100 mm length (Thermo Fischer Scientific). A binary mobile phase made of a) formic

Table 1. Physical and chemical main characteristics of coffee silverskin (CS) and brewer's spent grain (BSG) extracts.

Parameters	Unit	BSG	CS	LSD	
				0.05	0.01
pH	$[H^+]$	6.9* ± 0.1	5.6 ± 0.1	0.13	0.22
Electrical Conductivity	mS/cm	4.9 ± 0.3	4.6 ± 0.2	0.57	0.95
Total Nitrogen	g/L	1.2 ± 0.1	0.7 ± 0.2	0.35	0.57
Total Polyphenols	mg/L	353 ± 15	403 ± 22	44.0	73.0

*Each value is an average of three replications ± SE

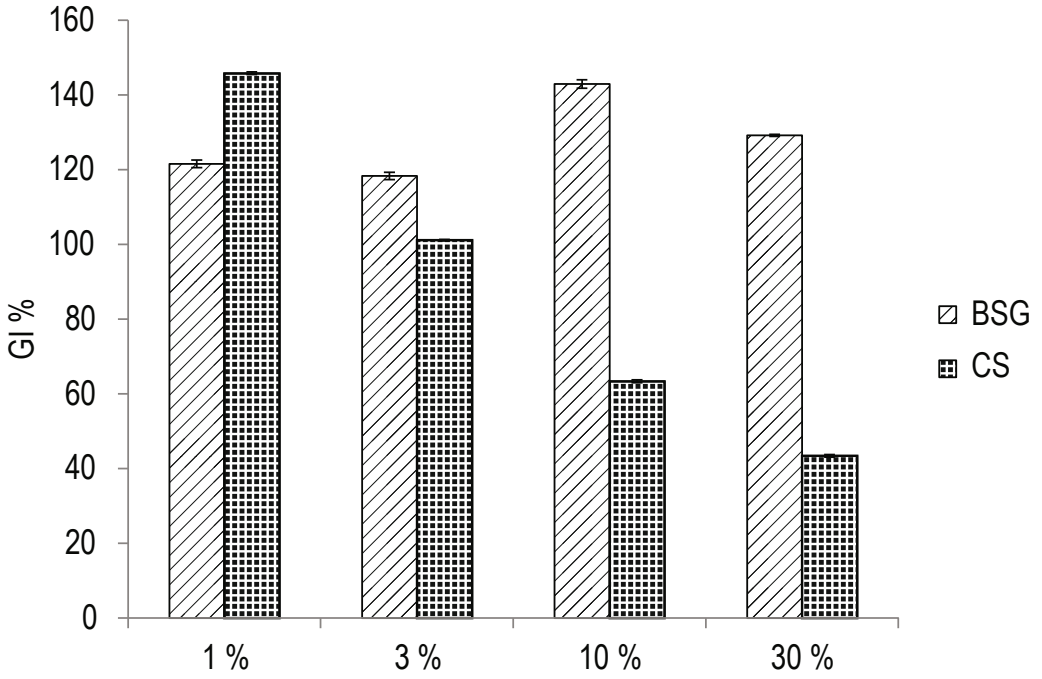

Fig. 1. Phytotoxicity test. Effect of different BSG and CS aqueous extract concentrations on germination index (GI) of garden cress seeds (*Lepidium sativum* L.).

acid aqueous solution at 0.1 % and b) formic acid in acetonitrile solution at 0.1 %, at a constant flow rate of 0.2 mL/min was used. The gradient program of solvent b was set to increase from 10 to 70 % in 20 min. The conditions of the MS system were the following: 320 °C for capillary temperature, 280 °C for source heater temperature, nebulizer gas N_2, collision gas Ar, sheath gas flow 35 psi, auxiliary gas flow 10 units, capillary voltage -2.8 kV, tube lens offset 78, 111 and 160 for Q1, Q2 and Q3, respectively. Calibration curves were performed using pure standard phenols solutions of chlorogenic and ferulic acid at concentration ranging from 2.5 mg/L to 20 mg/L. These calibrations, based on ion extracted chromatogram at m/z = $[M\text{-}H]^-$ from the total ion chromatogram, were used to obtain semi-quantitative data of the caffeoyl quinic and feruloyl quinic derivates compounds identified in the extracts.

Phytotoxicity tests

Phytotoxicity of the CS and BSG aqueous extracts was evaluated measuring the Germination Index (GI) of the garden cress seeds (*Lepidium sativum* L.) (Zucconi *et al*., 1981). *L. sativum* was exposed to the extracts diluted at 30 %, 10 %, 3 % and 1 %. GI was calculated according to the following formula:

$$GI = \frac{N_S \times E_S}{N_W \times E_W} \times 100$$

where N_s is the number of germinated seeds, E_s the root elongation measured in mm and N_w and E_w are the same parameters measured in the control treatment.

Preparation of infested soil and pot experiment

An Italian population of *Meloidogyne incognita* race 1 (Hartman & Sasser, 1985) was reared for two months on tomato [*Lycopersicum esculentum* Mill. (L.)] plants (cv. Marmande) in a glasshouse at 25 ± 2 °C. When large mature egg masses were formed, tomato plants were uprooted and their roots gently washed, to free them of adhering soil particles, and finely chopped. To estimate the numbers of eggs and second stage juveniles (J2s) in the chopped roots, ten 5-g root samples were suspended in a 1 % aqueous solution of sodium hypochlorite (NaClO) in 150 mL jars for 3 minutes, after which the eggs and J2s released in the suspension were counted (Hussey & Barker,1973). The roots were then thoroughly mixed with 4 kg of steam sterilized sandy soil (pH 7.9; sand = 85.7 %; silt = 7.1 %; clay = 7.2 % and organic matter = 0.6 %) and used as inoculum. Appropriate amounts of this inoculum were then thoroughly mixed with steam sterilized silty clay loam soil (USDA) in a concrete mixer to obtain a uniformly infested soil. Nematodes, eggs and J2s, were extracted from 8 soil samples to determine the initial population density corresponding to 3.17 eggs and J2s/mL soil (*Pi*). This infested soil in an amount of 6.5 L was then used to fill plastic pots (V = 7.5 L).

One month old seedling of tomato (landrace of Apulia Region) was transplanted into each pot. There were five replications for each treatment and pots were arranged on benches, in a glasshouse at 25 ± 2 °C, according to a randomized block design. During the experiment tomato plants were maintained randomizing the position of the blocks and at the same time repositioning each plant within a block every week, to avoid a block position effect

Table 2. Identification and quantification of compounds obtained by LC-MS/MS analysis of silver skin coffee extract (in brackets the relative abundance of each signal).

RT	$[M-H]^-$	MS^2	mg/L	Name *
1.44	191	85(100) 127(50)	54.78	QA
2.23	353	135(100) 191(80) 179(10)	3.43	3-CQA
3.22	353	191(100) 161(5) 173(6)	4.54	5-CQA
3.39	353	136(100) 191(60) 94(40) 173(30)	7.44	4-CQA
3.56	367	135(100) 193(10) 179(5) 118(5) 94(5)	2.26	3-FQA
4.01	367	367(100) 269(95) 287(40) 148(20) 349(15)	2.85	FQA1
4.67	367	367(100) 287(40) 243(40) 349(30)	2.02	FQA2
6.11	367	173(100) 134(80) 94(60) 193(15)	2.53	4-FQA
6.27	367	191(100) 135(40) 94(35) 193(15)	7.71	5-FQA
6.53	559	351(100)	0.36	3Si-4CQA
9.87	381	358(100) 363(74) 257(48) 273(35) 319(27) 363(25) 336(23)	4.25	3-DQA
12.34	397	397(100) 325(20) 219(10)	0.37	SiQA

*Q=quinic, F=Feruloyl, C=Caffeoyl, Si=Sinapoyl, D=Dimethoxycinnamoyl, A=Acid

and at the same time the factor position of the plant within the block. The experiment was performed twice. Plants received all the necessary maintenance (irrigation, fertilization, etc.). Plants were irrigated when it was necessary before their wilting. Hoagland solution (1 L/pot) was used for fertilization (2 times during the experiment) to avoid macro and micro elements deficiency (Hoagland & Arnon, 1950).

The pots were treated with CS and BSG aqueous extracts, obtained as described in the paragraph "Extracts preparation and characterization", at concentrations of 50 and 100 %. Untreated and Fenamiphos EC 240 (0.01 µL a.i./mL soil) treated pots were used as controls. Each pot received 1 L of extract, or nematicide suspension. CS and BSG treatments were applied twice: at plant transplant and 20 days later.

At the end of the experiment (2 months) plants were uprooted and height, fresh and dry top and root weights were recorded. Root gall index (RGI) was estimated according to a 0 – 10 scale, where 0 = no galls; 1 – 4 = galling of secondary roots only, 5 – 10 = galling of primary laterals and tap root, with 5 equal to 50 % of roots galled and 10 the maximum nematode infestation possible (Bridge & Page, 1980).

Final soil nematode population density was determined in each pot processing 500 mL soil by the Coolen's method (Coolen, 1979). *M. incognita* density in roots was assessed by cutting up each root

Table 3. Identification and quantification of compounds obtained by LC-MS/MS analysis of brewer's spent grain extract (in brackets the relative abundance of each signal).

RT	**$[M-H]^-$**	**MS^2**	**Structural hypothesis**
7.58	394	289(100) 333(88) 394(88) 305(82) 351(78) 271(36) 297(26)	Ca (289)
7.69	329	82(100) 247(99) 96(43) 163(37) 125(36) 148(33) 81(30) 173(28)	Co(163) Q frg(173) Dq(329)
8.2	265	123(100) 86(59) 175(45) 153(19) 168(16) 114(12) 106(11)	P(153)
8.68	357	163(100) 233(50) 151(10)	Co(163)
8.99	331	249(100) 153(32) 207(15) 234(13) 150(11)	P(153)
9.01	271	146(100) 148(59) 136(46) 176(20) 120(17) 163(11) 191(11)	Co(163) Q(191)
9.36	373	212(100) 283(53) 248(46) 191(43) 209(39) 194(35)	F(194) Q(191)
10.73	375	312(100) 191(81) 246(52) 187(48) 176(35) 219(24)	Q(191)
12.27	538	180(100) 414(59) 283(48) 206(28) 383(16) 184(11)	C(180)
12.94	480	173(100) 262(73) 306(32) 231(22) 480(20) 188(16) 204(15)	Q frg(173)
13.54	331	157(100) 314(74) 144(39) 153(23) 155(19) 138(18) 171(15)	P (153)
13.95	329	211(100) 222(44) 173(38) 212(38) 203(37) 163(16)	Co(163) Q(173) Dq(329)
15.58	317	153(100) 233(30) 112(28) 163(23) 133(19) 215(17)	Co(163) P(153)
17.19	541	230(100) 117(20) 194(15) 212(10) 153(5)	F (194) P(153)

Ca=catechin; Co=coumaric; Q=quinic; Dq=dimethylquercetin; P=protocatechuic; F=ferulic; C=caffeic; Frg=fragment.

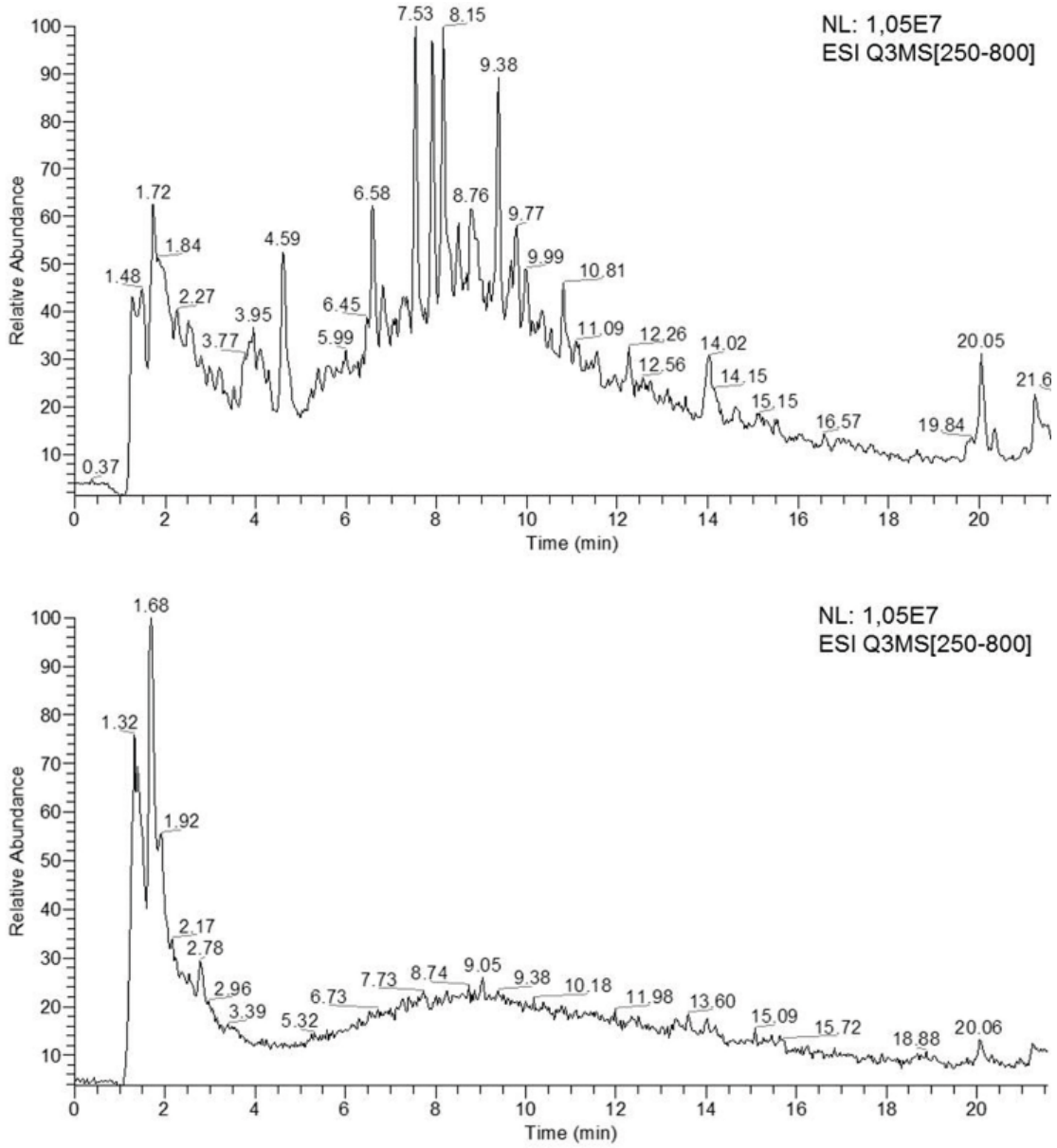

Fig. 2. Total ions chromatograms of silverskin coffee extract (top) and brewer's spent grain (bottom) obtained by LC-MS analysis.

system into small pieces and further comminuting them in a blender, containing 1 % aqueous solution of sodium hypochlorite for three periods of 20 sec (Marull & Pinochet, 1991). The water suspension was sieved on a 250 µm pore sieve over a 22 µm pore sieve. Nematodes and root debris gathered on the 22 µm pore sieve were separated by centrifugation (Beckman, Mod. Allegra X-12) at 2,000 rpm for five min in a magnesium sulfate solution of 1.16 specific gravity. Then eggs and juveniles in the water suspension were sieved again through the 22 µm pore sieve, sprayed with tap water to wash away the magnesium sulfate solution and collected in about 40-60 mL water. Eggs and juveniles in the water suspension were counted and final nematode population density (*Pf*) in each pot was determined by summing nematodes recovered from soil and roots. The nematode reproduction factor *r* was expressed as ratio between final and initial population density (*Pf*/*Pi*) of *M. incognita*.

Effects of CS and BSG treatments on Reactive Oxygen Species (ROS) and Chlorophyll levels

To test whether CS and BSG treatments at different concentration doses triggered an oxidative burst, the accumulation of ROS was quantified in tomato roots. ROS contents were determined following the method described in Melillo *et al*. (2006). Root portions were excised and pre-incubated for 30 min in potassium

Table 4. Effects of two different concentrations of aqueous solutions of coffee silverskin (CS) and brewer's spent grain (BSG) on the growth of tomato plants (landrace of Apulia Region) infested by the root-knot nematode *Meloidogyne incognita*.

Treatment	Dose (%)	Top weight (g) fresh			Top weight (g) dry			Height (cm)			Root weight (g)		
CS	50	76.1* ± 6.7	bc**	ABC	9.3 ± 0.8	bc	ABC	88.2 ± 4.4	ab	A	8.7 ± 0.7	b	AB
CS	100	80.7 ± 10.9	bcd	ABC	9.6 ± 1.2	bc	BC	91.4 ± 7	b	A	12.2 ± 0.7	c	BC
BSG	50	101.5 ± 6.9	d	C	12.4 ± 0.9	d	C	85.6 ± 2.6	ab	A	18.6 ± 1.5	d	D
BSG	100	94.4 ± 3.5	cd	BC	11.1 ± 0.6	cd	C	86.8 ± 5.6	ab	A	13.3 ± 1.4	c	C
Fenamiphos 240 EC (liquid formulation)	0.01 µL a.i./cm^3 soil	64.3 ± 11.1	ab	AB	7.2 ± 1.4	ab	AB	94.2 ± 7.5	b	A	4.7 ± 0.5	a	A
Untreated control	--	52.5 ± 4.5	a	A	5.8 ± 0.5	a	A	73.8 ± 5.9	a	A	12.3 ± 0.9	c	BC

* Each value is an average of 5 replications ± SE; **Data followed by the same letters in each column are not statistically different according to Least Significant Difference's Test (small letters for P= 0.05; capital letters for P= 0.01).

Table 5. Effects of different concentrations of aqueous solutions of coffee silverskin (CS) and brewer's spent grain (BSG) on the root-knot nematode *Meloidogyne incognita* infecting tomato plants (landrace Apulia Region).

Treatment	Dose (%)	Root gall index (0-10)			Eggs and juveniles/g root (x 1,000)			Eggs and juveniles/mL soil			Final population/mL soil (from roots and soil)			Reproduction rate *r = Pf/Pi*		
CS	50	3.8* ± 0.7	b**	B	19.8 ± 1.3	ab	A	14.8 ± 1.6	b	BC	42.9 ± 2.8	b	AB	13.5 ± 0.9	b	AB
CS	100	4 ± 0.3	bc	B	22.4 ± 1.5	b	AB	4.8 ± 0.7	a	A	52.9 ± 9.4	b	BC	16.7 ± 1.1	b	BC
BSG	50	4.6 ± 0.5	bc	B	35.2 ± 2.1	cd	CD	5.4 ± 1.3	a	A	107 ± 10.9	d	D	33.7 ± 3.4	d	D
BSG	100	4.2 ± 0.6	bc	B	29.3 ± 1.7	c	BC	16.8 ± 3.3	b	C	69.1 ± 11.4	bc	BC	21.8 ± 3.6	bc	BC
Fenamiphos 240 EC (liquid formulation)	0.01 µL/ cm^3 soil	1.4 ± 0.5	a	A	13.8 ± 2.9	a	A	5.8 ± 0.9	a	A	13.1 ± 1.2	a	A	4.1 ± 0.4	a	A
Untreated control	--	5.4 ± 0.2	c	B	40.3 ± 3.5	d	D	8.2 ± 1.8	a	AB	82.2 ± 13.6	cd	CD	25.9 ± 4.3	cd	CD

* Each value is an average of 5 replications ± SE; **Data followed by the same letters in each column are not statistically different according to Least Significant Difference's Test (small letters for P= 0.05; capital letters for P= 0.01).

phosphate buffer (20 mM, pH 6). Root tissues were homogenized (in a ratio of 1 mL/50 mg of tissue) inside a working solution containing 50 µM 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (Sigma, St Louis, MO, USA) dissolved in a potassium phosphate buffer 20 mM pH 6 with 0.2 g/mL of porcine liver esterase (Sigma) and then incubated for 30 min at 25 °C on a shaker. Fluorescence (E_x 488 nm, E_m 525 nm) caused by the oxidation of DCFH to DCF was measured by a fluorometer (GloMax-Multi Jr, Promega, Madison, WI, USA). For statistical purposes, fluorometry experiments were performed on six samples.

To verify the effect of CS and BSG treatments on chlorophyll contents, the following methods were used: a) indirect measures of chlorophyll content were recorded with a quick method using the SPAD-502 chlorophyll meter (Konica Minolta, Japan). For each plant ten measurements were recorded between the base and the apex of each leaf lamina and their average calculated as single SPAD value; b) 3 leaves of each treated or untreated plant were sampled and three disks, for each of them, were collected and immediately placed in vials containing 10 mL Dimethyl sulfoxide (DMSO). Chlorophyll extraction was obtained following the Tait and Hik's method (2003). Total chlorophyll content and its concentration were determined by UV-Vis spectrophotometer (Mod. Lambda 25 - Perkin Elmer). Contents of total chlorophyll and chlorophyll *a* and *b*, were assessed by using the equations described by Barnes *et al*. (1992). ROS contents and chlorophyll contents were determined 5 and 10 days after treatments and 25 days later after a second CS and BSG treatments.

Statistical analysis

Data from pot experiment, chlorophyll and SPAD assessments were statistically analyzed by analysis of variance (ANOVA). The Least Significant Difference's Test (LSD's Test) was used for post-hoc analysis of physical and chemical main characteristics of CS and BSG extracts. Student's *t*-tests ($P \leq 0.05$ and $P \leq 0.01$) was used for experimental design of ROS analysis in which we wished to make pairwise comparisons between treatments and their respective controls. Statistical analysis was performed using the Plot IT program Ver. 3.2 (Scientific Programming Enterprises, Haslett, MI, USA).

Results and Discussion

Extracts physical and chemical characteristics

The BSG extract showed a pH value close to neutrality (6.9) whereas a sub acid pH (5.6) was recorded for the CS extract (Table 1). No significant difference was observed for electrical conductivity. Total nitrogen content was significantly higher in BSG than in CS (about 70 %). Total polyphenols were significantly lower in BSG extract in comparison to CS extract (about 15 %) ($P < 0.05$).

Both CS and BSG extracts exerted a bio stimulating effect at concentration of 1 and 3 %, whereas at concentration of 10 and 30 % BSG increased its bio stimulating effect and CS approached the GI toxicity threshold of 40 % (Zucconi *et al*., 1981), remaining, however, in the non-toxic range (Fig. 1). It is interesting to note that BSG extract, at a concentration of 10 %, showed a stimulating activity close to 140 % of the control.

As showed in Figure 2 (top), LC-MS/MS analysis of polyphenols of CS extracts allowed to detect 77 signals. Only 12 of them were identified as quinic derivate considering their MS^2 spectra as compared to those reported by Clifford *et al*. (2003; 2006) and Jaiswal *et al*. (2010). The 3 isomers of caffeoylquinic acid, 3-CQA, 4-CQA and 5-CQA, were identified using their $[M-H]^-$ at *m/z* 353 and the diagnostic signals with relative abundances at *m/z* 179, 191 and 173, respectively. Similarly, the feruloyl quinc isomers, 3-FQA, 4-FQA and 5-FQA, were identified using *m/z* 367 $[M-H]^-$ and the diagnostic signals at *m/z* 193, 173 and 191, respectively. The quinic acid (QA), feruloyl quinic acid-1 (FQA1), feruloyl quinic acid-2

Table 6. Effect of coffee silverskin (CS) and brewer's spent grain (BSG) extracts soil treatments, at two concentrations (50 and 100%), on plant growth and reactive oxygen species (ROS) accumulation in tomato roots (landrace Apulia Region).

Treatment	Dose (%)	5 days after 1st treatment		10 days after 1st treatment		5 days after 2nd treatment	
		ROS content	Plant weight (g)	ROS content	Plant weight (g)	ROS content	Plant weight (g)
BSG	**50**	3,482 ± 125	4.96 ± 1.38	3,738 ± 69**	6.45 ± 0.86*	3,682 ± 77	30.83 ± 2.63
BSG	**100**	3,725 ± 71	3.37 ± 0.79	3,840 ± 50**	5.36 ± 1.05*	2,999 ± 82	28.97 ± 1.31
CS	**50**	3,444 ± 127	4.57 ± 0.82	3,325 ± 129	7.91 ± 1.44	3,392 ± 157	26.89 ± 2.04
CS	**100**	3,777 ± 165	4.11 ± 1.08	3,764 ± 130**	6.42 ± 1.18*	4,681 ± 139**	29.93 ± 0.49
Control		3,372[1] ± 182	4.91 ± 0.94	3,131 ± 148	11.00 ± 0.93	3,360 ± 203	27.13 ± 3.06

[1]Each value is an average of fluorescence units (FSU 50 mg^{-1} root fresh weight) of two experiments each containing six replications ± SE; Asterisks indicate statistically significant difference in comparison to the untreated control according to Student's *t*-test (* for P≤0.05, ** for P≤0.01).

(FQA2), 3-sinapoyl-4-caffeoyl quinic acid (3Si-4CQA), 3-dimethoxycinnamoyl quinic acid (3-DQA) and sinapoyl quinic acid (SiQA) were identified by partial matching with the expected monoisotopic mass and the diagnostic signals and reported in Table 2.

In Figure 2 (bottom), 35 signals detected in LC-MS/MS analysis of BSG extract were reported. Unfortunately, no one of them matched with those described by Quifer-Rada *et al*. (2015) and Munekata *et al*. (2016) for beer polyphenols and residues. Therefore, Table 3 reports a structural hypothesis about the possible nature of the molecules found in BSG.

Pot experiment

In Table 4 the effects of the CS and BSG extracts on the growth of tomato plants infested by *M. incognita* are reported. The nematode caused a significant reduction in fresh and dry top weight of tomato plants in comparison to CS and BSG treatments. Fresh

Table 7. Effect of different concentrations of coffee silverskin (CS) and brewer's spent grain (BSG) aqueous extracts on chlorophyll content (Chl) of leaves of treated or untreated (control) tomato plants (landrace of Apulia Region) at 5, 10 and 25 days after treatments.

15/11/2017 (5 days – after the first treatment)							
Treatment	**Dose (%)**	**Chl *a* (µg/cm²)**		**Chl *b* (µg/cm²)**		**Chl tot. (µg/cm²)**	
CS	**50**	27.6* ± 1.7	a**	7 ± 1.6	a	34.6 ± 3.2	a
CS	**100**	26.7 ± 4	a	7.3 ± 1.1	a	34.1 ± 5.1	a
BSG	**50**	28.9 ± 2.7	a	8.1 ± 0.7	a	37 ± 3.4	a
BSG	**100**	26.7 ± 2.9	a	7.7 ± 1.2	a	34.3 ± 4	a
Control		28.1± 1.3	a	7.9 ± 0.4	a	36.1 ± 1.7	a
20/11/2017 (10 days - after the first treatment)							
Treatment	**Dose (%)**	**Chl *a* (µg/cm²)**		**Chl *b* (µg/cm²)**		**Chl tot. (µg/cm²)**	
CS	**50**	29.4 ± 2	a	7.4 ± 0.3	a	36.7 ± 2.1	a
CS	**100**	31.4 ± 0.6	a	8.3 ± 0.4	a	39.7 ± 0.9	a
BSG	**50**	28.8 ± 1.9	a	7.8 ± 0.6	a	36.5 ± 1.7	a
BSG	**100**	29.3 ± 2.1	a	7.8 ± 0.7	a	37.2 ± 2.5	a
Control		32 ± 0.7	a	8.4 ± 0.4	a	40.5 ± 1.1	a
05/12/2017 (5 days - after the second treatment)							
Treatment	**Dose (%)**	**Chl *a* (µg/cm²)**		**Chl *b* (µg/cm²)**		**Chl tot. (µg/cm²)**	
CS	**50**	33.6 ± 1.7	a	11.4 ± 2.7	a	45.1 ± 4.4	a
CS	**100**	29.7 ± 0.7	b	7.9 ± 0.1	a	37.7 ± 0.7	a
BSG	**50**	34.3 ± 1.9	a	8.9 ± 0.7	a	43.2 ± 2.6	a
BSG	**100**	33.3 ± 0.2	a	9.1 ± 0.2	a	42.4 ± 0.4	a
Control		33.3 ± 2.2	a	9.5 ± 1.2	a	42.8 ± 3.4	a

* Each value is an average of 3 replications ±SE;** Data followed in each column by the same letter are not significantly different according to Least Significant Difference's Test (LSD's Test) (P≤0.05).

and dry top weights ranged between 52.5 and 101.5 g and 5.8 and 12.4 g, respectively. No statistical difference was observed between the 2 controls (Fenamiphos treated and untreated). All CS and BSG treatments did not differ from each other for plant fresh and dry top weights (P=0.01). These morphological parameters were not different in CS treated plants and in Fenamiphos treated plants (P=0.01) either. On the contrary, a significant difference was observed between the 2 BSG treatments and the Fenamiphos one (P=0.05). Both CS and BSG treatments had a stimulating effect on tomato growth compared to the untreated plants. Plant heights across treatments ranged between 85.6 cm and 94.2 cm, being not significantly different from those of control pots (73.8) at P=0.01.

Root weights in plants treated with BSG at a concentration of 50 % (18.6 g/pot) and Fenamiphos (4.7 g/pot) were significantly higher and lower, respectively, than those in the untreated control (12.3 g/pot) (Table 4). The higher root weight of tomato plants in comparison to Fenamiphos treated pots was due to the presence of numerous galls increasing root weight as already reported by D'Addabbo and Sasanelli (2005). All the other treatments did not influence root weight.

The nematological analysis pointed out that both CS and BSG treatments, independently from the dose, did not reduce root gall index (RGI), if compared to the untreated control (Table 5) (P=0.01). On the contrary, Fenamiphos was able to reduce the RGI to 1.4, a value significantly lower than those of all other treatments, including the untreated control, that spanned from 3.8 to 5.4.

Nevertheless, CS treatments were effective to reduce eggs and juveniles/g root by 50.9 and 44.4 % respectively, compared to the untreated control (Table 5) (P=0.01), irrespective of the concentration used. In addition, the observed nematicidal effect was no significantly different from that observed in Fenamiphos treated pots (P=0.01). CS treatments were more effective than BSG treatments in reducing nematode population on the roots (P = 0.05).

Soil nematode population density was lowered by CS 100 % (4.8 eggs and juveniles/mL soil) and BSG 50 % (5.4 eggs and juveniles/mL soil) treatments and was no statistically different from Fenamiphos (5.8 eggs and juveniles/mL soil) and the untreated control (8.2 eggs and juveniles/mL soil).

The final nematode population density, calculated summing nematodes from roots and soil, was reduced in CS 50 % and 100 % treatments by 47.8 and 35.6 %, respectively if compared to the untreated control. Interestingly, this parameter, in the CS 50 % treatment, was not significantly different (P = 0.05) from that recorded in Fenamiphos treated pots. By contrast, BSG treatments, at both concentrations, were not effective in reducing the final nematode population in comparison to the control.

The same results were obtained for the nematode reproduction factor (*Pf*/*Pi*). The lowest and the highest reproduction factors were recorded in Fenamiphos and BSG 50 % treatments, respectively (Table 5).

It is well known that substances with a high polyphenols content display a substantial nematicidal effect, the intensity of which is also related to the species of nematode concerned. Considering that CS and BSG extracts have both a high content of polyphenols, their different performances in assuring nematode control can be explained with their different polyphenols composition. This hypothesis is in line with the results of D'Addabbo *et al*. (2013), who studied the nematicidal activity of pure chlorogenic and caffeic acids and the extract of *Artemisia annua* on the nematodes *M. incognita*, *Globodera rostochiensis* (Woll.) Behrens and *Xiphinema index* Thorne *et* Allen. They found a high effect of these compounds on *G. rostochiensis*, a partial response on *X. index* and a low activity on *M. incognita*. Chlorogenic acid was able to elicit 100 % of juvenile mortality in *X. index* at a concentration of 125 µg/mL after 8 h of exposure, whereas caffeic acid produced the same result after 4 hrs of exposure, at the same concentration. The authors found a very little effect of these compounds against *M. incognita*, with the maximum observed effect after more than 24 h of exposure at the maximum concentration (500 µg/mL). The plant extracts were not able to produce the same nematicidal activity observed for pure chlorogenic and caffeic acids. However, *A. annua* extract was able to reduce significantly (50 %) the hatching percentage of eggs of *M. incognita* and *G. rostochiensis* in comparison to the control (distilled water). Considering these results, it is plausible that caffeoyl and feruloyl quinic derivates, identified in the CS extract, could be responsible for the observed nematicidal effect on the *M. incognita* population in our pot experiment.

Effects of CS and BSG treatments on ROS and Chlorophyll levels

In plants ROS are implicated as key signaling molecules in the regulation of numerous biological processes such as growth, development and responses to biotic and/or abiotic stimuli (Baxter *et al.*, 2014). ROS production was used as a phytotoxicity index of the tomato roots exposed to both doses of CS and BSG extracts. No ROS accumulation was detected in roots 5 days after treatments at the two applied doses (50 and 100 %) (Table 6) in comparison to the untreated plants. A significant increase (P=0.01) in ROS content was detected 10 days after BSG treatments in roots at both concentrations. In roots treated with 100 % of CS a significant ROS increase was recorded (Table 6). Twenty days after the first treatment, plants were newly treated with both extracts and ROS amount was evaluated 5 days later. A significant ROS accumulation was evident only in roots treated with 100 % CS compared to untreated and BSG-treated plants.

The treatments CS 100 %, BSG 50 % and BSG 100 % elicited the highest ROS values, which considerably affected the growth of tomato plants (Table 6). Five days after the second treatment plants recovered the loss of weight, compared to control. Because cell membranes are one of the major sites of ROS activity under environmental stress (Mittler, 2002), higher levels of ROS in CS- and BSG-treated plants might induce cellular damage leading to apoptic-like programmed cell death. Moreover, as high levels of ROS serve as substrates for synthesis of secondary metabolites

or as components enforcing the physical barrier of the cell walls their accumulation in treated roots alert plants to limit nematode infection.

Chlorophyll content (Chl) in all treated tomato plants did not differ from that of the control at 5 and 10 days after the first treatment (Table 7). No difference, at both observation times, was found in total chlorophyll, Chl *a* and *b*. After the second treatment, a significant difference was observed in chlorophyll *a* content in plants treated with CS 100 % (29.7 μg/cm^2) in comparison to all other treatments included the control (33.3 μg/cm^2) (Table 7). It is well known that ROS enhancement can cause the degradation of photosynthetic pigments and damage to photosynthetic machinery (Mittler, 2002). High ROS levels found after treatments may have directly or indirectly contributed to the decline in the observed chlorophyll levels.

Conclusions

Yield losses caused worldwide by root-knot nematodes require to find ecofriendly control strategies with low environmental impact (Stirling, 2014) considering that the control of these pests is meeting with difficulties as current national and international regulations are limiting the use of synthetic nematicides which exert serious detrimental effects on the environment (Sánchez-Moreno *et al*., 2009). In our study both tested by-product extracts were not phytotoxic to tomato plants as shown by the morpho-physiological parameters of the treated plants. All this makes CS extracts of economic relevance, as alternative products to be used in sustainable strategies for nematode management. The observed nematicidal effect of CS extract against *M. incognita* is related to the release of polyphenols (caffeoyl and feruloyl quinic derivates) from the coffee epidermis as demonstrated for other agro-industrial by-products as grape pomace (D'Addabbo *et al*., 2000) or olive mill wastes (Sasanelli *et al*., 2002).

Future research needs consist of the setup of techniques able to produce commercial CS extracts with a standardized composition and known nematicidal efficacy. However, further studies are also needed to investigate the effect of CS extracts on different nematode species and types of soils.

Conflict of Interest

Authors state no conflict of interest

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Two new species of the genus *Coomansinema* Ahmad and Jairajpuri, 1989 (Nematoda: Dorylaimida) with a key to its species

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Summary

Two new species of the genus *Coomansinema* Ahmad and Jairajpuri, 1989 are described and illustrated. *C. japonicum* n. sp. is characterized by having medium size body (L= 1.40 – 1.45 mm); lip region truncate with completely amalgamated lips; amphideal fovea goblet – shaped; 16 – 20 µm long odontostyle; 23 – 25 µm long odontophore; comparatively anterior position of the second pair of pharyngeal glands; amphidelphic female genital system; longitudinal vulva; males with 48 – 54 µm long spicules; 7 – 8 spaced ventromedian supplements and tail long filiform in female and short conoid in male. *C. longicaudatum* n. sp. is characterized by having medium size body (L= 1.1 – 1.3 mm); lip region truncate, continuous with completely amalgamated lips; amphideal fovea cup – shaped; 16 – 17 µm long odontostyle; 19 – 20 µm long odontophore; comparatively anterior position of the second pair of pharyngeal glands; amphidelphic female genital system; transverse vulva, intestinal – prerectum junction with a tongue – like structure and 210 – 269 µm long filiform tail. A key to its seven valid species is provided.
Keywords: *Coomansinema*; description; Japan; key to species; new species

Introduction

Ahmad and Jairajpuri (1989) established the genus *Coomansinema* and designated *C. dimorphicauda* as its type species reported from India. They differentiated the new genus on the basis of amalgamated, truncate and continuous lip region, without labial or post-labial sclerotization, odontostyle massive, slightly sinuate with thickened tip and wide lumen, anterior position of the second pair of pharyngeal glands and with sexual dimorphism in tail shape, female tail cupola-shaped with slightly dorsally bent terminal process and males with conoid-rounded without any process. Though *Coomansinema* lacks any labial sclerotization, but due to the position of its second pair of ventrosublateral pharyngeal gland nuclei and their orifices, this genus was placed under Thornenematinae Siddiqi, 1969. Recently, *Coomansinema* has been studied by Andrássy (2012) who accepted its position among the genera of the family Thornenematidae. Ahmad (1993) added a new species, *C. oryzae* with transverse vulva from paddy fields in India. Dhanam and Jairajpuri (2002) added two more species *C. alduri* and *C. digiticauda* from India, whereas, Ahmad and Shaheen (2004) further described a new species *C. brevicauda* with spike-like tail from Costa Rica, quite different from the other known species of *Coomansinema*. Andrássy (2012) reported a new species *C. taiwanense* from Taiwan with longest odontostyle and lower number of ventromedian supplements and discussed in detail the taxonomic position of the genus *Coomansinema*. Vinciguerra *et al.*, (2014) described new species *C. istvani* from forest in Ecuador.

Khan (1995), while redescribing *Timminema pakistanicum*, synonymized *Coomansinema* with *Timminema* without giving any valid ground of justification. Andrássy (2012) did not accept this

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synonymy. We fully agree with Andrássy's views and consider *Coomansinema* distinctly different from *Timminema*.
In the present paper two new species of this genus collected from Japan are described and illustrated. A key to species is also provided.

Materials and Methods

Soil samples were processed using Cobb's (1918) sieving and decantation and modified Baermann's funnel techniques. The nematodes were extracted and fixed in hot formalin-glycerol fixative, dehydrated by the slow evaporation method (Seinhorst 1959), and mounted in anhydrous glycerin. Permanent mounts were prepared using the paraffin wax ring method (de Maeseneer & d'Herde 1963). The measurements were taken with an ocular micrometer and drawings made using a drawing tube. Some of the best preserved specimens were photographed using a Nikon Eclipse 80i microscope and a Nikon DS digital camera. Raw photographs were edited using Adobe® Photoshop®.

Results

***Coomansinema japonicum* n. sp.**
(Figs. 1 & 2)
Measurements: See Table 1
Description: Adult: Moderately slender nematodes of medium size, 1.40 – 1.45 mm long. Body cylindrical, slightly curved ventrad upon fixation, tapering towards both ends but more so towards the posterior end because of the tapering long filiform tail. Cuticle three-layered, especially distinguishable at caudal region, a thinner outer layer bearing very fine transverse striations through the entire body, thicker intermediate layer with radial striations and thin inner layer; thickness 2 µm at anterior region and mid body, and 3 – 5 µm on tail. Lateral chord 6 – 10 µm wide at mid body, occupying about one-eighth to one-fifth (12 – 18 %) of mid-body diameter. Lip region truncate, continuous with body, 2.4 – 3.0 times as wide as high and about one-fourth (21 – 27 %) of body diameter at neck base; lips amalgamated; labial papillae not interfering with labial contour. Amphid fovea goblet-shaped, its aperture occupying about half of lip region diameter. Guiding ring simple, single, at 0.7 – 1.0 times lip region diameter from anterior end. Odontostyle cylindroid, rather robust, with distinctly thickened tip, 1.4 – 1.5 times the lip region diameter long, its aperture about one-third of its length, ventral arm slightly bent near middle giving a rather sinute appearance. Odontophore linear, rod-like, 1.0 – 1.5 times the odontostyle length. Pharyngeal expansion gradual; expanded portion 5.6 – 6.8 times as long as wide, 2.6 – 3.2 times as long as body diameter, and occupying about 45 – 50 % of total neck length. Nerve ring at 30 – 40 % of neck length from anterior end. Pharyngeal gland nuclei located as follows: D = 62 – 63 %; AS1 = 16 – 17 %; AS2 = 19 – 21 %; PS1 = 51 – 52 %; PS2 = 52 – 54 % as per Andrássy (1998); D0 = 53 – 59 %; DN = 56 – 61 %; DO – DN =2.3 – 4.5 %; S1N1 = 62 – 66 %; S1N2 = 69 – 77 %; S2N = 82 – 84 %; S2O = 84 – 85 % as per Loof and Coomans (1970). Cardia rounded conoid, gradually tapering to a fine pointed tip.
Female: Genital system didelphic-amphidelphic, both sexual branches almost equally developed, anterior 173 – 205 µm long or 11 – 13 % of body length and the posterior 195 – 255 µm long or 13 – 17 % of body length. Ovaries large sized, usually surpassing the sphincter level, the anterior measuring 44 – 144 µm and posterior 90 – 170 µm long; oocytes arranged first in two or more rows, then in a single row. Oviducts consisting a slender proximal part with traces of sperms, measuring 110 µm or 2.1 (anterior) and 90 µm or 1.7 (posterior) times the corresponding body diameter long; oviduct-uterus junction marked by a sphincter; uterus a short, simple, tube-like structure filled with sperms, measuring 97 µm or 1.8 (anterior) and 110 µm or 2.1 (posterior) times the corresponding body diameter long (n=1, all the other females being gravid). Vagina extending inwards, 22 – 26 µm or about two-fifths to half of the corresponding body diameter; *pars proximalis vaginae* 12 – 14 × 6 – 8 µm, with somewhat sigmoid walls and surrounded by weak musculature; *pars refringens vaginae* well developed with two triangular pieces with rounded edges, 7 − 8 × 4 − 5 µm, their combined width 14−15 µm and a third pentagon-shaped intermediate piece; *pars distalis vaginae* well developed, 4 – 5 µm long. Vulva a pre-equatorial longitudinal slit. Prerectum 2.1 – 3.5, rectum 1.5 – 1.8 anal body diam. long. Tail 8 – 11 anal body diam. long, tapering gradually behind anus into long filiform tail, terminus in some specimens dorsally bent otherwise ventrally; hyaline part 23 – 26 % of total tail length. Caudal pores two pairs, one lateral, another sub-dorsal.
Male: Slender nematodes of medium size, 1.12 – 1.33 mm. Genital system diorchic, with opposed testes. In addition to the ad-cloacal pair, situated at 6 – 7 µm from cloacal aperture, a series 7 – 8 regularly spaced ventromedian supplements starting at a distance of 45 – 51 µm from the ad-cloacal pair, each ventromedian 12 – 13 µm apart. Spicules total length 48 – 54 µm along the arc, 1.2 – 1.3 times that at the chord, 5.0 times the maximum width and 1.8 – 2.1 times the body diam. at the cloacal aperture. Curvature 126 – 139º. Dorsal contour regularly convex, ventral contour bearing prominent hump and hollow, the former located at 28 – 33 % of spicule total length from its anterior end. Head well developed, occupying 21 – 24 % of total length, its dorsal contour conspicuously curved and longer than the ventral. Median piece 10 – 12 times as long as wide, occupying 30 % of spicules maximum width, reaching spicule terminal tip. Posterior end 3 – 4 µm wide. Lateral guiding pieces, 8.6 – 10 times as long as wide and about one-fourth of spicules length. Tail convex conoid with broadly rounded terminus. Caudal pores two on each side.

Type habitat and locality: Soil collected from natural forest, Yaku town, Yakushima Island, Japan; 30º18'15" N 130º34'33.2" E collected by Dr. Mizukubo of National Agricultural Research Center, Tsukuba, Japan.

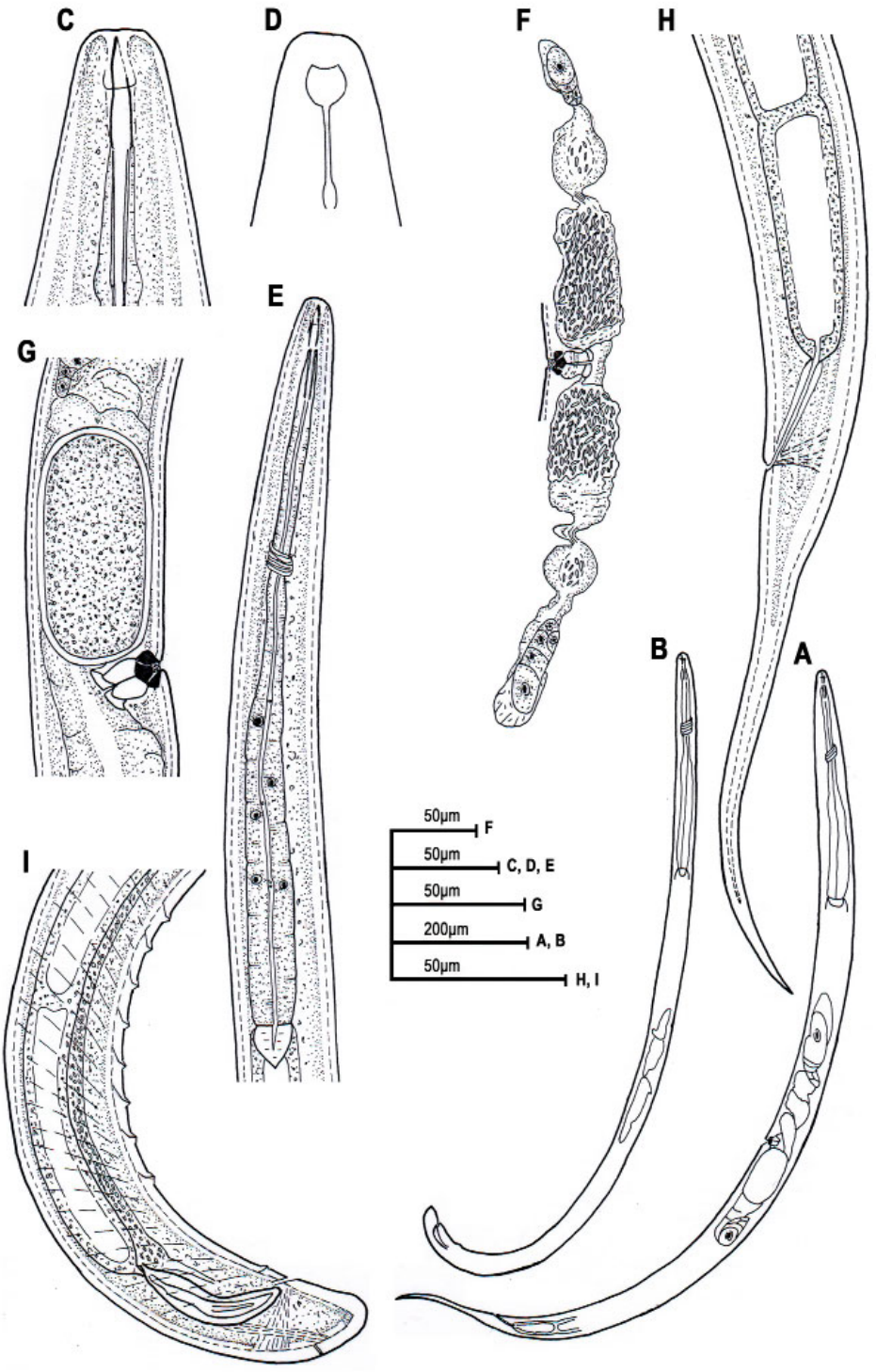

Fig.1. *Coomansinema japonicum* n. sp. (A) Entire female; (B) entire male; (C) anterior region; (D) anterior end showing amphid; (E) pharyngeal region; (F) female genital system; (G) vulval region with egg; (H) female posterior region; (I) male posterior region.

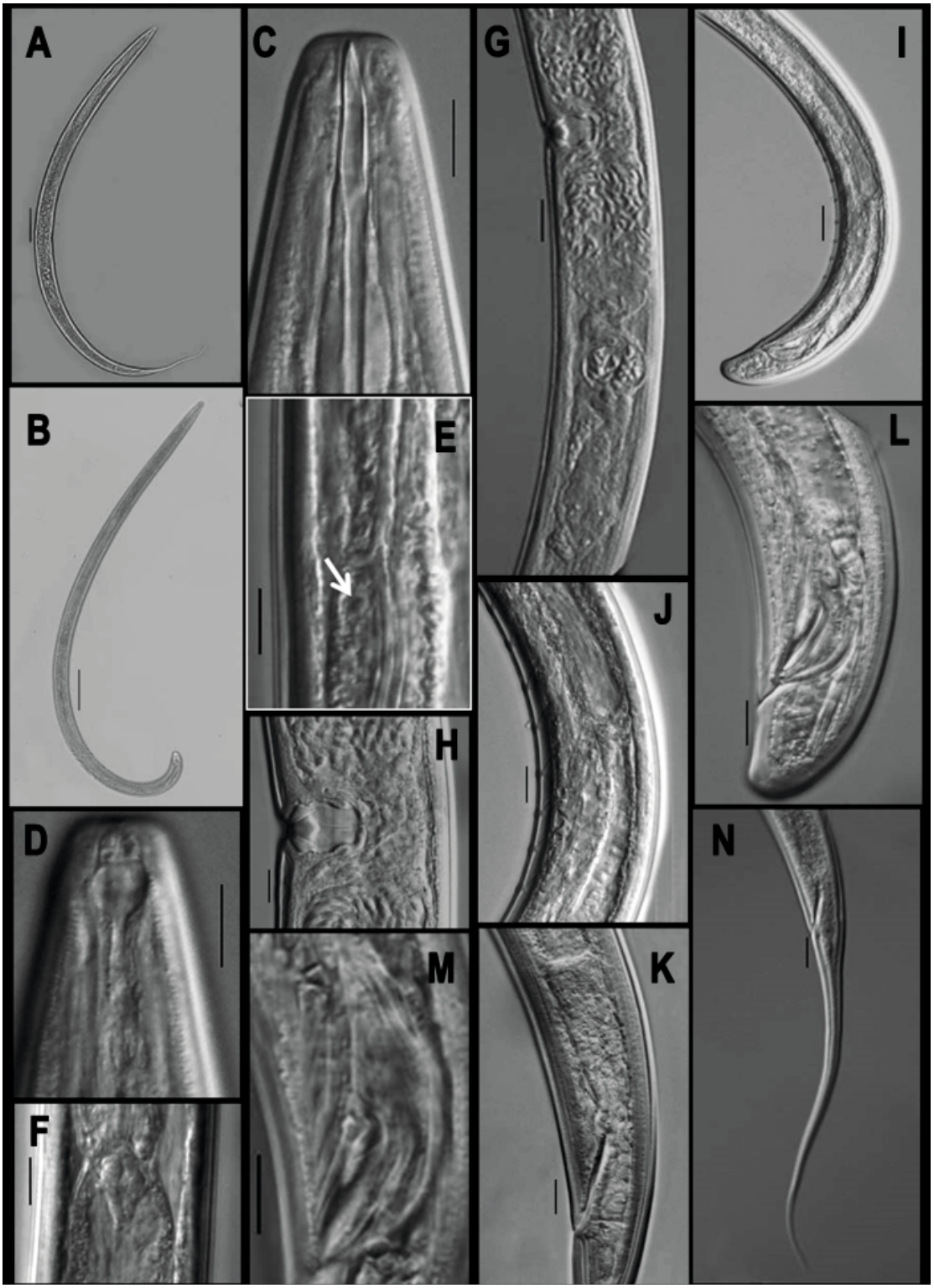

Fig.2. *Coomansinema japonicum* n. sp. (A) Entire female; (B) entire male; (C) anterior region; (D) anterior end showing amphid; (E) pharyngeal expansion arrow head pointing towards dorsal gland nuclei; (F) pharyngo-intestinal junction; (G) female genital branch (posterior); (H) vulval region; (I) male posterior region; (J) ventromedian supplements; (K) posterior region showing prerectum; (L) male caudal region; (M) male spicules; (N) female caudal region. (Scale bars: A, B = 100 µm; C, D, E, F, H, K, J, L, M = 10 µm; G, I, N = 20 µm).

Table 1. Measurements of *Coomansinema japonicum* n. sp.
(All measurements in µm except L in mm)

Characters	Holotype female	Paratype females	Paratype males
n	1	4	3
L	1.42	1.43 ± 0.21 (1.40 – 1.45)	1.23 ± 0.84 (1.12 – 1.33)
Body diameter at neck base	53	53.5 ± 3.4 (48 – 57)	38.6 ± 5.2 (34 – 46)
Body diameter at mid body	51	53.0 ± 3.7 (47 – 56)	39.0 ± 2.4 (36 – 42)
Body diameter at anus	21	22.7 ± 0.8 (22 – 24)	26.0 ± 0.8 (25 – 27)
a	28	27.2 ± 1.6 (25.7 – 29.7)	31.8 ± 0.4 (31.3 – 32.2)
b	4.0	4.5 ± 0.2 (4.0 – 4.6)	3.6 ± 0.1 (3.5 – 3.8)
c	6.8	6.8 ± 1.0 (6.0 – 8.5)	56.3 ± 0.7 (55.5 – 57.1)
c’	10	9.4 ± 1.1 (7.7 – 10.9)	0.80 ± 0.0 (0.80 – 0.88)
V	49	47.6 ± 1.2 (46.0 – 49.3)	–
G1	13.3	13.2 ± 0.8 (11.8 – 14.2)	–
G2	16.5	15.6 ± 1.6 (13.4 – 17.6)	–
Lip region diameter	12	12.5 ± 0.5 (12.0 – 13.0)	12.6 ± 0.5 (12.0 – 13.0)
Lip region height	5	4.3 ± 0.5 (4 – 5)	4.6 ± 0.5 (4 – 5)
Amphid aperture	6	6.1 ± 0.2 (6 – 6.5)	6.3 ± 0.5 (6 – 7)
Odontostyle length	19	17.7 ± 1.5 (16 – 20)	19.3 ± 0.5 (19 – 20)
Odontophore length	23	24 ± 1.0 (23 – 25)	22 ± 2.2 (20 – 25)
Guiding ring from anterior end	12	10 ± 0.0 (10 – 10)	10 ± 0.0 (10 – 10)
Nerve ring from anterior end	127	121.7 ± 2.0 (120 – 125)	124 ± 2.9 (120 – 127)
Neck length	355	321.2 ± 12.5 (308 – 337)	340.6 ± 16.1 (318 – 354)
Expanded part of pharynx	170	150.5 ± 3.6 (145 – 155)	155.3 ± 8.8 (143 – 163)
Cardia length	23	15.7 ± 1.6 (13 – 17)	14 ± 0.8 (13 – 15)
Anterior genital length	189	190.2 ± 11.4 (173 – 205)	–
Posterior genital length	235	221.2 ± 23.3 (195 – 255)	–
Vaginal depth	26	24.5 ± 1.5 (22 – 26)	–
Vulva from anterior end	700	684.2 ± 20.6 (665 – 719)	–
Prerectum length	48	74.2 ± 20.6 (52 – 78)	90 ± 8.2 (80 – 100)
Rectum length	38	37.7 ± 1.1 (36 – 39)	43.3 ± 0.9 (42 – 44)
Tail length	210	213.7 ± 26.8 (170 – 240)	22 ± 1.6 (20 – 24)
Spicules length	-	–	50.6 ± 2.5 (48 – 54)
Lateral guiding pieces	-	–	30.3 ± 3.7 (26 – 35)
Ventromedian supplements	-	–	7.6 ± 0.5 (7 – 8)

Type specimens: Holotype female and a paratype male on slide *Coomansinema japonicum* n. sp./1; paratype females and males on slides *Coomansinema japonicum* n. sp./2-4; deposited in the nematode collection of the Department of Zoology, Aligarh Muslim University, Aligarh.
Etymology: The new species is named *Coomansinema japonicum* n. sp. because it is recorded from Japan.

Diagnosis and relationships: *Coomansinema japonicum* n. sp. is characterized by having 1.40 – 1.45 mm (female) and 1.12 – 1.33 mm (male) long body; truncate, continuous lip region with completely amalgamated lips; amphid fovea goblet-shaped, guiding ring single, comparatively anterior position of the second pair of pharyngeal glands; amphidelphic female genital system; longitudinal vulva and long filiform tail 170 – 240 µm and males with dorylaimoid spicules 48 – 54 µm long; 7 – 8 equally spaced ventromedian supplements and short conoid tail with rounded terminus.
The new species differs from all the known species of the genus *Coomansinema* in having long filiform tail. However, in the presence of longitudinal vulva, this new species comes close to *C. dimorphicauda* and *C. taiwanense*, but differs from the former in having large body (1.4 – 1.45 *vs* 1.25 µm), shorter odontostyle (16 – 20 *vs* 22 µm) and longer *vs* shorter digitate tail (c= 6 – 8.5 *vs* 43; c'= 7.7 – 10.9 *vs* 1.0). From latter, it differs in having shorter body (1.4 – 1.45 *vs* 1.5 – 1.88 mm) narrow lip region (12 – 13 *vs* 17 – 19 µm), shorter odontostyle (16 – 20 *vs* 26 – 28 µm), anterior position of vulva (V= 46 – 49.3 *vs* 54 – 60), smaller spicules (48 – 54 *vs* 64 – 70 µm) and long filiform tail (c= 6 – 8.5 *vs* 26 – 41; c'= 7.7 – 10.9 *vs* 1.1 – 1.7). From *C. brevicauda*, it differs by having narrow lip width (12 – 13 *vs* 15 – 17 µm), longitudinal vulva (*vs* transverse), longer tail (*vs* tail initially cupola then strongly narrowed to filiform process). From *C. istvani* the new species differs in having shorter odontostyle (16 – 20 *vs* 20 – 27 µm), longitudinal vulva (*vs* transverse) and longer tail 170 – 240 *vs* 17 – 31 µm (c= 6 – 8.5 *vs* 35.8 – 68.8; c'= 7.7 – 10.9 *vs* 0.6 – 1.0).

***Coomansinema longicaudatum* n. sp.**
(Figs. 3 & 4)
Measurements: See Table 2
Description: Female: Moderately slender nematodes of medium size, 1.1 – 1.3 mm long. Body cylindrical, slightly curved ventrad upon fixation, tapering towards both ends but more so towards the posterior end because of the tapering long filiform tail. Cuticle three-layered, especially distinguishable at caudal region, where it consists of thinner outer layer bearing very fine transverse striations through the entire body, thicker intermediate layer with radial striations and thin inner layer; thickness 1.5 – 2.0 µm in the anterior region, 2.0 µm at mid body and 3.0 µm on tail. Lateral chords 6 – 9 µm wide at mid body, occupying about one-seventh to one-fifth (15 – 21 %) of mid body diameter. Lip region rounded, continuous with body, 2.2 – 2.7 times as wide as high and about one-third (28 – 31 %) of body diameter at neck base; lips amalgamated. Amphid fovea cup-shaped, its aperture occupying about half to three-fifths (50 – 63 %) of lip region diameter. Guiding ring single, at 0.8 – 1.0 times lip region diameters from anterior end. Odontostyle cylindroid, rather robust, with distinctly thickened tip, 1.3 – 1.7 times the lip region diameter long, its aperture about one-third of its length, ventral arm slightly bent near middle giving a rather sinute appearance. Odontophore linear, rod-like, 1.1 – 1.3 times the odontostyle length. Anterior region of pharynx enlarging gradually at 57 – 60 % of neck length; basal expansion 5.5 – 6.5 times as long as wide, 3.1 – 3.2 times as long as body diameter, occupying about 40−48 % of neck length. Pharyngeal gland nuclei located as follows: D = 62 – 65 %; AS1 = 10 – 12 %; AS2 = 16 – 18 %; PS1 = 46 – 47 %; PS2 = 50 – 53 % as per Andrássy (1998); DO= 58 – 62 %; DN = 61 – 63 %; DO-DN = 2.4 – 3.9 %; S1N1 = 69 – 72 %; S1N2 = 71 – 75 %; S2N = 81 – 84 %; S2O = 83 – 85 % as per Loof and Coomans (1970). Nerve ring at 38 – 40 % of total neck length. Cardia first rounded conoid, then gradually tapering to a fine rounded terminus. Genital system didelphic-amphidelphic; both sexual branches almost equally well developed, anterior 85 – 99 µm long or 7 – 8 % of total body length and the posterior 90 – 118 µm long or 7 – 9 % of body length. Ovaries small sized, usually not surpassing the sphincter level, measuring anterior 30 – 43 µm and the posterior 40 – 56 µm long; oocytes arranged first in two or more rows, then in a single row. Oviducts consisting a slender proximal part and a well developed *pars dilatata,* measuring 42 – 50 µm or 1.0 – 1.3 (anterior) and 46−48 µm or 1.2−1.7 (posterior) times the corresponding body diameter long. Oviduct-uterus junction marked by a sphincter. Uterus a short, simple, tube-like structure without trace of sperms, measuring 42 – 52 µm or 1.0 – 1.3 (anterior) and 41 – 53 µm or 1.0 – 1.2 (posterior) times the corresponding body diameter long. Vagina extending inwards, about two-fifths to one-half (40 – 50 %) of corresponding body diameter; *pars proximalis vaginae* 10 – 12 × 6 – 7 µm with somewhat sigmoid walls, surrounded by weak musculature; *pars refringens vaginae* well developed, comma-shaped, 5 − 6 × 2 − 3 µm, their combined width 11 × 12 µm, distal part of both close to each other but their proximal part far apart; *pars distalis vaginae* well developed, 5 – 6 µm long. Vulva a transverse slit. Intestinal-prerectum junction with a well developed conical tongue-like structure. Prerectum 1.2 – 1.9, rectum 1.3 – 1.6 anal body diam. long. Tail long filiform, 9 – 11 anal body diam. long; hyaline part 29 – 31 % of total tail length. Caudal pore two pairs, one lateral, another sub-dorsal.
Male: Not found.
Type habitat and locality: Tall grasses from Koibuchimachi, Mito C, Ibaraki Prefecture, Japan; 36°20'29"N 140°26'48"E; collected by Dr. Masaki Araki on 07.12. 2011.
Type specimens: Holotype female on slide *Coomansinema longicaudatum* n. sp. /1; paratype females on slides *Coomansinema longicaudatum* n. sp. /2-8; deposited with the nematode collection of the Department of Zoology, Aligarh Muslim University, India.
Etymology: The new species is named *C. longicaudatum* n. sp. because of its long tail.

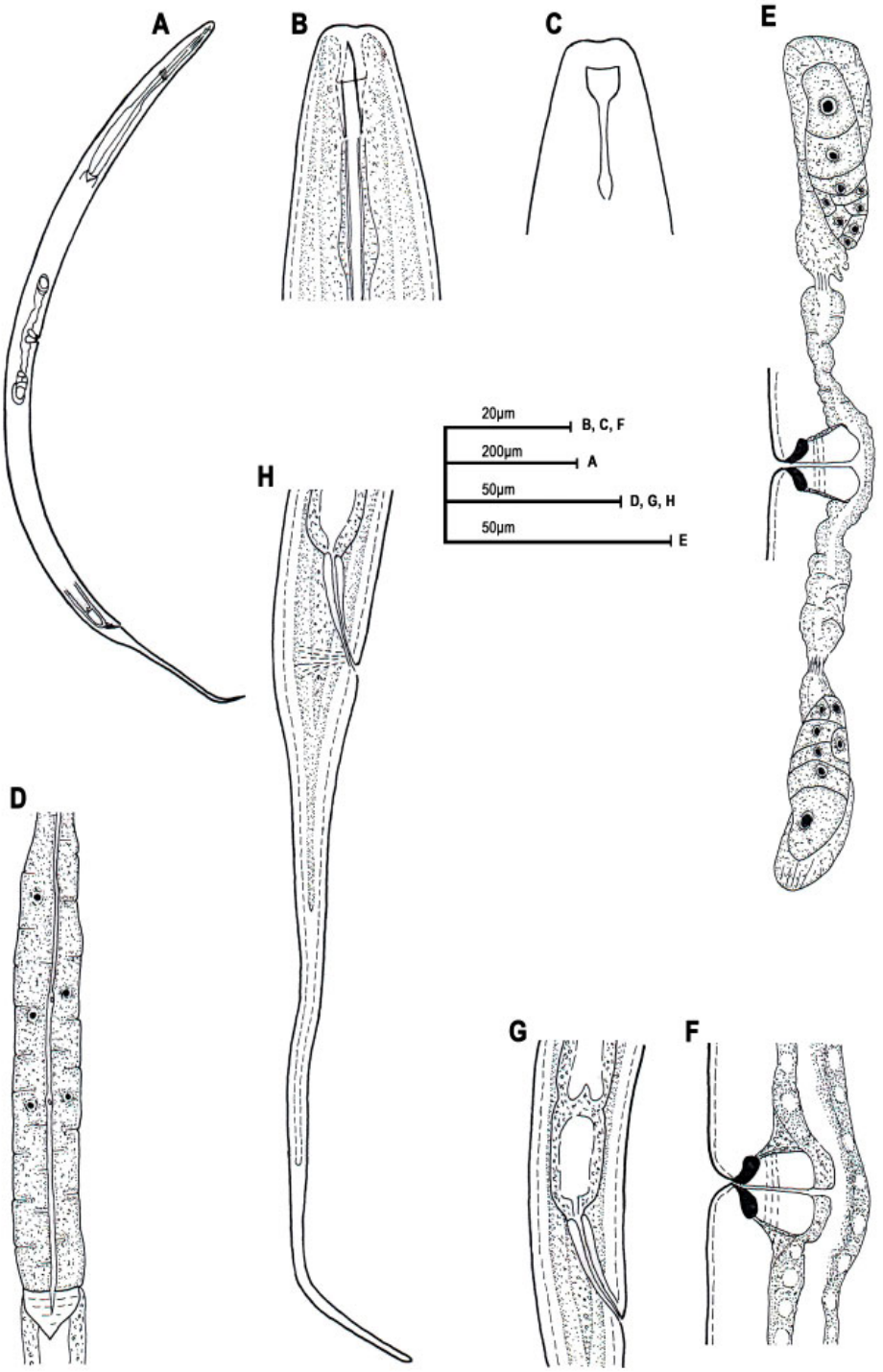

Fig.3. *Coomansinema longicaudatum* n. sp. (A) Entire female; (B) anterior region; (C) anterior end showing amphid; (D) pharyngeal region; (E) female genital system; (F) vulval region; (G) posterior region showing prerectum; (H) female posterior region.

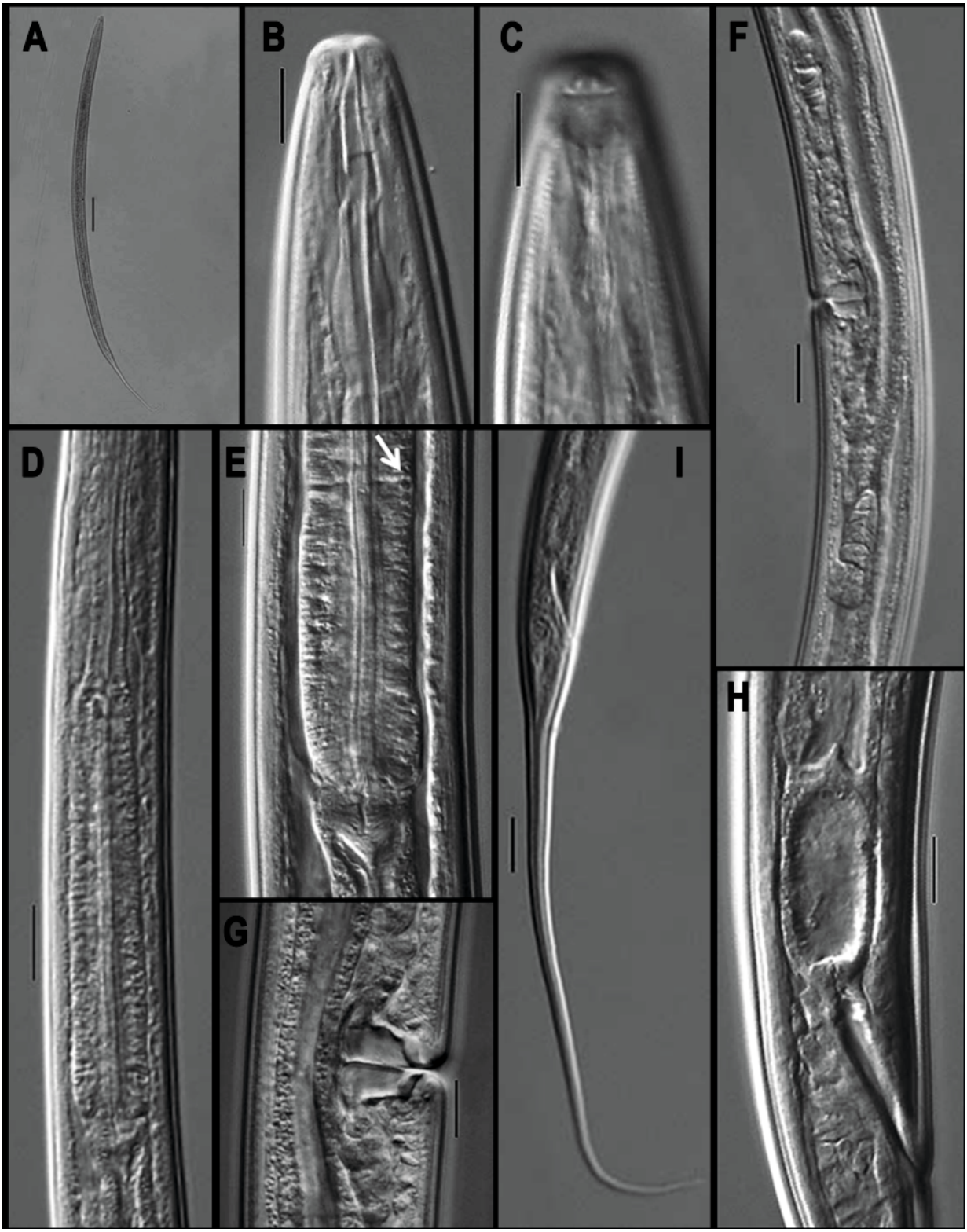

Fig.4. *Coomansinema longicaudatum* n. sp. (A) Entire female; (B) anterior region; (C) anterior end showing amphid; (D) expanded part of pharynx (E) pharyngeal base showing subventral gland nuclei and pharyngo-intestinal junction (arrow head pointing towards S2N); (F) female genital system; (G) vulval region; (H) posterior region showing prerectum; (I) caudal region. (Scale bars: A = 100 µm; B, C, E, G, H = 10 µm; D, F, I = 20 µm).

Table 2. Measurements of *Coomansinema longicaudatum* n. sp.
(All measurements in µm except L in mm)

Characters	Holotype female	Paratype females
n	1	8
L	1.23	1.23 ± 0.66 (1.12 – 1.35)
Body diameter at neck base	39	37.9 ± 1.7 (35 – 40)
Body diameter at mid body	40	38.9 ± 1.5 (37 – 41)
Body diameter at anus	22	22.8 ± 1.0 (21 – 24)
a	33.3	31.9 ± 1.1 (29.6 – 33.4)
b	4.1	4.3 ± 0.2 (3.9 – 4.5)
c	5.0	5.3 ± 0.2 (5.0 – 5.6)
c’	11.1	10.2 ± 0.6 (9.1 – 11.2)
V	44.2	43.8 ± 1.7 (41.8 – 47.5)
G1	8.0	7.6 ± 0.4 (7.1 – 8.4)
G2	8.7	8.4 ± 0.5 (7.5 – 9.1)
Lip region diameter	11	11.1 ± 0.3 (11 – 12)
Lip region height	4	4.5 ± 0.5 (4 – 5)
Amphid aperture	6	6.5 ± 0.5 (6 – 7)
Odontostyle length	16	16.9 ± 0.3 (16 – 17)
Odontophore length	20	19.6 ± 0.5 (19 – 20)
Guiding ring from anterior end	10	9.6 ± 0.7 (9 – 11)
Nerve ring from anterior end	115	115.5 ± 1.7 (113 – 118)
Neck length	298	290.1 ± 7.2 (283 – 306)
Expanded part of pharynx	122	123.1 ± 2.7 (118 – 127)
Cardia length	14	15.5 ± 0.9 (14 – 17)
Anterior genital length	99	93.1 ± 4.3 (85 – 99)
Posterior genital length	107	103.8 ± 9.1 (90 – 118)
Vaginal depth	20	17.9 ± 0.8 (17 – 19)
Vulva from anterior end	544	541.3 ± 16.7 (507 – 568)
Prerectum length	35	35.1 ± 4.1 (30 – 40)
Rectum length	32	33.8 ± 1.6 (32 – 37)
Tail length	245	232.3 ± 16.7 (210 – 269)

Diagnosis and relationships: *C. longicaudatum* n. sp. is characterized by having 1.1 – 1.3 mm long body; lip region truncate, continuous with amalgamated lips; amphideal fovea cup-shaped; 16 – 17 µm long odontostyle; 19 – 20 µm long odontophore; comparatively anterior position of the second pair of pharyngeal glands; amphidelphic female genital system; transverse vulva; intestinal-prerectum junction with a tongue-like structure and 210 – 269 µm long filiform tail.

The new species closely resembles *C. japonicum* n. sp. in having long filiform tail in females but distinctly differs from it in the presence of a transverse vulva (*vs* longitudinal). It further differs from it in the shape of amphid (cup-shaped *vs* goblet-shaped), shorter odontostyle (*vs* odontostyle 16 – 20 µm), shorter prerectum (*vs* prerectum 35 – 108 µm) and in the absence of males (*vs* presence).

Remarks. Ahmad and Jairajpuri (1989) placed the genus *Coomansinema* in the subfamily Thornenematinae Siddiqi, 1969 mainly because of anterior position of its second pair of ventrosublateral pharyngeal glands and their orifices. In the shape of its lip region, the nature of odontostyle and the position of second pair of subventral pharyngeal glands, *Coomansinema* closely resembles the genus *Opisthodorylaimus* Ahmad and Jairajpuri, 1982, except for having didelphic-amphidelphic females. All the *Opisthodorylaimus* species possess mono-opisthodelphic female genital system. Carbonell and Coomans (1986) while revising the genus *Opisthodorylaimus* recorded anterior uterine branch from completely absent (*O. filicaudatus*), mostly reduced to a uterine sac showing different degree of degeneration (*O. cavalcantii*) to anatomically complete (*O. paracavalcantii*) but never functional. Although, Gagarin (2004) described *O. major,* an amphidelphic species from fresh water habitat in Russia, it is quite different from other known species of *Opisthodorylaimus* and do not fit in the generic diagnosis of *Opisthodorylaimus* and hence Andrássy (2007) rightly considered it a species *incertae sedis*. As of today the placement of these two new species seems most justified in the genus *Coomansinema* rather than *Opisthodorylaimus* till sequence data on species representing both these genera is not available.

In the presence of long filiform tail, the two new species also resembles the long-tailed dorylaim genera *Paradorylaimus* Andrássy, 1969 and *Laimydorus* Siddiqi, 1969. However, they distinctly differs from both in their characteristic wide, massive odontostyle, distinctly thickened at tip, and anterior position of S2N. As regards the tail shape, *Coomansinema* has a short conoid tail with slight projection at tip in the type species to characteristic elongation after the short conoid portion (cf. *C. oryzae*, *C. brevicauda*) and now the two newly described species has long filiform tail. Similar diversity in tail shape is quite common in the genus *Mesodorylaimus* Andrássy, 1959 and several other dorylaim genera.

Andrássy (2012) doubted the validity of *C. alduri* Dhanam and Jairajpuri, 2002 as the differences from *C. oryzae* reported in original description of *C. alduri* appear to be irrelevant. He also considered the position of *C. digiticauda* Dhanam and Jairajpuri, 2002 under *Coomansinema* rather doubtful because of the shape of its lip region being quite different from *Coomansinema* pattern, less anterior position of the second pair of pharyngeal glands, longer prerectum and a non-offset tail peg. We concur with Andrássy (2012) and the two species *C. alduri* and *C. digiticauda* are considered as *species inequirendae*.

Key to species of genus *Coomansinema.*

1. Female tail long filiform, usually more than 8 anal body width long.....2
   - Female tail shorter, usually less than 4 anal body width long.....3
2. Vulva transverse; c = 5.0-5.5; c'= 9.0-11.2.....*longicaudatum* n. sp.(Japan)
   - Vulva longitudinal; c = 6.0-8.5; c'= 7.7-10.9.....*japonicum* n. sp. (Japan)
3. Vulva longitudinal.....4
   - Vulva transverse.....5
4. Female 1.25 mm long; odontostyle 22 µm long; c'=1.0; ventromedian supplements in male 12-15.....*dimorphicauda* Ahmad & Jairajpuri, 1989 (India)
   - Female 1.5-1.8 mm long; odontostyle 26-28 µm long; c' =1.1-1.7; ventromedian supplements in male 7-8.....*taiwanense* Andrássy, 2012 (Taiwan)
5. Tail cupola-shaped with a very short finger-like, dorsally bent blunt process, female 0.9-1.5 mm long; odontostyle 20-27 µm long; c'=0.6-1.0.....*istvani* Vinciguerra, Orselli & Clausi, 2014 (Ecuador)
   - Tail cupola-shaped with a long, narrow, dorsally bent process.....6
6. Female 1.7-1.9 mm long; c= 17-20; c'= 2.4-3.2; long tongue like structure present at intestine-prerectum junction; ventromedian supplements in male 12-13.....*oryzae* Ahmad, 1993 (India, Ecuador, Peru)
   - Female 1.3-1.6 mm long; c= 20-39; c'= 1.7-2.2; tongue like structure absent at intestine-prerectum junction; ventromedian supplements in male 10.....*brevicauda* Ahmad and Shaheen, 2004 (Costa Rica, Ecuador, Peru)

Conflict of Interest

All authors have no potential conflict of interest pertaining to this submission to *Helminthologia.*

Acknowledgments

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Case Report

Multiple parasitic infestation in a nine-month-old patient: a case report

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Summary

We are reporting the case of a nine-month-old Pakistani female with complaint of growth retardation who presented multiple intestinal parasitic infections. Probably because of contamination with fecal matter, the initial microscopic examination of the urinary sample revealed the presence of eggs of *Enterobius vermicularis*, cysts of *Entamoeba coli*, and an organism similar to mites. Stool samples were obtained after two weeks and microscopic investigation confirmed the presence of *Enterobius vermicularis* eggs, cysts of *Entamoeba coli*, and hookworm eggs. The patient was immediately subjected to mebendazole therapy associated with trimethoprim-sulfamethoxazole, to which she responded well. Follow-up stool re-examinations performed 15 and 30 days after the treatment tested negative for all parasitic ova and cysts. This study reflects the importance of considering multiple parasitic infestations in low socio-economic populations and highlights the need of improving poor hygienic conditions to prevent such infections, in particular in children.
Keywords: polyparasitism; *Enterobius vermicularis*; hookworm; *Entamoeba coli*; infestations

Introduction

Intestinal parasites are widely distributed around the world with and infestation rates varying depending on the country of residence and the age of the exposed subjects (Manganelli *et al*., 2012; Gyang *et al*., 2017). Globally, more than 3.5 billion people are infected by intestinal parasites, including soil-transmitted helminthes, such as *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm, and protozoa such as *Giardia intestinalis* and *Entamoeba* spp. (Hotez *et al*., 2009; Manganelli *et al*., 2012). Higher prevalence rates of intestinal parasitic infections have been recorded in developing countries, which are considered endemic for most of these parasitic infections. Indeed, poverty, lack of access to clean water, poor hygiene, malnutrition, and hot and humid climate are the most common risk factors for the widespread of intestinal parasites (Hotez *et al*., 2009). In particular, over 800 million preschool- and school-age children live in areas where parasite prevalence and transmission are elevated (Harhay *et al*., 2010; Zemene and Shiferaw, 2018) and their yet not fully developed immune system makes them more susceptible to parasitic infections (Harhay *et al*., 2010; Zemene and Shiferaw, 2018). Before the year 2000, Italy was considered an endemic area for numerous parasites (e.g. *Entamoeba* spp., *G. duodenalis*, *Dientamoeba fragilis*, *T. trichiura*, *Strongyloides stercoralis*, *Ancylostoma duodenale*, *A. lumbricoides*, *Hymenolepis nana*, *Taenia* spp, *Echinococcus granulosus*, and *Enterobius vermicularis*) (Belli *et al*., 2014). Currently, in Italy, the infection rates due to parasites are low, even though only limited epidemiological data have been collected. Crotti *et al.* (2013) showed that the most common intestinal parasites identified in Italy between 2005 and 2008 were *S. stercolaris* and *E. vermicularis*, among heltminthes, and *G. intestinalis* and *Entamoeba* spp., among protozoa. In endemic countries, intestinal parasitosis rep-

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resents a social and economic burden (Abou-Shady *et al*., 2011; Manganelli *et al*., 2012), while in industrialized countries the major groups at risk of parasitosis are immigrants and nomad populations (Manganelli *et al*., 2012; Barnes *et al*., 2017). Depending on the parasite, transmission can occur via direct person-to-person contact or because of contact with a contaminated source (i.e. food, water, soil) (Hotez *et al*., 2009; Someshwaran *et al*., 2015; Gyang *et al*., 2017). Co-infection with two or more parasites is accidental and is associated with increased risk of morbidity, higher mortality rates and susceptibility to other infections (Supali *et al*., 2010; Gyang *et al*., 2017). In children, parasitic infestations can be responsible for malabsorption, leading to growth and cognitive development retardation (Manganelli *et al*., 2012). Pre-school and school age children thus present higher risk of worse health outcomes due to polyparasite infestations, compared to monoparasitosis (Supali *et al*., 2010). The aim of this case report was to show that poor socio-economic conditions and living in disadvantageous conditions of immigrants can significantly compromise health status also in developed countries, and the efforts to improve hygienic conditions and sanitation can decrease the vulnerability, in particularly of children, to parasitic infestations.

Ethical Approval and/or Informed Consent

Informed consent has been obtained from all individuals included in the study.

Case presentation

On June 21th, 2018, the urine sample from an outpatient nine-month-old female was received at the laboratory of Desio Hospital (Lombardy, Italy). The child was born in Italy from Pakistani parents arrived in Italy a few months before, had a medical history of growth retardation without the diagnosis of other diseases. The urine sample, contaminated with fecal matter, was analysed by microscopy. The analysis revealed the presence of *E. vermicularis* eggs (Fig. 1A) and *Entamoeba coli* cysts (Fig. 1B). Surprisingly, the analysis of the urinary sediment revealed also the presence of an organism similar to mites (Fig. 1C). However, after cutaneous examination, skin lesions indicating the invasion of ectoparasites were not reported. There was no eosinophilia in the peripheral blood, and bacteriological urine test was not required. The child was receiving artificial milk. Although parents and pediatrician were promptly informed about parasitic infestations, a fecal specimen of the patient was obtained only two weeks after the first examination. On July 6th, three stool samples preserved in the Universal Fixative solution (UNIFIX®, Medical Chemical Corporation, Torrance, CA) were sent to the Microbiology laboratory, and, after concentration, examined for ova and parasite (O & P). The presence of cysts of the protozoan *E. coli* (Fig. 1D) and eggs of the nematode *E. vermicularis* (Fig. 1E) was confirmed. Additionally, hookworm eggs measuring 55 – 60 µm in length and 35 – 40 µm in width were also observed (Fig. 1F). Collectively, the patient presented a co-infection with three parasites: two helminthes and a protozoan. No parasites were found in the stool samples obtained from the parents. Mebendazole (100 mg once a day for three days, repetition of the regimen one week later) associated with trimethoprim and sulfamethoxazole (100 mg + 800 mg for 15 days) were immediately started and, 15 and 30 days after the treatment, microscopic stool re-examination revealed no parasitic ova or cysts.

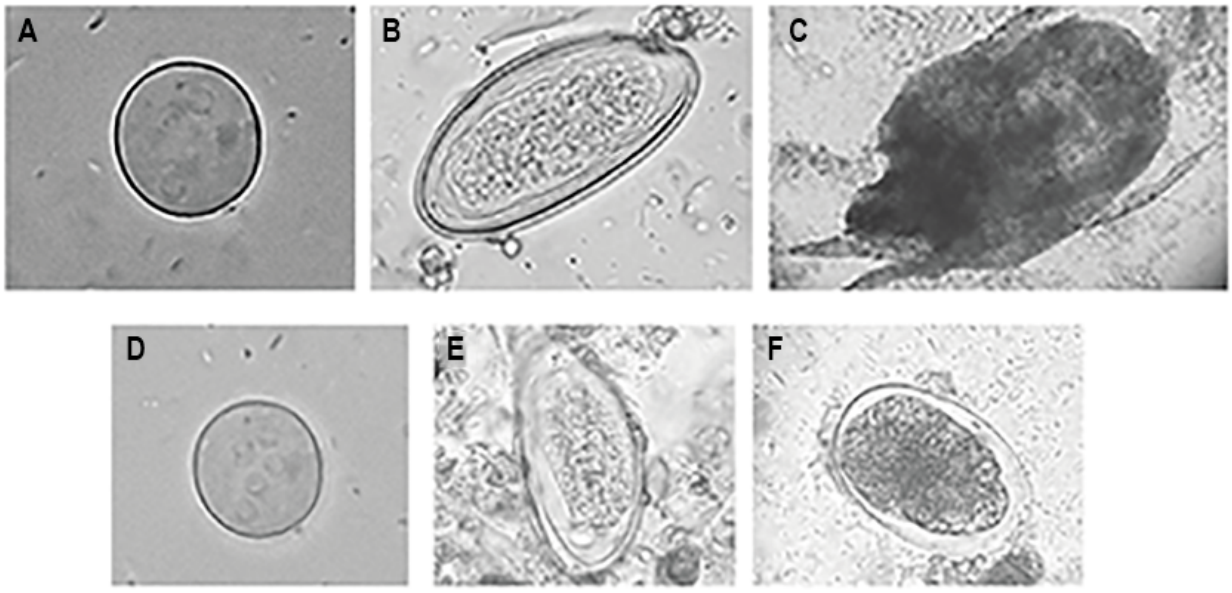

Fig. 1. Urine sediment analysis: (**A**) Cyst of *Entamoeba coli*; (**B**) Egg of *Enterobius vermicularis*; (**C**) Organism similar to mites. Microscopic stool examination: (**D**) Cyst of *Entamoeba coli*; (**E**) Egg of *Enterobius vermicularis*; (**F**) Egg of hookworm.

Discussion

The risk for parasitic infections, at both individual and community level, is associated with a complex and multivariate group of demographic, biological, social, environmental and behavioral factors. In endemic countries, poor hygienic conditions represent the most important risk factor for the diffusion and acquisition of intestinal parasitic infections (Hotez *et al*., 2009; Someshwaran *et al*., 2015; Gyang *et al*., 2017). Repeated infections with the same or different parasites are a common occurrence, and the simultaneous infection with multiple organisms can occur. In particular, polyparasitism increases morbidity and susceptibility to other infections (Manganelli *et al*., 2012; Someshwaran *et al*., 2015; Gyang *et al*., 2017; Wesolowska *et al*., 2018). The expansions in people travelling and immigration has contributed to increase the number of cases reported outside endemic areas (Manganelli *et al*., 2012; Barnes *et al*., 2017; Wesolowska *et al*., 2018). In several areas considered non-endemic, such as Europe, USA, Gulf States, migration from developing countries contributed to an increased number of parasite infections (Abu-Madi *et al*., 2010; Norman *et al*., 2015a, 2015b).

In Italy, among the immigrant communities coming from Eastern Europe, Africa, Asia, and Central and South-America, the prevalence of intestinal parasite was 2.6 times higher than that of non-immigrant groups (Masucci *et al*., 2011). In particular, Manganelli *et al*. (2012) observed that, among children aged between 0 and 15 years of European, African, Asian, and South-American origin, 15 % were infected by parasites, and prevalence rate increases when they live in shacks or if poor sanitary conditions persist, even after a longer stay in Italy.

The case here presented underlies that the simultaneous parasitic infections can also occur in immigrant children from zero to one-year old, especially during weaning, when the immune system is yet immature (Simon *et al*., 2015). It is very difficult to demonstrate if those infections were acquired locally or were imported, however, *Enterobius vermicularis* and *Entamoeba* spp. are two of the most common parasites detected in Italy (Crotti *et al*., 2013). In Pakistan, the commonest intestinal parasitic infestations are due to *Ascaris lumbricoides*, *Giardia intestinalis*, *Entamoeba* spp., and in minor prevalence to *Enterobius vermicularis* and hookworms (Ullah *et al*., 2014). However, a close relationship between socio-economic conditions and parasitism exists. The presence of organism similar to mites, hookworm and *E. vermicularis* eggs, and cysts of *Entamoeba* spp. confirms that poor sanitary and environmental hygiene can favor contamination and interpersonal parasites transmission, even in developed countries. In fact, the transmission of hookworm and *E. vermicularis* can either occur through contaminated soil or via fecal-oral route, while *Entamoeba* spp. can be acquired through contaminated food and water. Moreover, malnutrition leads children to grow at rates below normal values, making them more vulnerable to infections with one or more parasites, although this possible association is still under debate (Manganelli *et al*., 2012; Someshwaran *et al*., 2015; Gyang *et al*., 2017).

Conclusion

Improving socio-economic conditions could be useful to safeguard people, particularly children, from parasitic infections. Although difficult to perform, mass or periodic stool examinations in endemic areas as well as among high-risk groups in industrialized countries could be helpful to achieve an early diagnosis and reduction in transmission. Improving health education, environmental and personal hygiene, and nutrition quality appear as preventive measures that could contribute to control the risk of parasite transmission and infection. Finally, any reported case of intestinal parasite infestations in children enhance the knowledge on epidemiology, persistence and risk factors.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Case Report

First report of heartworm (*Dirofilaria immitis*) infection in an imported dog in Lithuania

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Summary

Over the past decade, increasing numbers of autochthonous cases of heartworm infection have been reported in the countries of Eastern Europe where previously only imported cases were described. In this report we have described the first clinical case of *Dirofilaria immitis* infection in an imported dog in Lithuania.

In 2018, a 5-year-old male Spanish greyhound (Spanish galgo) was imported to Lithuania from southern Spain and referred to a small animal veterinary clinic in Vilnius for wellness screening. Circulating microfilariae and female antigens of *D. immitis* were detected using the Knott's test and SNAP 4Dx Plus Test (IDEXX Laboratories, Portland, USA). The diagnosis was confirmed using molecular analysis. Treatment according to the guidelines recommended by the American Heartworm Society was applied. This is the first confirmed report of canine heartworm infection in an imported dog in Lithuania. Heartworm-infected dogs transported to North-Eastern Europe from endemic areas could act as microfilarial reservoirs for the local mosquito population, which could increase the risk of spreading the disease.

Keywords: Dirofilariasis; Heartworm; *Dirofilaria immitis*; Lithuania

Introduction

Among all filarial species the most relevant for dogs in Europe are *Dirofilaria repens* and *Dirofilaria immitis*. Adult nematodes of *D. repens* most often are found in subcutaneous tissues, whereas *D. immitis* is the causal agent of canine and feline cardiopulmonary dirofilariasis (McCall *et al*., 2008). Both species are zoonotic, responsible for human ocular/subcutaneous (*D. repens*) and pulmonary (*D. immitis*) dirofilariasis. Although humans are a less suitable host for these parasitic nematodes due to specific immune response that destroys the worm in most cases (Simón *et al*., 2005), in the past two decades the number of infections in humans has been rising (Pampiglione & Rivasi, 2000; Fuehrer *et al*., 2016).

Dogs infected with *D. immitis* usually develop severe life-threatening symptoms. Progression of the disease is chronic. Firstly, adults develop in the vascular and pulmonary system and eventually in the right chambers of the heart (McCall *et al*., 2008).

The infective larvae (L3) of both *D. repens* and *D. immitis* are transmitted by more than 70 mosquito species, including some species of the Lithuanian mosquito fauna (Cancrini *et al*., 2006; Bernotienė, 2012). Looking at the biological life cycle of parasites, both species require the same time interval and temperature for incubation in same mosquito species (Genchi *et al*., 2009).

Over the past decade, increasing numbers of autochthonous cases of heartworm infection have been reported in the following countries of Eastern Europe where previously only imported cases

* – corresponding author

were described: Belarus (Şuleşco *et al*., 2016), Czech Republic (Svobodová *et al*., 2006), Hungary (Jacsó *et al*., 2009; Tolnai *et al*., 2014), Poland (Świątalska & Demiaszkiewicz, 2012), Slovakia (Svobodova *et al*., 2005; Miterpáková *et al*., 2008) and Russia (Kartashev *et al*., 2011). Rapid spread of this parasitic infection in non-endemic regions is caused by several factors. Climate change plays the essential role in the spreading of vector-borne diseases in Europe (Genchi *et al*., 2005). The risk season for the transmission of the disease due to more suitable conditions for vector development is becoming longer (Genchi *et al*., 2009).

Perhaps the most important factor for the spread of dirofilariasis into new areas is the increased movement of infected dogs due to the simplification of human and animal traveling rules and regulations in Europe (Genchi *et al*., 2011).

A growing number of vector-borne pathogens (such as *Babesia canis* and *Anaplasma phagocytophilum*) has been observed in recent years in Lithuania (Radzijevskaja *et al*., 2008, 2017; Paulauskas *et al*., 2014), including *D. repens* (Jankauskaitė *et al*., 2011; Paulauskas *et al.,* unpublished). According to the observations of veterinary practitioners, the highest incidence of canine subcutaneous dirofilariasis (*D. repens*) is registered in the central and western parts of Lithuania.

The aim of this report is to describe the clinical case of *D. immitis* in the dog imported from Spain and therefore to draw attention of veterinary practitioners and owners that the presence of *D. immitis* infected dogs could influence the spread of canine heartworm disease in Lithuania and other areas in North-Eastern Europe.

Material and Methods

Case history and observations

In January 2017, a 5-year-old male Spanish greyhound (Spanish galgo) was found free ranging in Cádiz (province of southern Spain) by a Lithuanian family. The dog was owned by local hunters, but after a leg trauma it was not suitable for hunting. The dog had lost significant amount of its body fat and muscle mass, weighed 13 kg (normal weight of a male Spanish greyhound dog is 27 – 29 kg) and was lethargic and exercise-intolerant. In April 2018, the dog arrived in Lithuania. In May 2018, the dog was referred to a small animal veterinary clinic in Vilnius for wellness screening.

Serology

Serology for circulating female (*D. immitis*) antigens and tick-transmitted pathogens (*Borrelia burgdorferi*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Anaplasma phagocytophilum* and *Anaplasma platys*) was performed using SNAP 4Dx Plus Test (IDEXX Laboratories, Portland, USA).

Microfilaria

The Knott's test was used to detect circulating microfilariae. One ml of EDTA blood was mixed with 9 ml of 2 % formalin in a 12 ml tube and centrifuged for 5 minutes. The supernatant was poured off and two drops of the sediment was transferred to a slide covered with a coverslip and examined with a microscope using low power magnification.

PCR

A molecular analysis was performed to confirm the diagnosis. Genomic DNA was extracted from 200-µl aliquots of EDTA blood (taken from *Vena cephalica* of the examined dog) using the GeneJet Whole Blood Genomic DNA Purification kit (Thermo Fisher Scientific, Lithuania) according to the manufacturer's instructions. Species identification was based on amplification of partial internal transcribed spacer 2 (ITS-2) region of the ribosomal DNA using the panfilarial primer set DIDR-F1 and DIDR-R1 which allow to differentiate between *D. immitis* and five other filariae found in dogs (Rishniw *et al*., 2006). PCR amplification was carried out in a total volume of 20 µl containing 1X PCR buffer, 1.5 mM MgCl2, 250 µM dNTPs, 0.5 µM of each primer, 1 U of Taq polymerase (Thermo Fisher Scientific, Lithuania) and 1 µl of DNA template. Amplification was performed as follows: denaturation at 94 °C for 2 min and 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 60 °C), and extension (30 s at 72 °C) and a final extension (7 min at 72 °C). The amplification product was detected after electrophoresis in 1.5 % of agarose gel, revealed by staining with ethidium bromide and visualized under UV light. The PCR product was purified using a commercial kit (GeneJet Gel Extraction Kit, Thermo Fisher Scientific, Lithuania) and analysed by sequencing (performed at Macrogen Europe, Amsterdam). The obtained sequence was compared to those available in the GenBank database by using the Basic Local Alignment Tool (BLAST) analysis and Mega 6.0 software. Sequence obtained in this study was aligned with sequences derived from GenBank using CLUSTAL W algorithm. The phylogenetic tree was generated using maximum likelihood method (ML) with 1000 bootstraps replications.

Following the confirmation of the diagnosis, echocardiography and chest radiography were carried out to evaluate the patient prognosis.

Treatment

Treatment using the guidelines recommended by the American Heartworm Society (Nelson *et al*., 2014) was applied.

Adjunct therapy

The routine dose of prednisone at 0.5 mg/kg twice daily for the first week and 0.5 mg/kg once daily for the second week, 0.5 mg/kg every other day for the third and fourth weeks and doxycycline at 10 mg/kg twice daily for 4 weeks was applied. To kill susceptible larval stages milbemycin oxime (0.5 mg/kg) at day 30 of treatment was administered orally and continued monthly (Nelson *et al*., 2014).

Adulticide therapy

Following the adjunct therapy, three-dose regimen of melarsomine (one injection of 2.5 mg/kg body weight followed at least one

month later by two injections of the same dose 24 hours apart) was used (Nelson *et al*., 2014).

Ethical Approval and/or Informed Consent

Animal care and handling were carried out in accordance with institutional guidelines.

Results

During the clinical examination, no symptoms of heartworm disease were detected. Thoracic radiograph and echocardiography did not show any identifiable abnormalities commonly found with heartworm disease and appeared fairly normal, most likely due to an early asymptomatic stage of infestation with *D. immitis*.
In ELISA negative results were reported for *B. burgdorferi*, *E. canis*, *E. ewingii*, *A. phagocytophilum*, *A. platys* antibodies and positive for circulating female (*D. immitis*) antigens. Low numbers of microfilaria were found in the examined blood sample using the Knott's test.
Visualization of the PCR product of the examined sample by gel electrophoresis demonstrated DNA fragment of the expected size for *D. immitis* (542 bp). The sequence analysis of partial ITS-2 region revealed that the sequence had 99 – 100 % identity (with 1- to 3 nucleotides difference) with the *D. immitis* sequences deposited in GenBank, thereby confirming the *D. immitis* diagnosis. The phylogenetic analysis based on partial ITS-2 region sequence (Fig. 1) shows phylogenetic relationship of *D. immitis* obtained in present study and other filarial nematodes. Partial ITS2 region sequence was submitted to the GenBank database under the accession number MH663471.

Using the recommended treatment within 12 hours of the first injection of melarsomine, tenderness at the injection site, fever, lethargy and tremor were noted and after 24 hours the symptoms completely disappeared. Two weeks after the first injection of melarsomine, the dog started to gain weight, became more active and started to tolerate exercises. No side effects were noticed after the second and third melarsomine injection. Knott's test ant circulating female antigen test will be repeated at day 120 and 271 from starting treatment.
Information about this case are summarized in Table 1.

Table 1. Summarized case information.

Species	Domestic dog
Breed	Spanish greyhound
Age	5 year old
Imported from	Spain
Imported in	Lithuania
Weight before treatment	13.0 kg
Weight after treatment	21.0 kg
Symptoms	• Lethargic • Exercise-intolerant
Knott's test	Positive
Serology	Positive
PCR	Positive
Adjunct therapy	• Prednisone (0.5 mg/kg) • Doxycycline (10 mg/kg) • Milbemycin oxime (0.5 mg/kg)
Adulticide therapy	• Melarsomine (2.5 mg/kg)

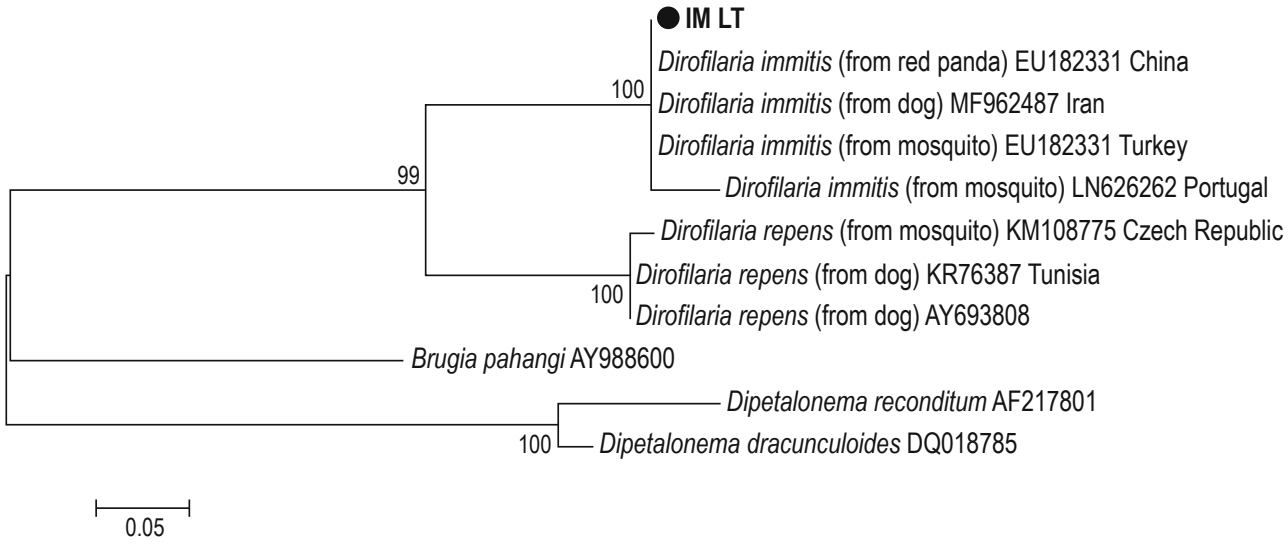

Fig. 1. The phylogenetic tree generated using maximum likelihood (ML) method, based on partial ITS-2 sequences. Maximum Likelihood bootstrap values associated with the branches. Sequence of *D. immitis* obtained in this study are marked with black circle (accession number MH663471).

Discussion

Previous research and published reports suggest that the geographical range of canine cardiopulmonary dirofilariasis has expanded in Europe in the past decade due to increasing movement of dogs and global changes in climate (Genchi *et al*., 2005). Many reports about imported cases of dirofilariasis have been published in different Central and Eastern European countries in the past decade and a few years later those areas became endemic for heartworm disease (Genchi *et al*., 2011).

Despite that both species, *Dirofilaria repens* and *Dirofilaria immitis,* require the same conditions for development, subcutaneous dirofilariasis spreads faster in new non-endemic areas than heartworm disease (Genchi *et al*., 2011). There are several explanations for it. Firstly, heartworm disease most often ends lethally due to adult worm development in the cardiovascular system, for that reason, infected dogs have shorter period of transmitting the disease, than the dogs infected with *D. repens*. Secondly, most cases of the subcutaneous dirofilariasis infection are asymptomatic, therefore dogs infected with *D. repens* could be the source of infection for several years. Dogs infected with *D. immitis* often develop clinical signs and are referred to the veterinary clinic where appropriate treatment is provided and transmission of the disease is prevented (Genchi *et al*., 2011).

Despite the fact that the period suitable for heart worm transmission in Lithuania is short (Genchi *et al*., 2005), previously reported autochthonous cases of subcutaneous dirofilarias and knowledge that both filarial species require the same temperature and the same time interval for incubation in vector suggest that an increase in the autochthonous heartworm cases in this area is possible. Furthermore, numerous suitable vector species (such as *Culex pipiens* s.l.*, Anopheles maculipennis* s.l.*, Aedes vexans*) for parasite development and transmission are found in Lithuania (Bernotienė, 2012).

Due to the complicated parasite life cycle, heartworm disease is chronic and asymptomatic in the primary stage of development. Veterinary clinicians in non-endemic areas lack experience in identifying the disease, therefore most cases are under diagnosed, microfilaremic dogs often do not get appropriate treatment and become the source of infection to the local mosquito population (McCall *et al*., 2008).

This is the first case report of heartworm infection in Lithuania in the dog imported from Southern Europe confirmed by serological, cytological and molecular methods. On the basis of this report it can be stated that heartworm-infected dogs transported to Lithuania from parasite endemic areas could act as donors of microfilariae to local mosquito species. Protocols of periodic heartworm antigen testing, in particular for traveling dogs, enable diagnosing early stages of heartworm disease and preventing transmission of microfilariae. A clear understanding of the biological life cycle of *D. immitis,* importance of asymptomatic dog treatment, and disease prevention in healthy dogs are critical to stop the spread of the disease in previously non-endemic areas.

Conflict of interest statement

Authors state no conflict of interest.

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Case Report

First report of *Sarconema eurycerca* (Filarioidea) in mute swan (*Cygnus olor*) in Poland – the case report

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Summary

Mute swans (*Cygnus olor*) of the Anatidae family are common in wetlands of Europe. They winter in Africa, Asia and some parts of Europe. The species is exposed to many pathogens in its places of residence, including parasites possibly introduced from tropical countries by other species of birds that take long wanderings and occupy a similar ecological niche. One such case is the infection of the *Sarconema eurycerca*, nematode belonging to the Filarioidea family. It invades the bird's myocardium and, according to some authors, this nematode may be one of the main causes of swans' deaths. The material for the present study was an approximately 2 year old female mute swan, which during the flight fell suddenly to the ground in Pomorskie Voivodeship (Poland, 53°50'18"N 18°12'54"E) in November. During the examination and medical observation, weakness, diarrhea and infestation with lice were found. The cause of its eventual death was attributed to a failure of the circulatory system. Post mortem, two abscesses with diameters of 2-3 cm were found in its liver parenchyma. Three nematodes were visible in the epicardium and many more in myocardium. Upon cutting open the heart, small yellowish foci, about 1 mm in diameter were scattered over valves. On the basis of morphological features, infection by *Sarconema eurycerca* was concluded. As far as we are aware, in Poland there were no earlier reports of this parasite infecting a swan.
Keywords: case report; Cygnini; hearth worms; myocardium; nematode; waterfowl

Introduction

Mute swans (*Cygnus olor*) are partially migratory water birds belonging to the Anatidae family. They are quite common around water bodies in Poland. In recent years, swans wintering in Poland have also been observed, both on non-freezing inland waters and on the Baltic coast, e.g. in the Gulf of Gdańsk (Bzoma & Meissner, 2005). Migratory flights occur in February – May and September – December. Swans are large birds: adults measuring 144 – 158 cm measuring from the beak to the end of the tail, with a wing span of 2 – 2.5 m. They over-winter in northern Africa, central and southern Asia and in some parts of Europe (Wieloch, 1991). The resident breeding population of these birds is estimated at approximately 5,000 – 6,000 pairs. Their diet consists mainly of plant food with the addition of crustaceans and other invertebrates. They can consume up to 10 kg of aquatic plants per day (Minnesota Department of Natural Resources). Mute swans are precocial birds, hence the cygnets are looked after by both the male and the female. In Poland, as by The Birds Directive (Directive 2009/147 / EC of 30 November 2009) they are subject to strict species conservation and require active protection. Unfortunately though, the mute swan is susceptible to an array of pathogens, both those endemic to its breeding grounds and those acquired from tropical countries during their own migration or by other birds, e.g. by ducks (Gaidet

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et al., 2010; Takekawa *et al*., 2010). Among the most dangerous swan pathogens are parasites. From the group of endoparasites, the most frequently detected are: *Trichomonas* spp., *Eimeria* spp., Schistosomatidae, *Echinoparyphium recurvatum*, *Orchipedum tracheicola*, *Wardoides nyrocae*, *Amidostomum anserinus*, *Capillaria* spp., *Echinuria uncinata*, *Sarconema eurycerca*, *Filicollis anatis* and *Polymorphus minutis* (Pennycott, 1998; 1999; Ballweber, 2004). In turn, in the group of ectoparasites, mainly infections of lice (Mallophaga) are diagnosed, e.g. *Anaticola cygnopsis*, *Ciconiphilus pectiniventris*, *Trinoton querquedulae* and *Trinoton anserinum* (Lapage, 1961; Cohen *et al*., 1991; Ballweber, 2004).
Most relevant to the present report is the *S. eurycerca* nematode of the Filarioidea family which occurs in many species of wild waterfowl including swans and geese (Cole, 1999; Bartlett, 2008). Adult worms settle in the myocardium, probably in the coronary veins, within the epicardium, myocardium and endocardium. The female nematode gives birth to microfilaria, which then circulate in the bird's peripheral blood, from where they can be taken up by ectoparasites. From blood-collecting arthropods, microfilaria enter the final host in which they migrate to the myocardium. There, they reach sexual maturity (Wehr, 1939). An important vector, as well as an intermediate host for the cardiac worm *S. eurycerca,* are the aforementioned lice *Trinoton querquedulae* and *T. anserinum* (Wehr, 1939), as confirmed in the studies by Seegar *et al*. (1976), and. According to some authors, *S. eurycerca* may be one of the main causes of swan deaths (Ballweber, 2004).

Material and Methods

The object of the present study was a female mute swan weighing 8.20 kg at the age of about two years as indicated by grey tips of the alulas and by the absence of annulus. According to witnesses, the bird suddenly fell to the ground while flying over the village of Czubek, the municipality of Kaliska, Pomorskie Voivodeship (53°50'18"N 18°12'54"E). The swan was recovered alive and was transported to the Pomeranian Center for Rehabilitation of Wild Animals "Ostoja" in Pomieczyn. The swan stayed and was treated at the Pomeranian Center from November 9, 2017 to the day on which the bird was found dead on November 18, 2017. At the Center, the swan had no contact with other animals; it was lodged in solitary confinement in a covered aviary for water birds, with easily washable floors, a swimming pool with drain, rubber mats and artificial grass. During the stay at Pomeranian Rehabilitation Center for Wild Animals full clinical examination was performed.
During the stay, the following treatment was used: Insectin (permetrine cis / trans 25:75, 10 mg / g, external: Biowet; Puławy; Poland), Betamox L.A. (amoxicillin 150mg / ml; ScanVet; Skiereszewo; Poland, 300.0 mg *subcutaneous* every 24 hours), Orungal (itraconazole 100mg; Janssen-Cilag International NV; Beerse; Belgium, 50.0 mg *per os* every 24 hours). The bird did not feed on its own, therefore it was fed with esophageal feeding tube two times a day with a mix of rescue feeds Dr Ziętek for animals feeding on seeds and plants (Manufacturer - Ambulance of small mammals Dr Ziętek, Lublin, Poland) along with supplementation with Oro-Digest and Probi-Zyme (Versele-Laga, Deinze, Belgium, acc. manufacturer's recommendations).
A full postmortem examination was performed in Pomeranian Rehabilitation Center for Wild Animals "Ostoja" according to standard guidelines (Van Riper & Van Riper, 1980). After the detection of parasites, the heart was transported to Department of Parasitology and Invasive Diseases, for further diagnosis. No other tissues were collected. During heart autopsy, nematodes were dissected from epicardium and fixed in 70 % ethanol for further testing. Parasites species were identified based on their morphological characteristics under a Leica M165C stereoscopic microscope (Leica Microsystems GmbH, Wetzlar, Germany) (×40 magnification). The scientific publications available in Pubmed were helpful in identifying the parasites (Wehr, 1939; Holden & Sladen, 1968; Bekir *et al*., 2015). Descriptive statistic of nematodes length (Mean – *M*; Median – *Me*; Standard Deviation – *SD*; Standard Error – *SE*: Coefficient Interval 95 % – *CI 95 %*; Variance – *V*) was calculated using the Statistica 13.1 program.

Ethical Approval and/or Informed Consent

The swan was handled according to good veterinary practice and Polish veterinary regulations. Pomeranian Center for Rehabilitation of Wild Animals "Ostoja" in Pomieczyn has permission from the Ministry of the Environment for treating and holding wild animals (DZPWG.6520.21.2015.mk).

Results and Discussion

At the examination, no bone fractures were detected. Numerous but minor abrasions around the right elbow and right foot, caused by falling, did not require skin care procedures, and analgesics were not used. In the feathers, lice infestation was present but species identification was not performed. There were some cornified plantar masses on the bird's feet. During continued observation, the following symptoms were recorded: weakness and diarrhea (only at the beginning of the stay, probably caused by stress related to the transport to the Center) and abnormal for the species reactions to environmental stimuli and to the presence of humans, in the form of excessive vocalization and aggression.
The cause of the swan's death 10 days after arrival at the Center was a failure of the circulatory system. Post mortem, two abscesses with diameters of 2 – 3 cm were found in its liver parenchyma. Three nematodes were visible in the epicardium (see Fig. 1), and numerous nematodes were present in the myocardium. Upon cutting open the heart, small yellowish foci about 1 mm in diameter were scattered over valves. Pancarditis and dilated cardiomyopathy within the ventricles were demonstrated during cardiac section examination. The official pathological diagnosis was avian parasitic pancarditis.

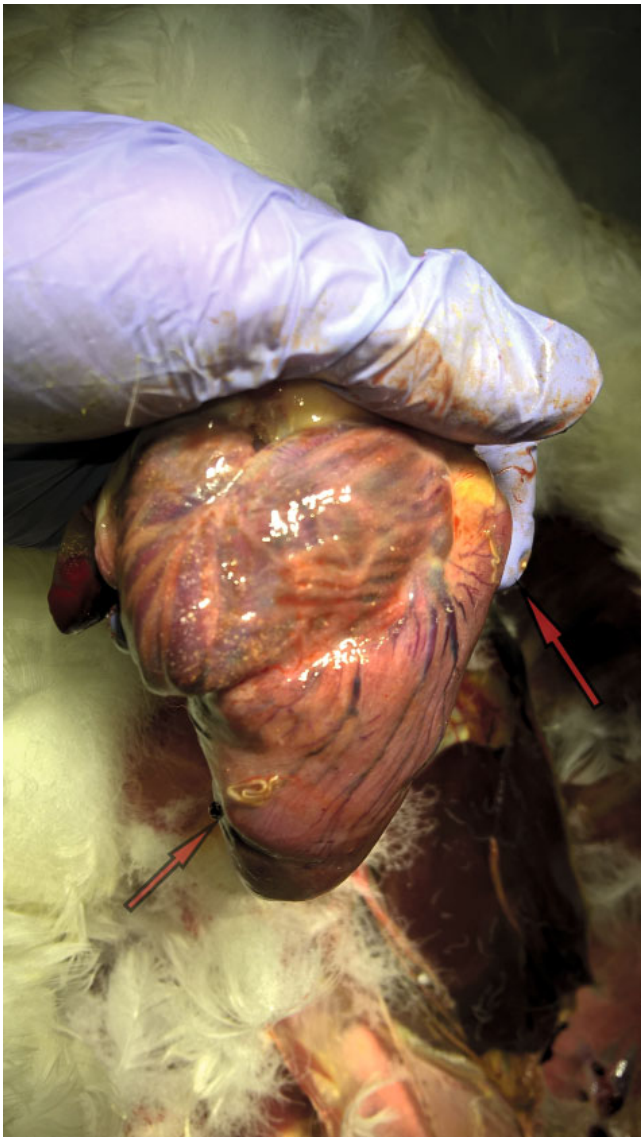

Fig.1 A photo from a anatomopathological study of a mute swan showing the heart with *Sarconema eurycerca*. The nematodes were marked with arrows.

The nematodes mean long was *M*= 6,87 cm (*Me*= 6.9; *SD*= 0.35; *SE*=0.20; *CI 95 %*= 5.99 – 7.74; *V*= 5.11). On the basis of morphological features, it was established that the nematodes belong to the *Sarconema eurycerca* species. Most likely, this nematode infection of the heart led to the fall of the bird (as a result of cardiorespiratory distress) and, subsequently, to its death from heart failure. *Sarconema eurycerca* has been most often observed in North America and in Asia. It was first identified and described in the USA in the states of Washington, Wisconsin and Utah in whistling swan *Cygnus columbianus* (Wehr, 1939). In 1975, *Sarconema eurycerca* infections in Canada were recorded by MacNeil (1975) in whistling swan and independently by Irwin who found that the prevalence of this parasite in an Ontario swan population amounted to over 50 % in the same species. Decades later, the nematode was reported in Japan (Yoshino *et al*., 2009) and Korea (Woo *et al*., 2010). Soon thereafter, Saparov *et al*. (2013) reported 15 cases of the infection of *S. eurycerca* in the wild Anseriformes in Uzbekistan, and one year later Bekir *et al*. (2014) described the first case in Turkey.

The available literature provides also evidence of the migration of this nematode towards Europe. The first case report of *S. eurycerca* diagnosed in Europe came from England (Boughton, 1965). Forty years later, the nematode was diagnosed in Austria (Khayal *et al*., 2010) and in the Netherlands (de Bruijn, 2009). Currently, there are no reports of this parasite from Central or Northern Europe. In particular, it is our understanding that the parasite has never before been recognized in Poland (Kavetska, 2008).

The reports from Western Europe pointed to a similar course of infection and its consequences for swans as in our case. Kluge (1967) described *S. eurycerca* pancarditis with yellowish foci scattered over the epicardial and endocardial surfaces of the heart and throughout the 1 – 2 mm myocardium. Similar changes were observed in our case. According to available literature the most important changes in the histopathological picture of the heart during the *S. eurycerca* infection include: scattered foci with zigzag pattern of myocardial fibres, numerous basophilic granules in their sarcoplasm, interstitial fibrosis throughout the myocardium, focal areas of chronic inflammation characterized by fibrin deposition, local mineralization within the epicardium, as well as endocardium and myocardium necrosis (Kluge, 1967; Woo *et al*., 2010). In studies on physiological effects of *S. eurycerca* on birds, a significant reduction in body weight was observed regardless of age and gender (Seegar, 1979). In our case, we also found an approximate 30 % reduction in the bird's body weight (8.2 kg), as the normal range for 2 year old mute swan females is 11 – 12 kg. Due to the location of the parasite, detection of the infection is difficult. So far, no diagnostic methods or treatment have been developed against *S. eurycerca*, which in future may pose a threat to native swan populations, and also creates the possibility of endemic sites for this parasite in Europe. The detection of the infection of *S. eurycerca* in northern Poland indicates the need for monitoring wild birds, especially swans, in the direction of infection with this nematode. It is also advisable to pay attention to the occurrence of similar cases in other areas of Northern and Central Europe.

Conflict of Interest

Authors state no conflict of interest

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Research Note

Helminths of mustelids with overlapping ecological niches: Eurasian otter *Lutra lutra* (Linnaeus, 1758), American mink *Neovison vison* Schreber, 1777, and European polecat *Mustela putorius* Linnaeus, 1758

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Summary

This study presents the helminthological data on three mustelid species with overlapping ecological niches in Lithuania. In general, 14 helminth species or higher taxa were reported from all mustelids: *Isthmiophora melis*, *Strigea strigis* metacercariae, *Pseudamphistomum truncatum*, *Alaria alata* mesocercariae, *Phyllodistomum folium*, *Opisthorchis felineus*, *Metametorchis skrjabini*, *Mesocestoides* sp., *Taenia martis*, *Aonchotheca putorii*, *Crenosoma schachmatovae*, *Eucoleus aerophilus*, *Molineus patens*, and Nematoda g. sp. The largest number of helminths was detected in *M. putorius* (11) and *N. vison* (10) from wetlands; 7 helminths were detected in *M. putorius* from forests, and 8 in *N. vison* and 4 in *L. lutra* from water bodies. Habitat-related differences were found in the abundance and prevalence of *E. aerophilus* in *M. putorius*. *M. putorius* has higher indices of infection by *I. melis*, *S. strigis* metacercariae, and *E. aerophilus* compared to *N. vison* in wetlands. Differences in the abundance and prevalence of *P. truncatum* among *N. vison* and *L. lutra* in water bodies have been observed. Helminths detected in *N. vison* in the present study are native European parasites.
Keywords: *Lutra lutra*; *Neovison vison*; *Mustela putorius*; helminths

Introduction

The Eurasian otter *Lutra lutra* (Linnaeus, 1758) (subfamily Lutrinae), the American mink *Neovison vison* Schreber, 1777 and the European polecat *Mustela putorius* Linnaeus, 1758 (subfamily Mustelinae) are mammals belonging to the family Mustelidae, with different affinity to the aquatic environment.

It is thought, that *L. lutra* originated in Asia and spread into Europe at the latest Pleistocene and early Holocene (Willemsen, 1992). Due to the loss of the riparian habitat, water pollution, polychlorinated biphenyls (PCBs) concentrations, hunting, declining food resources and road traffic accidents, *L. lutra* population declined in all of its distribution during the 20th century (MacDonald & Mason, 1988; Lodé, 1993b; Roos *et al*., 2015). Today *L. lutra* is listed as "Near Threatened" though it has one of the widest distributions of all Palearctic mammals (cover Europe, Asia and North Africa) (Roos *et al*., 2015). *L. lutra* is semi-aquatic mustelid found in a variety of aquatic habitats (Mason & MacDonald, 1986), whose diet consists mainly of aquatic prey (Bonesi *et al*., 2004). The species has been the subject of several more detailed helminthological studies, mainly in Belarus (Shimalov *et al*., 2000; Anisimova, 2002), Poland (Górski *et al*., 2010), Ukraine (Korol *et al*., 2016), United Kingdom (Fahmy, 1954; Jefferies *et al*., 1990; Weber, 1991; McCarthy & Hassett, 1993; Sherrard-Smith *et al*., 2015b), Germany (Schuster *et al*., 1988), and southwest Europe (Torres *et al*., 2004). Parasites act as a factor which could have an impact on the otter population dynamics, therefore the knowledge of the parasites may be useful for protecting the species.

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N. vison was introduced to Europe at the beginning of 20th century from North America for the purpose of fur farming (Brzeziński & Marzec, 2003). As a result of escapes, deliberate releases, and farm damages by 1990s the feral *N. vison* population was registered almost in all European countries (Bonesi & Palazon, 2007; Lecis *et al*., 2008). Due to competition for food resources and space, *N. vison* is considered as an invasive species which could have an impact on the decline or disappearance of the European mink *Mustela lutreola* (Linnaeus, 1761) population in Europe (Maran & Henttonen, 1995). More recently *N. vison* has been considered as having a negative impact on the populations of *L. lutra* and *M. putorius* (MacDonald & Harrington, 2003; Melero *et al*., 2012). *N. vison* as well as *L. lutra* are semi-aquatic mustelids, however *N. vison* exploits both aquatic and terrestrial prey (Bonesi *et al*., 2004). The study of parasites related to the *N. vison* invasion in new regions is important due to possibility of introduction of new parasites to endemic host and transfer of endemic parasites to a new host. The impact of the introduced *N. vison* on parasite transmission has been studied (e.g., Ivanov & Semenova, 2000; Sherrard-Smith *et al*., 2015a; Martínez-Rondán *et al*., 2017).

M. putorius occurs throughout the Western Palearctic (Mitchell-Jones *et al*., 1999). In the last century its population sharply declined across Europe due to increase in human activities (Baghli *et al*., 2005). Today the species is listed as Least Concern in the IUCN Red List of Threatened Species (Skumatov *et al*., 2016). In northern and central Europe *M. putorius* is known to occupy a variety of habitat types: rivers, marshes, forests, woodland, farms, and villages (Jędrzejewski *et al*., 1993; Lodé, 1994; Baghli *et al*., 2005). According to Rondinini *et al*. (2006) the species is strongly associated with riparian areas in mainland Europe. Based on that, all three mustelid species (*L. lutra*, *N. vison*, and *M. putorius*) could present competition among them, because they exhibit overlap in diet and habitat preference (Lodé, 1993b; Bonesi *et al*., 2004).

No studies of *L. lutra* helminths from Lithuania have been reported to date. There is also poor documentation of *N. v*ison and *M. putorius* parasites in this country. Earlier, unidentified nematodes and *Isthmiophora melis* (Schrank, 1788) Lühe, 1909 were reported in *M. putorius* (Maldžiūnaitė, 1959; Kazlauskas & Prūsaitė, 1976). Larvae of *Trichinella* Railliet, 1895 and *Alaria alata* (Goeze, 1782) were also documented in this host species (Grikienienė *et al*., 2001; Senutaitė & Grikienienė, 2001). Helminths of mustelids, including *N. vison* and *M. putorius* were reported by Nugaraitė *et al*. (2014).

The aim of this study was to explore the helminth communities of three mustelids with overlapping ecological niches: *L. lutra*, *N. vison*, and *M. putorius*.

Material and Methods

Carcasses of 6 *L. lutra*, 59 *N. vison*, and 27 *M. putorius* were collected in different localities of Lithuania between 2013 and 2017 (Fig. 1). *N. vison* and *M. putorius* were hunted by hunters and collected from car accidents, while *L. lutra* individuals were collected only from car accidents. Mustelids were assigned to

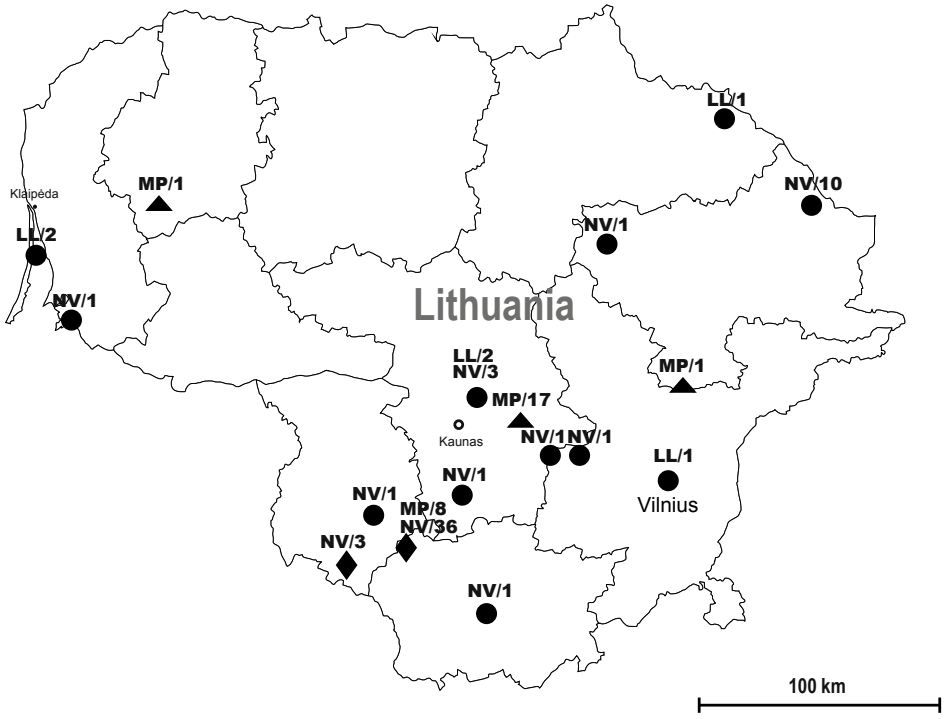

Fig. 1. Collection sites of *Lutra lutra* (LL), *Neovison vison* (NV), and *Mustela putorius* (MP) in Lithuania.
(● – water bodies; ▲ – forests; ♦ – wetlands). The numbers on the map indicate the numbers of collected animals.

the closest habitat and grouped as follows: (1) forests – *M. putorius* (n = 18); (2) wetlands – *N. vison* (n = 39) and *M. putorius* (n = 8); and (3) water bodies (rivers, lakes, and lagoons) – *N. vison* (n = 20) and *L. lutra* (n = 6). All carcasses were stored at -20°C until examination. Mustelids were examined using the method of total helminthological dissection of individual organs (Ivashkin *et al*., 1971). Frontal sinuses, connective tissue between the muscle fibres, trachea, lungs, heart, liver, gall bladder, kidney, urinary bladder, and entire gastrointestinal tract (stomach, small and large intestines) were analysed. Parasites were collected and stored in 70 % ethanol until studied.

Temporary preparations were used for nematode morphological identification, while trematodes and cestodes were identified using permanent preparations. The identification was based on publications of Kozlov (1977), Sidorovich (1997), Kostadinova & Gibson (2002), Vieira *et al*. (2012), and Kontrimavičius (1969).

Helminthological terms were used according to the recommendations of Bush *et al*. (1997). The 95 % confidence intervals for prevalence were calculated as described by Rojtman & Lobanov (1985). Differences in the abundance and prevalence were tested using the Mann-Whitney U test and the Fisher's exact test respectively.

Ethical Approval and/or Informed Consent

The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. The study protocol no.

2017.03.22 No. 13. was approved by License of Environmental Protection Agency (EPA).

Results

Fourteen species or higher taxa of helminths were found in three species of mustelids from all habitats, i.e., 10 helminths in *N. vison*, 12 in *M. putorius*, and 4 in *L. lutra* (Table 1). The largest number of helminths was detected in *M. putorius* (11) and *N. vison* (10) from wetlands; 7 helminths were detected in *M. putorius* from forests, 8 in *N. vison* and 4 in *L. lutra* from water bodies. *N. vison* and *M. putorius* from wetlands shared 9 helminths; *M. putorius* from forests and wetland shared 6 helminths.

The abundance and prevalence of *Eucoleus aerophilus* (Creplin, 1839) in *M. putorius* from wetlands was higher than in *M. putorius* from forests (p = 0.003/0.004 respectively). The abundance and prevalence of *Strigea strigis* (Schrank, 1788) metacercariae (p = 0.014/0.019) and *E. aerophilus* (p = 0.030/0.011) in *M. putorius* was higher (p<0.05) that in *N. vison* from wetlands. The abundance of *I. melis* in *M. putorius* was also higher that in *N. vison* (p = 0.010) from wetlands. *N. vison* from different habitats shared 8 helminth species, but differences in the abundance and prevalence of all helminth species were insignificant at p>0.05.

The abundance and prevalence of *Pseudamphistomum truncatum* (Rudolphi, 1819) in *L. lutra* from water bodies was higher that in *N. vison* (p = 0.000/0.002). *A. alata* mesocercariae was detected only in mustelids collected from wetlands. All three mustelids shared *I. melis* and *P. truncatum.*

Discussion

Of 14 helminths found and discussed in the present study, most are reported in at least one of three species of mustelids in other countries (Table 2), with the exception of *Phyllodistomum folium* (Olfers, 1816) and *Crenosoma schachmatovae* Kontrimavičius, 1969.

P. folium was collected from *L. lutra* stomach. *P. folium* is a characteristic parasite of the northern pike *Esox lucius* Linnaeus, 1758, yet it is found in other fish as well (Bykhovskaya-Pavlovskaya & Kulakova, 1987). The occurrence of *P. folium* in *L. lutra* results from feeding on definitive host-fishes of the fluke.

C. schachmatovae have been described in stoat (*Mustela ermine* Linnaeus, 1758) from Karelia, Russia (Kontrimavičius, 1969). In Lithuania, *C. schachmatovae* have been reported in *N. vison, M. putorius*, and stone marten (*Martes foina* (Erxleben, 1777)) (Nugaraitė *et al*., 2014).

Cestodes of the genus *Mesocestoides* were not determined to the species level due to the poor preservation status of the specimens, however most measurements coincided with those of *Mesocestoides lineatus* (Goeze, 1782). This tapeworm is documented in other countries in *M. putorius*, *N. vison*, and *L. lutra* (Table 2).

Habitat-related differences were found in the abundance and prevalence of *E. aerophilus* in *M. putorius*. *E. aerophilus* has both a direct (by ingestion of larvated eggs) and indirect (by ingestion of oligochaetes containing larvae) life cycle (Anderson, 2000). The diet of *M. putorius* has not been studied in Lithuania, however according to other authors *M. putorius* does not feed on earthworms (Hammershøj *et al*., 2004; Malecha & Antczak, 2013). Therefore, the direct life cycle is likely the main mechanism of transmission of *E. aerophilus* to the host. Conditions for the survival of eggs are better in wetlands, consequently, wetlands offer a better environment for the life cycle realization.

In wetlands *M. putorius* and *N. vison* shared 9 helminths and, compared to *N. vison, M. putorius* was more parasitized by *I. melis*, *S. strigis* metacercariae, and *E. aerophilus*. Sharing the dens between *N. vison* and *M. putorius* may increase the risk of infection with the same species of helminths. Both mustelids do not have specialized diets, are generalist predators and can easily exploit different food resources (Bartoszewicz & Zalewski, 2003; Malecha & Antczak, 2013). According to Lodé (1993a) in wetlands *N. vison* mainly preys on fish and birds, whereas *M. putorius* consumes more rodents and amphibians. Amphibians, as an important component of the diet of *M. putorius*, were also suggested by other authors (Jędrzejewski *et al*., 1993; Hammershøj *et al*., 2004). So, the difference in diet could be a reason why *M. putorius* were more parasitized with helminths in whose life cycle amphibians play a role (i.e., *I. melis*, *A. alata*, and *S. strigis*). Transmission of *A.*

Table 1. Helminths of *Lutra lutra*, *Neovison vison*, and *Mustela putorius* from different habitats in Lithuania.
A – mean abundance, SD – standard deviation, P, % – prevalence (95 % confidence intervals)

		Lutra lutra		*Neovison vison*		*Mustela putorius*	
	Habitat	**A ± SD**	**P, %**	**A ± SD**	**P, %**	**A ± SD**	**P, %**
Trematoda							
	F					258.7 ± 400.7	77.7 (55.9 – 93.6)
Isthmiophora melis	Wa	4.5 ± 2.8	50.0 (14.1 – 85.9)	34.3 ± 49.9	70.0 (48.2 – 87.9)		
	We			46.4 ± 60.1	77.0 (62.3 – 88.8)	449.5 ± 656.5	87.5 (57.0 – 100.0)
	F					24.6 ± 30.2	61.1 (37.8 – 82.0)
Strigea strigis metacercariae	Wa			5.7 ± 11.5	30.0 (12.1 – 51.8)		
	We			6.1 ± 14.9	28.2 (15.1 – 43.5)	13.3 ± 15.0	75.0 (40.9 – 97.1)
	F					1.0 ± 3.9	16.6 (3.4 – 37.2)
Pseudamphistomum truncatum	Wa	8.3 ± 11.6	66.7 (27.8 – 95.4)	0.3 ± 1.1	30.0 (12.1 – 51.8)		
	We			1.9 ± 5.4	17.9 (7.5 – 31.7)	0.3 ± 1.0	12.5 (0.0 – 43.0)
Alaria alata mesocercariae	We			-	7.6 (1.5 – 18.2)	-	12.5 (0.0 – 43.0)
*Phyllodistomum folium**	Wa	3.3 ± 8.2	16.7 (0.0 – 53.5)				
Opisthorchis felineus	We					0.1 ± 0.3	12.5 (0.0 – 43.0)
Metametorchis skrjabini	We					1.1 ± 3.1	12.5 (0.0 – 43.0)
Cestoda							
Mesocestoides sp.	Wa			0.05 ± 0.2	5.0 (0.0 – 18.9)		
	We			0.07 ± 0.2	7.6 (1.5 – 18.2)	0.1 ± 0.3	12.5 (0.0 – 43.0)
Taenia martis	We			0.02 ± 0.1	2.5 (0.0 – 10.0)	0.1 ± 0.3	12.5 (0.0 – 43.0)
Nematoda							
	F					32.5 ± 100.7	44.4 (22.5 – 67.6)
Aonchotheca putorii	Wa			22.1 ± 48.0	50.0 (33.0 – 76.0)		
	We			10.6 ± 27.7	33.3 (19.3 – 49.0)	31.7 ± 43.4	50.0 (17.5 – 82.5)
	F					3.2 ± 13.4	16.6 (3.4 – 37.2)
Crenosoma schachmatovae	Wa			0.8 ± 2.7	15.0 (3.0 – 33.9)		
	We			0.3 ± 1.0	10.2 (2.7 – 21.8)	1.7 ± 4.5	25.0 (2.9 – 59.1)
	F					0.05 ± 0.2	5.5 (0.0 – 20.8)
Eucoleus aerophilus	Wa			3.1 ± 13.1	10.0 (1.0 – 26.9)		
	We			1.4 ± 4.3	15.3 (5.8 – 28.5)	2.5 ± 3.9	62.5 (28.1 – 91.0)
	F					2.8 ± 8.6	22.2 (6.4 – 44.1)
Molineus patens	Wa			2.0 ± 7.7	20.0 (5.7 – 40.2)		
	We			0.7 ± 3.1	12.8 (4.2 – 25.2)		
Nematoda g sp.	Wa	1.6 ± 4.1	16.7 (0.0 – 53.5)				

Forests (F) – *M. putorius* (n = 18); Wetlands (We) – *N. vison* (n = 39); *M. putorius* (n = 8); Water bodies (Wa) – *N. vison* (n = 20); *L. lutra* (n = 6); *: Fish-specific trematode

alata and *S. strigis* usually occurs through amphibians (Kontrimavičius, 1969; Shultz & Gvozdev, 1972), while *I. melis* is transmitted through both amphibians and freshwater fishes (Dönges, 1964; Radev *et al*., 2009). Reptiles, birds and small mammals can serve as paratenic hosts for *A. alata* (Kontrimavičius, 1969). For these two species, mustelids are paratenic hosts (Kontrimavičius, 1969) and are likely infected when they eat second intermediate hosts or, for *A. alata*, second intermediate or paratenic hosts.

Differences in the abundance and prevalence of *P. truncatum* among *N. vison* and *L. lutra* in water bodies have been observed. The life cycle of *P. truncatum* include two intermediate hosts; the first intermediate host is freshwater *Bithynia* snails and second is the Cyprinidae fish species (Hawkins *et al*., 2010). Fish is the major prey category in the diet of *L. lutra* (biomass 75.28 %) in Europe (Krawczyk *et al*., 2016). Despite that the diets of *L. lutra* and *N. vison* overlap (Bonesi *et al*., 2004) and both mustelids feed on intermediate hosts of *P. truncatum*, *L. lutra* is likely to consume more fishes than *N. vison*.

M. putorius and *N. vison* from wetlands harboured the richest helminth communities compared with other habitats. Such differences may be related to the differences in the composition and abundance of intermediate hosts among different habitats and the conditions for surviving of free-living stages of parasites which are better in wet environment.

In the present study *I. melis* and *P. truncatum* were detected in all three mustelid species. Detection of *I. melis* and *P. truncatum* in all mustelids is closely associated with their living environment and diet. Introduced *N. vison* and native *L. lutra* are semiaquatic

Table 2. Helminth species of the present study reported in *Lutra lutra* (LL), *Neovison vison* (NV), and *Mustela putorius* (MP) in other countries.

Helminths	Host	Country / Source
I. melis	LL	Germany (Schuster *et al*., 1988), Belarus (Sidorovich & Anisimova, 1999; Shimalov *et al*., 2000)
	NV	Germany (Zschille *et al*., 2004); France (Torres *et al*., 2008); Belarus (Shimalov & Shimalov, 2001); Caucasus (Itin & Kravchenko, 2016)
	MP	Germany (Kontrimavičius, 1969); Bulgaria (Kostadinova & Gibson, 2002), Hungary (Sugár & Matskási, 1978), Poland (Sołtys, 1962; Malczewski, 1964; Kontrimavičius, 1969); Belarus (Shimalov & Shimalov, 2002); Russia (Morozov *et al*., 1939; Kontrimavičius, 1969); Lithuania (Maldžiūnaitė, 1959; Kazlauskas & Prūsaitė, 1976); Former Czechoslovakia (Kontrimavičius, 1969; Mituch, 1972)
S. strigis metacercariae	MP	Belarus (Shimalov & Shimalov, 2002)
P. truncatum	LL	United Kingdom (Simpson *et al*., 2005; Sherrard-Smith *et al*., 2015b, 2016); Poland (Hildebrand *et al*., 2011); Belarus (Sidorovich *et al*., 1997; Shimalov *et al*., 2000); Germany (Schuster *et al*., 1988); Ukraine (Korol *et al*., 2016); Denmark; France; Germany; Sweden (Sherrard-Smith *et al*., 2016); Ireland (Hawkins *et al*., 2010)
	NV	England and Wales (Sherrard-Smith *et al*., 2015a, 2016); Ireland (Hawkins *et al*., 2010); Denmark (Skov *et al*., 2008); Volga Delta (Ivanov & Semenova, 2000); Caucasus (Itin & Kravchenko, 2016); Belarus (Sidorovich & Anisimova, 1997; Shimalov & Shimalov, 2001)
	MP	Belarus (Anisimova, 2002; Shimalov & Shimalov, 2002); Russia (Morozov *et al*., 1939; Kontrimavičius, 1969)
A. alata mesocercariae	LL	Poland (Górski *et al*., 2010); Belarus (Sidorovich *et al*., 1997; Shimalov *et al*., 2000)
	NV	Germany (Zschille *et al*., 2004); Caucasus (Itin & Kravchenko, 2016); Belarus (Shimalov & Shimalov, 2001); Volga Delta (Ivanov and Semenova, 2000)
	MP	Germany, Italy (Kontrimavičius, 1969); Belarus (Shimalov and Shimalov, 2002); Russia (Kontrimavičius, 1969; Kruchkova et al., 2008); Lithuania (Grikienienė *et al*., 2001)
O. felineus	LL	Germany (Schuster *et al*., 1988); Belarus (Shimalov *et al*., 2000)
	NV	Belarus (Shimalov & Shimalov, 2001)
	MP	Belarus (Shimalov & Shimalov, 2002)
M. skrjabini	MP	Gorky Oblast, Russia (Morozov, 1939)
M. lineatus	LL	Belarus (Shimalov *et al*., 2000)
	NV	Germany (Zschille *et al*., 2004); Caucasus (Itin & Kravchenko, 2016)
	MP	Belarus (Anisimova, 2002; Shimalov & Shimalov, 2002); Former Czechoslovakia (Mituch, 1972)
T. martis	LL	Germany (Schuster *et al*., 1988)
	NV	Iberian Peninsula (Torres *et al*., 2006); Germany (Zschille *et al*., 2004)
	MP	Belarus (Shimalov & Shimalov, 2002); Germany (Kontrimavičius, 1969); Former Czechoslovakia (Mituch, 1972)
A. putorii	LL	Poland (Górski *et al*., 2010); France, Spain, Portugal (Torres *et al*., 2004); Belarus (Sidorovich *et al*., 1997; Shimalov *et al*., 2000); Latvia (Vismanis & Ozolins, 1998)
	NV	France (Torres *et al*., 2008), Iberian Peninsula (Torres *et al*., 2006); Caucasus (Itin & Kravchenko, 2016); Belarus (Shimalov & Shimalov, 2001); Spain (Martínez-Rondán *et al*., 2017)
	MP	Belarus (Anisimova, 2002; Shimalov & Shimalov, 2002); Russia (Morozov, 1939); Poland (Górski *et al*., 2006); Iberian Peninsula (Torres *et al*., 1996); France (Torres *et al*., 2008); Belgium (Bernard, 1969); Former Czechoslovakia (Mituch, 1972)
E. aerophilus	MP	Russia (Morozov, 1939; Kontrimavičius, 1969; Kruchkova *et al*., 2008); Belarus (Shimalov & Shimalov, 2002); France (Torres *et al*., 2008)
M. patens	NV	Germany (Zschille *et al*., 2004); Iberian Peninsula (Miquel *et al*., 1993-1994; Torres *et al*., 2006); France (Torres *et al*., 2008); Belarus (Shimalov & Shimalov, 2001); Spain (Martínez-Rondán *et al*., 2017)
	MP	Belarus (Shimalov & Shimalov, 2002); Russia (Morozov, 1939; Kontrimavičius, 1969); Iberian Peninsula (Torres *et al*., 1996); France (Durette-Desset & Pesson, 1987; Torres *et al*., 2008); Belgium (Bernard, 1969); Switzerland (Mermod *et al*., 1983)

mustelids which coexist in many riparian habitats. Moreover, some studies suggest *M. putorius* preference for the aquatic environment (Jędrzejewski *et al*., 1993; Zabala *et al*., 2005; Rondinini *et al*., 2006). Diets of these mustelid species overlap and include intermediate hosts of these two flukes. *L. lutra* diet consists mainly of aquatic prey, *N. vison* and *M. putorius* exploit both aquatic and terrestrial prey (Bonesi *et al*., 2004).

In general, the helminths community of *M. putorius* was richer (12 helminths) compared with *N. vison* (10 helminths), and *L. lutra* (4 helminths). The helminth fauna of *L. lutra* in Lithuania is probably richer, however results in the present study are derived from small number of animals examined. It is related with *L. lutra* protection status in Europe. Despite the fact that its population is widely distributed across Lithuania (covers 95 % of the territory) (Baltrūnaitė *et al*., 2009), hunting has been prohibited since 1975 (Mickevičius, 1993; Baranauskas *et al*., 1994).

The richer helminth fauna of *M. putorius* is probably related with a wide variety of habitats used by this species.

Introduction of *N. vison* in Lithuania occurred after World War II as the result of releases from fur farms in Kaliningrad Oblast, Russia and Lithuania and introduction of animals from Tatarstan (Russia) (Prūsaitė *et al*., 1988; Balčiauskas, 1996). The invasion of *N. vison* in new regions may have led to the introduction of new parasites and their transfer to endemic hosts. Helminths of *N. vison* in North America have been reported by numerous authors (e.g., Beaver, 1941; Zabiega, 1996; Foster *et al*., 2007). A checklist of helminths in *N. vison* from Montana was reported by Barber and Lockard (1973). Some helminth species (i.e., *Metorchis albidus* (Braun, 1893), *Aonchotheca mustelorum* (Cameron & Parnell, 1933) (syn. *Capillaria mustelorum*), and *Baylisascaris devosi* (Sprent, 1952) (syn. *Ascaris devosi*)) detected in *N. vison* from Belarus are supposed as species arrived with this animal from its native area, i.e. North America (Anisimova, 2004).

All helminths detected in *N. vison* in our study are native European parasites, which are also common parasites of a wide range of European mustelids and other mammals. All helminth species found in *N. vison* are also found in *M. putorius*. Helminthological studies in other European countries show similar results that *N. vison* is parasitized by native parasites, e.g. in Spain (Torres *et al*., 2006; Martínez-Rondán *et al*., 2017) and France (Torres *et al*., 2008). Invasive *N. vison* could lose their original parasites, because feral populations come from animals raised in fur farms, where parasite cycling is aggravated. Invasive species are likely to accumulate parasites in the environments inhabited by closely related species (Parker & Gilbert, 2007). In our case infection of invasive *N. vison* with native parasites can be facilitated by contacts with taxonomically closely related native mustelids (e.g. *M. putorius,* etc.) and parasites being widespread generalist with a wide host range. It is worth mentioning that the ranges of some parasite species found in our study include invasive and natural range of *N. vison* (i.e., Palearctic and Nearctic). Helminths found by us are known in *N. vison* from North America: *I. melis* (Beaver, 1941), *A. putorii* (Zabiega, 1996; Foster *et al*., 2007), and *M. patens* (Foster *et al*., 2007). From all above we can conclude that all three studied mustelids exchange helminths and have common species. Helminths community structure is influenced by habitat. Our results show that the epidemiological role of invasive *N. vison* is in the maintenance of the life cycles of native parasites.

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Conflict of Interest

Authors state no conflict of interest.

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Research Note

Is *Amphiorchis* (Digenea: Spirorchiidae) an exclusive parasite of sea turtles?

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Summary

The side-necked turtle *Hydromedusa tectifera* commonly inhabits the tributary streams of the Rio de La Plata and occasionally is found in brackish waters within the estuary of the Rio de La Plata. Few studies have been conducted on its parasitic fauna, especially in Argentina. In the present work *Amphiorchis* sp. is registered for the first time in a freshwater turtle, expanding the knowledge about the specificity of the genus that until now was considered inhabiting only marine turtles.
Keywords: *Amphiorchis*; Argentina; Diplostomida; Freshwater turtles; *Hydromedusa tectifera*; Platyhelminthes; Sea Turtles; Spirorchiidae; South America; Trematoda

Introduction

Spirorchiid trematodes are parasites of freshwater and marine turtles inhabiting the circulatory and lymphatic system. The infection by members of this family may resulted in severe tissue damage in the form of inflamatory granulomatous reactions due eggs deposition in the bloodstream and accumulated in various tissues can lead the animal to death (Santoro et al., 2017). Among Spirorchiidae, *Amphiorchis* Price, 1934 is composed of seven valid species parasites exclusively of sea turtles. Species if this genus have been parasitizing heart, lung and circulatory system of green turtle *Chelonia mydas* L. and hawksbill turtle *Eretmochelys imbricata* L. (see Table 1).

Life cycles of spirorchiid are known for freshwater species (i.e. Turner and Corkum, 1977), whereas little is known for marine species of *Amphiorchis* having only one record for turtles in captivity in which gasteropods (Vermetidae) act has intermediate hosts from which the cercariae are released and actively infect their definitive host, the sea turtle *Caretta caretta* (Cribb et al., 2017).

In the present work *Amphiorchis* sp. is registered for the first time in a freshwater turtle, the side-necked turtle *Hydromedusa tectifera* Cope, 1870 in Argentina, expanding the knowledge about the specificity of the genus that to date has been considered inhabiting only marine turtles.

Material and Methods

In November 2017, a carcass of a road-killed side-necked turtle with evidence of multiple shell fractures found on a road near Martin stream (34°55'42"S, 58°03'30"W, datum: WGS84), Buenos Aires province, Argentina. The turtle was placed in a recipient containing 10 % formalin solution for further analysis in the laboratory. The viscera were observed under the stereomicroscope (Leica M60®) in search of cysts or lesions in the tissue and then with the help of tweezers and scissors the content was examined in search of parasites. The parasites found were collected and preserved in 70 % ethanol.

Digeneans were stained with hydrochloric carmine, dehydrated in a graded ethanol series, cleared in eugenol, and mounted in natural Canada balsam for their morphological study using a polarizing

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Table 1. Hosts and localities of *Amphiorchis* species reported in the literature.

Host	Species	Locality	Reference
Chelonia mydas	*A. amphiorchis*	USA	Price (1934)
	A. indicus	India	Gupta and Mehrotra (1981)
		Brazil	Werneck and Silva (2013)
	A. solus	India	Simha and Chattopadhyaya (1970)
		Costa Rica	Santoro *et al*. (2006; 2007)
		Brazil	Werneck *et al*. (2008); Werneck and Medeiros (2016)
	A. stacyi	USA	Werneck and Greiner (2018)
Eretmochelys imbricata	*A. amphiorchis*	Puerto Rico	Fischthal and Acholonu (1976)
	A. caborojoensis	Puerto Rico	Fischthal and Acholonu (1976); Dyer *et al*. (1995)
		Brazil	Werneck *et al*. (2008); Dutra *et al*. (2012)
	A. indicum	India	Simha and Chattopadhyaya (1978)
	A. lateralis	Japan	Oguro (1938)
Hydromedusa tectifera	*Amphiorchis* sp.	Argentina	Present report

microscope (Olympus BX51®). The drawings were made with the aid of a camera lucida. Measurements are given in micrometers unless otherwise indicated. Additionally, photographs were taken with a Q-Imaging Go-3 digital camera. Specimens were deposited in the Helminthological Collection of the Museo de La Plata MLP-He 7505. The analyzed host was deposited in the Herpetological Collection of the Museo de La Plata, number R-6514. The field study was carried out under permission issued by Dirección de Flora y Fauna of the Buenos Aires Province (Disp. 69/2016). The turtle were cared in accordance with the Guidelines for use of live amphibians and reptiles in field and laboratory research.

Taxonomic keys and specific literature were used for morphological identification and morphometric comparisons, respectively (Price, 1934; Oguro, 1938; Simha & Chattopadhyaya, 1970, 1978; Fischthal & Acholonu, 1976; Gupta & Mehrotra, 1981; Platt, 2002; Werneck & Greiner, 2018). Specimens here found were determined by comparison with available voucher specimens collected by one of the authors of this manuscript (M. Werneck). Those specimens included *A. caborojoensis* (Helminthological Collection of the Biosciences Institute (CHIBB), numbers: 1392, 1406 and 6211), *A. solus* (CHIBB, numbers: 4044 and 7843), *A. indicus* (CHIBB, numbers: 4046, 4048, 4991 and 4995) and *A. stacyi* (U.S. National Parasite Collection, number: 1482618) (Werneck et al. 2008, 2011; Dutra et al. 2012; Werneck & Silva 2013; Werneck & Medeiros 2016; Werneck & Greiner 2018).

Results

Two trematode specimens identified as "*Amphiorchis* sp." were recovered from the duodenum and are described below.

Spirorchiidae Stunkard, 1921
Amphiorchis Price, 1934
Amphiorchis sp. (measures based on a single intact specimen) (Figs. 1, 2)

Body thin and elongate 1.37 mm long by 166 wide, posterior end rounded; oral sucker terminal and large, 61 long by 48 wide; ventral sucker not observed; esophagus 440 long, sinuous, bifurcating in two caeca just posterior to the beginning of the vitelline follicles; left caeca (745) longer than right caeca (679); testes in tandem, large with few lobes, oval in shape, occupying the intracaecal area; anterior testis 94 long by 69 wide, between caecal bifurcation and external seminal vesicle, posterior testis 115 long by 66 wide, located between the ovary and end of caeca; ovary with few lobes, 94 long by 60 wide, occupying the area between the testes, closer to the posterior testis; external seminal vesicle small and oval-shaped, 37 long by 57 wide, just posterior to the anterior testis; cirrus sac between the testes, enclosing an internal seminal vesicle, prostatic cells and cirrus; genital pore diestral to the ovary; vitellarium formed by large rounded follicles occupying both the intracaecal and extracaecal areas, the fields start before the caecal bifurcation extending to near the end of body and are

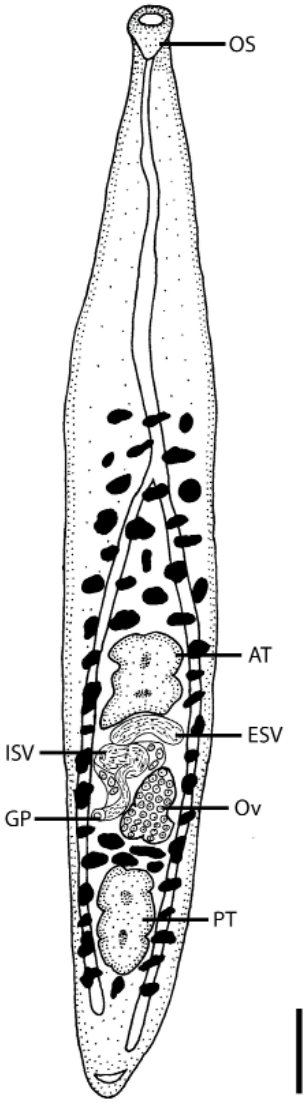

Fig. 1. Schematic illustration of *Amphiorchis* sp. **OS:** Oral sucker, **AT:** Anterior testis, **ESV:** External seminal vesicle, **ISV:** Internal seminal vesicle, **OV:** Ovary, **GP:** Genital pore, **PT:** Posterior testis. Scale bar 50µm.

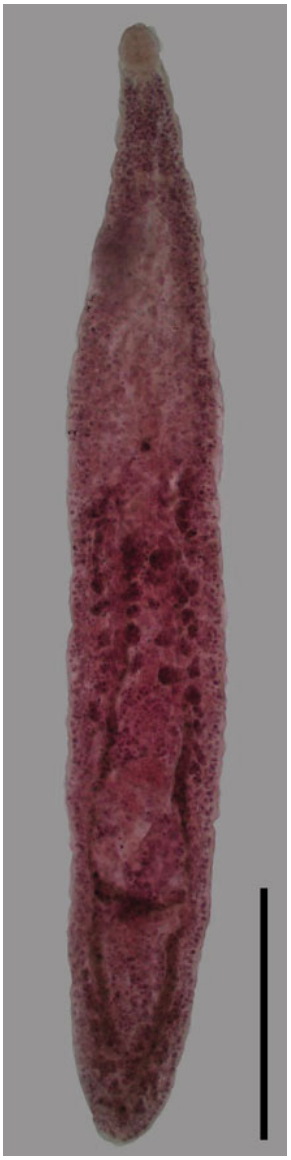

Fig. 2. Microphotograph of *Amphiorchis* sp. Scale bar 200 µm.

ventral to the caeca; not eggs observed.
New host: *Hydromedusa tectifera* Cope, 1870
New locality: Martin Stream (34°55'42" S, 58°03'30" W, datum: WGS84), Buenos Aires, Argentina
Location: duodenum
Although we have observed significant differences with the seven previously identified species of the genus (see discussion below) we prefer to maintain a more conservative description regarding a possible new species, for the following reasons: I) few specimens found, which does not guarantee an adequate analysis in the description of a new species, although some species have been described based on few specimens before, and some others are still completely lost scientifically (e.g. *A. lateralis*, *A. indicum*); II) it was not possible performs the genetic analysis of the present specimens due to the previous fixation of the host, although genetic analyses of *Amphiorchis* species are scarce and only few 28S ribosomal RNA sequences of undetermined *Amphiorchis* spp. exist in GenBank (see Cribb *et al.*, 2016).
In this way we prefer to identify the two specimens found only at the genus level and being described in this note as *Amphiorchis* sp.

Remarks
Amphiorchis sp. differs from *A. amphiorchis*, *A. caborojoensis*, *A. indicus* and *A. lateralis* by the presence of a longer esophagus occupying the first third of body. Also, it can be distinguished by *A. amphiorchis*, *A. caborojoensis* and *A. solus* by having lobed testes. Additionally, differs from *A. solus* and *A. caborojoensis* for not having a caeca loop and a constriction at level of acetabulum.

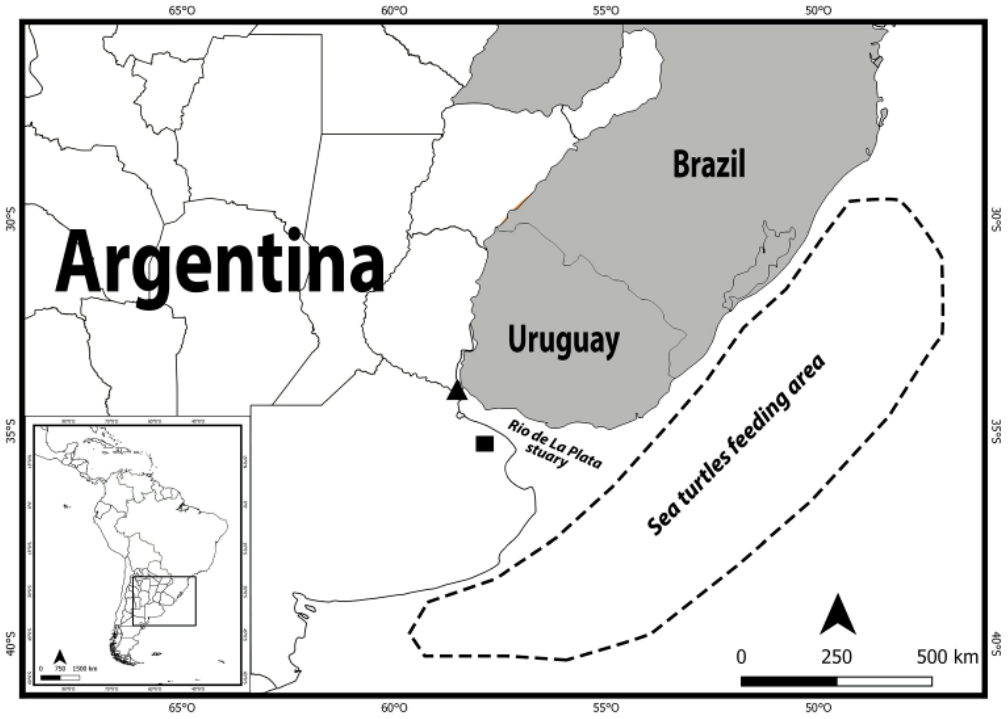

Fig. 3. South America map showing the new record of *Amphiorchis* sp. (black square), the record of *Dermochelys coriacea* within the estuary of the Rio de la Plata (black triangle), the estuary of the Rio de la Plata, and the feeding area of the marine turtles.

In addition, in the present specimens the vitellaria occupies both intracaecal and extracaecal fields in almost entire body, whereas in *A. amphiorchis* it is extracaecal after the posterior testicle (Price, 1934). *Amphiorchis* sp. differs from *A. lateralis* because caeca ends asymmetrically whereas they end symmetrically in *A. lateralis* (Oguro, 1938). The caeca extends beyond the testes in *Amphiorchis* sp. whereas they end immediately after the posterior testicle in *A. solus* (Simha & Chattopadhyaya, 1970). Present specimens differs from *A. caborojoensis* by having a considerable distance between the second testis and the end of body, meanwhile in *A. caborojoensis* it is very close to the posterior end (Fischthal & Acholonu, 1976). *Amphiorchis* sp. possess the genital pore lateral to the ovary, and the caeca end in an asymmetrical position, in contrast in *A. indicum* the genital pore is posterior to the ovary and the caeca end symmetrically (Simha & Chattopadhyaya, 1978). Despite *Amphiorchis* sp. shares the same asymmetry in the length of the caeca than *A. indicus*, the former has less dense vitellaria and have not so voluminous posterior testis. The testes in *Amphiorchis* sp. are located in the posterior half of body, whereas in *A. indicus* the testes are located near to the caecal bifurcation and the anterior testis is clearly located in the anterior half of body (Mehrotra, 1973; Gupta & Mehrotra, 1981). Finally, *Amphiorchis* sp. differs from *A. stacyi* because the vitelline follicles begin at the level of the caecal bifurcation and they are smaller than those in *A. stacyi*. Also, in present specimens the caeca are asymmetric, whereas in *A. stacyi* they are symmetric (Werneck & Greiner, 2018).

Discussion

Considering that the genus *Amphiorchis* had only been registered for sea turtles since its description by Price (1934) (see table 1), the finding of these specimens in a freshwater turtle is very striking. The Martin stream flows to the Rio de La Plata, which forms a large estuary (see Fig. 3) with mixtures of fresh and salt waters and flows into the sea at the north of Buenos Aires province, which is a path of constant exchange between the two environments (Guerrero, 1998). There are records of sea turtles in the rivers that flow into the Rio de la Plata (i.e. *Dermochelys coriacea* (Vandelli), and also there are records of *H. tectifera* specimens in estuarial environments with marine influence areas near Buenos Aires province coast (Bona *et al.*, 2009; Carman *et al.*, 2011). This point of contact could be one of the ways in which marine parasite species diverged in freshwater species, gradually adapting to the physical and chemical changes that occur in this transition, differentiating and conquering empty niches.

The presence of *Amphiorchis* sp. in *H. tectifera* represents the first record of the genus in a species of freshwater turtle and extends the geographic range since it was not registered in any marine species near the Argentine coasts.

The features of specimens here describe would indicate the presence of a new species of *Amphiorchis*. However, it is necessary to found more and well preserved mature specimens for could be able to corroborate this hypothesis.

Amphiorchis sp. here found is smaller in size than other species in the genus. It could be explained by the size of the host, given that *H. tectifera* does not exceed 35 cm, whereas the sea turtles are bigger. It is known that there is a positive correlation between the body size of host and length of parasite (Poulin, 2007).

Although it is possible to hypothesize that the presence of *Amphiorchis* sp. in a freshwater turtle is accidental, the fact that the cercarie actively infects its definitive host and that, despite not having eggs, the specimens found were mature, it is feasible to propose that *H. tectifera* acts as definitive host of this *Amphiorchis* species. In *H. tectifera* there were reported four species of digeneans, *Cheloniodiplostomum testudinis* (Dubois, 1936) and *Cheloniodiplostomum argentinensis* Palumbo and Diaz, 2018 in Argentina, *Nematophila grandis* (Diesing, 1839) in Paraguay, and *Pseudotelorchis devincenzii* (Catto and Amato, 1993) and *Telorchis platensis* Mane-Garzon and Gil, 1961 in Uruguay (Fernandes & Kohn, 2014; Palumbo & Diaz, 2018). The presence of *Amphiorchis* sp. in *H. tectifera* represents the sixth digeneans species reported for this turtle species being the third in Argentina.

Ethical Approval

All animals were cared in accordance with the Guidelines for use of live amphibians and reptiles in field and laboratory research (Am. Soc. Ichth. and Herpt., 1987).

Conflict of Interest

Authors state no conflict of interest.

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Research Note

Cercarial fauna of freshwater snails in selected agricultural areas in Laguna, Philippines

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Summary

Freshwater snails serve as one of trematodes' intermediate hosts. Previous studies on trematode larval stages in the Philippines have largely focused on species with public health importance. This study sought to investigate the prevalence of cercarial morphotypes in several freshwater snail species found in different habitat types (rice field, irrigation canals, and residential area) in selected agricultural areas in Los Baños and Bay in Laguna. Cercarial emergence was induced through exposure to artificial light. A total of 2,720 freshwater snails were collected and were represented by seven species, namely, *Melanoides tuberculata* Muller 1774 (n = 1229), *Radix quadrasi* von Moellendorf (n = 630), *Tarebia granifera* Lamarck, 1816 (n = 417), *Pomacea canaliculata* Lamarck 1819 (n = 257), *Vivipara angularis philippinensis* Nevill (n = 18), *Stenomelania* sp. (n = 104), *Thiara scabra* Muller 1774 (n = 65). A 2.57 % over-all prevalence was recorded; the infected snail species were *M. tuberculata* (2.21 %), *R. quadrasi* (0.21 %), *T. granifera* (0.11 %). Four cercarial morphotypes, namely, Parapleurolophocercous cercaria (1.80 %), Virgulate xiphidiocercaria (0.26 %), Megaluruous cercaria (0.29 %), and Echinostome cercaria (0.22 %) were recovered from the infected snail species. Prevalence of cercarial infection was significantly different ($p < 0.05$) among habitat types.
Keywords: Trematodes; cercarial infection; freshwater snails; Laguna, Philippines

Introduction

Digenetic trematodes, commonly known as flukes, demonstrate a heteroxenous life cycle which includes various intermediate hosts for their development (Roberts & Janovy, 2009). Different species of freshwater snails have received considerable attention as they harbor larval stages of trematodes known to have both public health and veterinary importance (Mohammed *et al.*, 2016). Since the larval development in snails is obligatory, the distribution of these freshwater snail species dictates the occurrence of different trematode taxa in a locale (Hechinger & Lafferty, 2005). For instance, the freshwater snail, *Oncomelania hupensis quadrasi* Möllendorf, 1895, has been studied for infection of the strain of *Schistosoma japonicum* in the Philippines (Pesigan *et al.*, 1958a; Madsen *et al.*, 2008). Likewise, attention has been given to the control of these snail host species (Pesigan, *et al.*, 1958b; Ohmae, *et al.*, 2003). However, there is a dearth of recent information on cercarial fauna of other freshwater snail species in the country. Researches on cercarial prevalence are deemed important to survey other snail species that may serve as intermediate hosts of other trematodes. Hence, this study generally sought to investigate the prevalence of cercarial types in freshwater snail species in selected agricultural areas in Laguna, Philippines: Los Baños and Bay. Specifically, it aimed to examine the snail species present for cercarial infection, to morphologically identify the cercarial species that will be recovered from the snail samples, and to compare their prevalence among the different habitat types.

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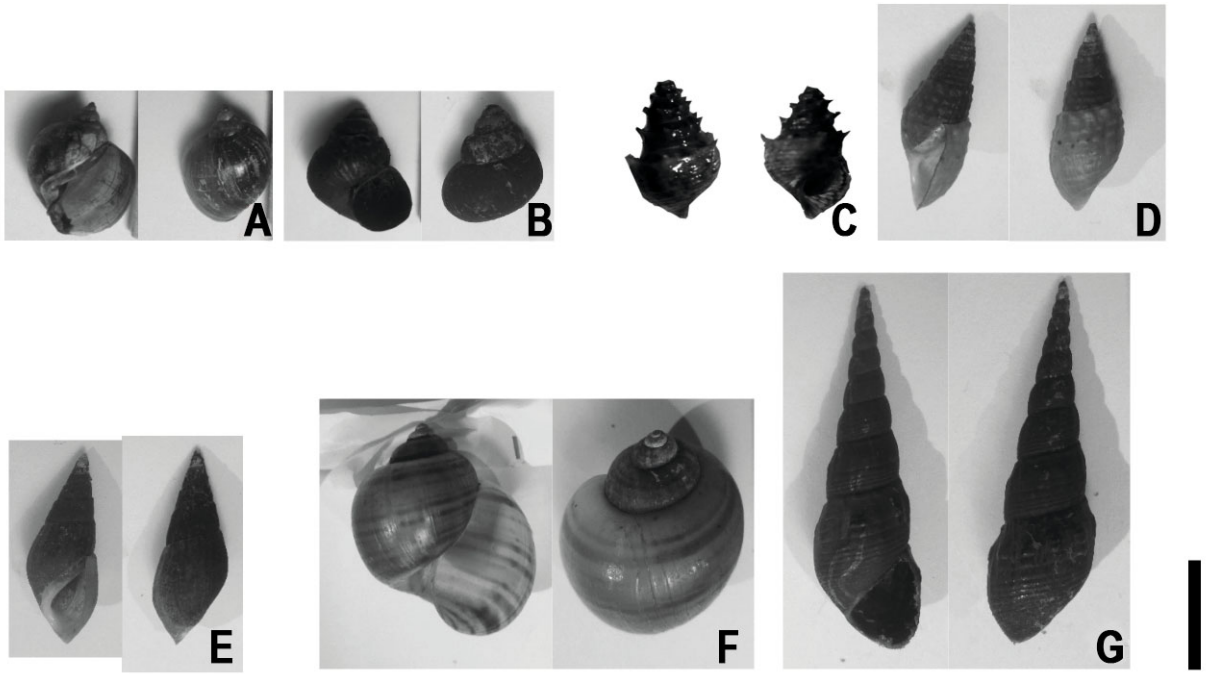

Fig. 1. Freshwater snail species collected in Los Baños and Bay, Laguna: (A) *Radix quadrasi* von Moellendorf, (B) *Vivipara angularis philippinensis* Nevill, (C) *Thiara scabra* Muller, 1774, (D) *Tarebia granifera* Lamarck, 1816, (E) *Stenomelania* sp., (F) *Pomacea canaliculata* Lamarck, 1819, (G) *Melanoides tuberculata* Muller 1774. (Scale bar = 1 cm)

Materials and Methods

Study site

This study was conducted in two municipalities in Laguna, Philippines: Los Baños and Bay. Los Baños (14° 09' 53.1" N, 121° 15' 21.8" E) is classified as a first-class urban municipality, while Bay (14° 10' 58" N, 121° 17' 5" E) is a second-class coastal municipality. Both municipalities generate income from agriculture and fishery. Moreover, both municipalities are part of the Mount Makiling Forest Reserve that has a wide range of ecosystems serving as habitats to diverse flora and fauna.

Sample collection

Semi-purposive sampling was done based on the following criteria: (1) having or being situated near an agricultural land, (2) presence of snails, and (3) having consent from owners. Selected sampling sites were of different habitat types – (1) rice fields, (2) irrigation canals, and (3) residential areas/ houses near the rice fields. A total of six (6) collection sites were selected representing each of the habitat types**.** Collection was conducted from 06:00 to 09:00 AM by hand picking and representatives were sent to UPLB Museum of Natural History for species identification. The snail samples were brought to the laboratory for processing.

Sample processing

Snail samples were individually placed in 50mL glass containers filled to half with dechlorinated tap water and were exposed to artificial light for six hours during daytime (08:00 AM to 02:00 PM) at room temperature. The water in each container was checked for the presence of cercaria every two hours. Snails that did not shed cercaria on the first exposure were subjected to the same procedure for second trial on the following day. The snails that were negative for cercarial emergence after two trials of light exposure were subjected to crushing method. Briefly, the snails were crushed and the hepatopancreas of each snail was isolated and squashed onto a glass slide. A drop of 0.95 % physiological saline solution was added to the sample. The samples were viewed under a compound microscope to check for the presence of cercaria. Cercarial morphotypes were identified using the classification key of Frandsen and Christensen (1984) for cercarial species emerging from African snails and the illustrations provided by Chontananarth and Wongsawad (2013) for cercarial species emerging from snails collected in Thailand.

Data Analysis

Prevalence was computed using the formula:

$$\%\ Prevalence = \frac{number\ of\ infected\ snails}{total\ number\ of\ snails} \times 100$$

To compare cercarial prevalence among habitat types, Chi-square test was employed using Quantitative Parasitology version 3 (Rózsa, *et al*., 2000). Values were considered statistically significant if $p < 0.05$.

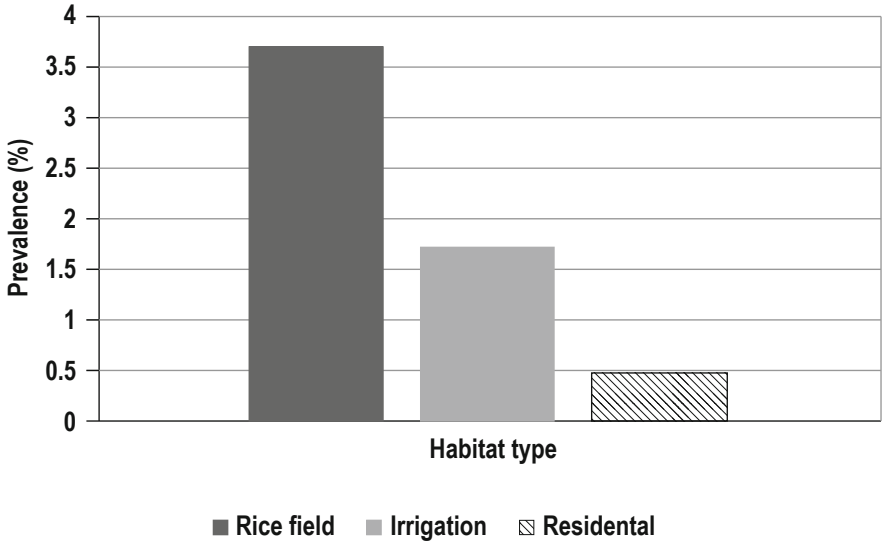

Fig. 2. Cercarial prevalence among habitat types

Ethical Approval and/or Informed Consent

The conducted research included animal subjects that are not covered by the institutional guidelines for the care and use of animals; hence, ethics clearance is not required.

Results and Discussion

A total of 2, 720 freshwater snails represented by seven species, namely, *Melanoides tuberculata* Muller 1774 (n = 1229), *Radix quadrasi* von Moellendorf (n = 630), *Tarebia granifera* Lamarck, 1816 (n = 417), *Pomacea canaliculata* Lamarck 1819 (n = 257), *Vivipara angularis philippinensis* Nevill (n = 18), *Stenomelania* sp. (n = 104), *Thiara scabra* Muller 1774 (n = 65) were collected (Fig. 1).

Of the total samples, 1541 snails were collected in rice fields, 579 in irrigations, and 600 in residential areas near the rice fields. Overall, only 70 (1.56 %) of the snails collected were found positive for cercarial infection. Highest cercarial infection was recorded in rice fields, accounting to 3.70 % of the total collected snails in all rice fields surveyed. On the other hand, snails from irrigation canals and residential areas had 1.73 % and 0.5 % cercarial prevalence, respectively (Fig. 2). Chi-square test revealed that the prevalence of cercarial infection differed significantly (p = 0.0001). The differences in the cercarial prevalence among the three habitat types surveyed may be due to various possible reasons. Although environmental factors were not measured in this study, parameters such as water temperature (Studer & Poulin, 2013), pH (Candia *et al.*, 2015), vegetation cover (Koprivnikar *et al.*, 2007), and leaf litter (Luth *et al.*, 2016) were found to influence snail community and trematode prevalence in an area. It is also important to note that the presence of other animals that may serve as final hosts for these trematodes may also be one reason for prevalence of infection. Interestingly, more rodents, amphibians, and some species of migratory birds have been observed in the rice fields than in the irrigation canals and household areas surveyed in this study.

In general, freshwater snail species have been extensively studied due to their role as intermediate hosts of several trematode species known to cause diseases to humans and domestic animals. Members of the *Melanoides* genus have been recorded as hosts of various trematodes of medical and veterinary importance. For instance, numerous researches on *M. tuberculata* have been conducted as it harbors different trematode parasites such as *Philophthalmus gralli* (Pinto & Melo, 2010), *Centrocestus formosanus* (Paula-Andrade *et al.*, 2012; Dos Santos *et al.*, 2013; Najet *et al.*, 2014; Yousif *et al.*, 2016)*, Haplorchis taichui* and *H. pumilio* (Krailas *et al.*, 2011), and various types of cercarial species (Devkota *et al.*, 2011; Duangduen *et al.*, 2014).

In this study, highest cercarial infection was observed in *M. tuberculata* (2.21 %), followed by *R. quadrasi* (0.21 %), and *T. granifera* (0.11 %). Cercariae were putatively classified into four morphotypes, namely, Virgulate xiphidiocercaria, Parapleurolophocercous cercaria, Echinostome cercaria, and Megalurous cercaria (Fig. 3) based on the taxonomic classification key for cercariae by Frandsen and Christensen (1984) and illustrations provided by Chontananarth and Wongsawad (2013). Among the infected snails, *Melanoides tuberculata* was found to harbor three types of cercariae: Parapleurolophocercous cercaria (3.74 %), Megalurous cercaria (0.65 %), and Echinostome cercaria (0.49 %). However, no co-infection per individual was noted among the *M. tuberculata* snails examined. On the other hand, *T. granifera* was found infected with Parapleurolophocercous cercaria while Virgulate xiphidiocercaria was recovered from *R. rubiginosa*.

Prevalence of the cercarial types in the infected snail species is summarized in Table 1. Virgulate xiphidiocercaria exhibits unique

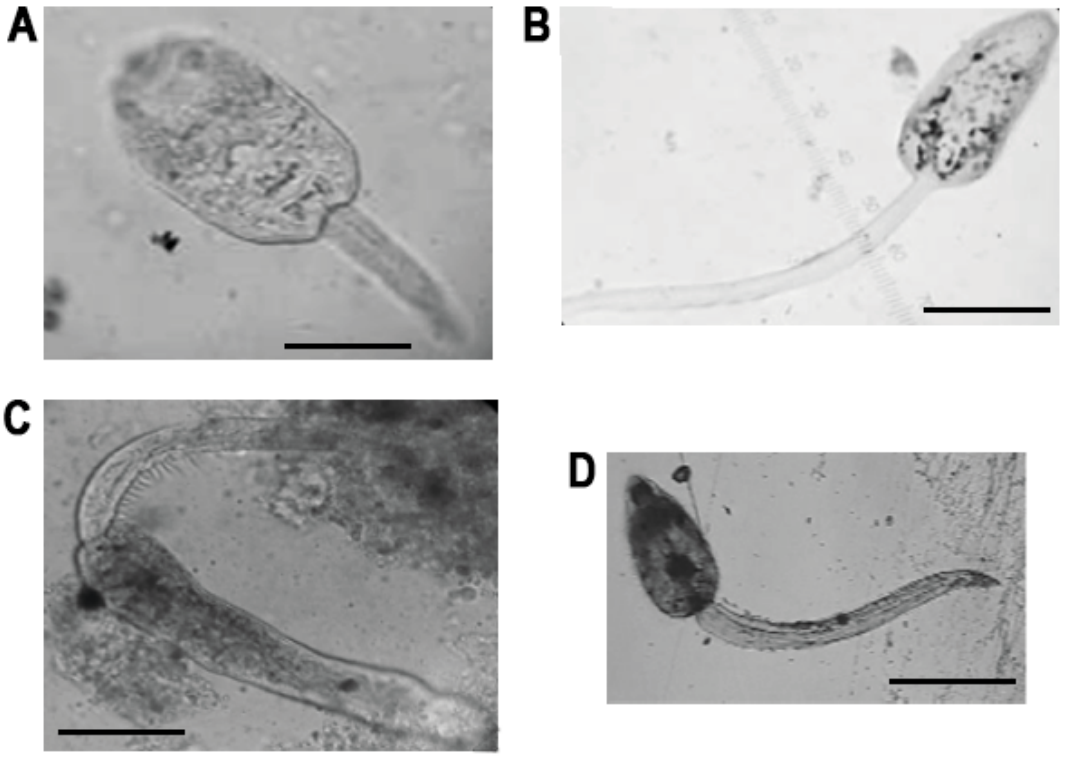

Fig. 3. Cercarial types found in snail species: (A) Virgulate xiphidiocercaria, (B) Parapleurolophocercous cercaria, (C) Megalurous cercaria, and (D) Echinostome cercaria. (Scale bar = 20 uM)

features such as its small size, a tail shorter than the body and has no dorsoventral finfold, a bilobed or pyriform virgula organ in the oral sucker region, and a ventral sucker smaller than the oral sucker. Parapleurolophocercous cercaria has distinct features including unforked tail with well-developed finfolds, absence of ventral sucker, presence of eyespots, absence of adhesive organs at posterior end of the body, and few cystogenous glands. Echinostome cercaria can be distinguished by its unforked tail, ventral sucker on mid-ventral surface of the body, oral sucker without stylet but surrounded by a spiny collar, and numerous cystogenous glands in the body. Megalurous cercaria is characterized by an elongated body with yellowish-brown granules, minute spines covering the posterior half of the body, bifurcated esophagus located in the middle of pharynx and ventral sucker, ventral sucker slightly larger than the oral sucker and located medially on the body, sub-terminal oral sucker with complex muscular apparatus, and long, elastic, and slender tail with adhesive gland cells at the tip. These cercarial morphotypes are characteristic larval stages of various trematode families. Virgulate xiphidiocercariae can develop into intestinal trematodes in the family Lecithodendriidae parasitizing bats, birds, and amphibians. Parapleurolophocercous cercaria is commonly produced by members of the family Heterophyidae which include species of intestinal trematodes known to infect birds and mammals. Echinostome cercariae are produced by species belonging to the family Echinostomatidae which is comprised by various species of intestinal parasites of birds, reptiles, and mammals. Megalurous cercaria is the characteristic larval stage of the avian eye trematodes in the family Philophthalmidae (Frand-

Table 1. Prevalence of the total cercarial types among infected snail species.

Snail species	No. of snail examined	No. of infected snails (% prevalence)			
		Cercarial type			
		Virgulate xiphidiocercaria	Parapleurophocercous cercaria	Megulurous cercaria	Echinostome cercaria
M. tuberculate	1229	–	46 (3.74)	8 (0.65)	6 (0.49)
R. rubiginosa	630	7 (1.11)	–	–	–
T. granifera	417	–	3 (0.72)	–	–

sen & Christensen, 1984; Chontananarth & Wongsawad, 2013).
The present study provides baseline data for cercarial morphotypes infecting freshwater snails in selected sampling sites in Los Baños and Bay in Laguna, Philippines. The result of the study revealed 2.57 % over-all prevalence of cercarial infection among the snail species collected in different habitat types. Interestingly, prevalence was significantly different among habitat types, with most of the infected snails recovered in rice fields. The snail species namely, *M. tuberculata, R. quadrasi*, and *T. granifera,* were found to be infected with one or more cercarial morphotypes. The cercariae were putatively identified as Virgulate xiphidiocercaria, Parapleurolophocercous cercaria, Echinostome cercaria, and Megalurous cercaria. These are known to be characteristic larval stages of diverse trematode parasites known to infect a wide range of vertebrates including humans.
Although this revealed a low cercarial prevalence, monitoring and survey should still be done to determine the presence of trematodes to raise awareness regarding the potential public health and veterinary importance. Due to the lack of recent published information on the cercarial prevalence in the Philippines, surveillance in other parts of the country is recommended to document the cercarial types infecting freshwater snail species inhabiting the different locales. Moreover, other identification protocols, such as the use of scanning electron microscopy and molecular biological methods, should be employed to further characterize the cercariae and to identify the possible adult trematode species that may develop from them.

Conflict of Interest

Authors state no conflict of interest.

Acknowledgments

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