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The activity of hydrolytic enzymes in the digestive system of Acanthobdellida, Branchiobdellida and Hirudinida (Annelida, Clitellata) – considerations on similarity and phylogeny

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Abstract
Activities of nineteen hydrolases were measured in the digestive systems of predatory and blood-feeding true leeches (Hirudinida) and their closest relatives, Branchiobdellida and Acanthobdellida. Hydrolase activities were analyzed in different parts of the digestive systems: the species-specific anterior part, i.e. jaws, pharynx or proboscis, crop and intestine. The results obtained suggest that food digestion and possible absorption predominate in the intestine of most of the studied Hirudinida and *A. peledina*, whereas in *B. astaci* these processes take place in the anterior part of the digestive system and crop. In Erpobdellidae and *Piscicola respirans*, the activity of acid and alkaline phosphatases, N-acetyl-β-glucosaminidase, leucine and valine arylamidases, and α-fucosidase was also detected in the anterior part of the digestive system. We also detected differences in enzyme occurrence between the studied species, which are probably connected with their different food preferences. Moreover, the presence of the whole spectrum of enzymes in predatory leeches and the absence of trypsin and α-chymotrypsin activity in the crop of all the leeches support the hypothesis that the leech ancestor was a blood-feeder. Our study showed that "Rhynchobdellida" constitute a paraphyletic group which confirms the previous results based on molecular phylogenetics, while Arhynchobdellida appears to be a non-monophyletic group which is not consistent with previous molecular results.

Keywords: *Food preferences, hydrolases, blood feeding, digestive system, phylogeny, leeches*

Introduction

Research on enzymes can have alternative character; either enzymes are studied to understand the physiological abilities of an organism, or it can be a perspective challenge for taxonomists as a method of description of new species (Ayala & Powell 1972).

The digestive system of ectoparasitic Clitellata, i.e. Branchiobdellida, Acanthobdellida and Hirudinida, similarly to other invertebrates is composed of three distinct regions: the foregut, midgut and hindgut (Fernández et al. 1992). The proboscis, jaws and pharynx are ectodermal regions of the foregut, while the midgut is formed by four regions: the esophagus,

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crop, posterior crop caecum and intestine. The hindgut is a short tube-shaped structure composed of the rectum and anus. They are lined with simple epithelium (Jennings & Gelder 1979; Rost-Roszkowska et al. 2012). The precise ultrastructure of the digestive cells in the midgut of Hirudinida has been analyzed only in five species: *Hirudo medicinalis* (Hirudinidae), *Haementeria depressa*, *Helobdella triserialis* and *Theromyzon rude* (Glossiphoniidae) as well as *Piscicola geometra* (Piscicolidae) (Hammersen & Pokahr 1972a, 1972b; Fernández et al. 1992; Rost-Roszkowska et al. 2012, 2015). Apart from general morphological and functional descriptions (summarized by Gelder & Williams 2015) more detailed studies devoted to the digestive system structure and digestion in Branchiobdellida come from *Cambarincola macrodonta* (Jennings & Gelder 1979). Similarly, studies on the Acanthobdellida digestive system were mainly focused on its general morphology (Epshtein 1966; summarized by Bielecki et al. 2014b).

Numerous morphological and molecular analyses have confirmed that ectoparasitic and predatory clitellates, i.e. true leeches (Hirudinida), Branchiobdellida and Acanthobdellida, form a monophyletic taxon among Clitellata with Lumbriculida as their sister group (Purschke et al. 1993; Martin 2001; Siddall et al. 2001; Erséus & Källersjö 2004; Marotta et al. 2008; Rousset et al. 2008; Martinez-Ansemil et al. 2012; Tessler et al. 2018). However, the sister relationships between these three leech-like taxa are still unresolved (Siddall et al. 2001; Erséus & Källersjö 2004; Marotta et al. 2008; Urbisz & Świątek 2013; Phillips et al. 2019 for more details see Discussion).

Leeches (Hirudinida), a taxon grouping approximately 1000 species (Govedich & Moser 2015), seem to be one of the most derived groups of annelids (Sawyer 1986; Bielecki 1997; Erséus & Källersjö 2004; Sket & Trontelj 2008; Bielecki et al. 2011a, 2011b, 2012a, 2012b, 2013; Cichocka et al. 2015; Elliott & Dobson 2015). Generally, leeches are known for their blood-feeding habits. However, different feeding strategies have evolved among these animals, i.e. ectocommensalism, predation, scavengery and sanguivory (Sawyer 1986; Apakupakul et al. 1999; Sket & Trontelj 2008). Leeches can attack and swallow small animals, such as earthworms, but generally feed on soft animal tissues and body fluids including blood (Pawłowski 1936, 1968; Lukin 1976; Sawyer 1986; Bielecki 1988a, 1988b). A wide variety of animals can be prey for leeches, e.g. earthworms (Pawłowski 1936, 1968), molluscs (Daniels & Sawyer 1975; Klemm 1975; Sawyer 1986), fish (Bielecki 1997; Williams & Burreson 2006; Utevsky 2007; Kovalenko & Utevsky 2015; Adamiak-Brud et al. 2016), amphibians (Van der Lande & Tinsley 1976), reptiles, water birds (Davies & Wilkialis 1981; Bielecki et al. 2009; Buczyński et al. 2014), and mammals (Hong et al. 1999). There are also reports showing opportunistic feeding of leeches on amphibian and fish eggs, but such habits seem to be occasional and therefore they are usually overlooked (Davies & Govedich 2001; Light et al. 2005).

Branchiobdellidans are obligate ectosymbionts of crayfish. In fact, they are omnivorous animals feeding on different organisms occurring on their host exoskeleton. Their guts may contain algae, diatoms, ciliates, nematodes and oligochaetes, as well as branchiobdellidans, insect larvae and host haemolymph; some of them parasitize crayfish gills (Gelder & Williams 2015). Free living branchiobdellidans have not been observed (Sawyer 1986; Gelder & Williams 2015).

Two described acanthobdellidan species (*Acanthobdella peledina* and *Paracanthobdella livanowi*) are regarded as temporary ectoparasites of salmonid fish, feeding on fish blood and soft tissues from the fish body wall. After feeding, acanthobdellidans detach from the host and live freely; especially *P. livanowi* specimens were frequently found unattached to the fish in the local environment (Livanow 1906; Epshtein 1966; Sawyer 1986; Erséus & Källersjö 2004; Kaygorodova et al. 2012; Utevsky & Shedko 2013a; Utevsky et al. 2013b; Bielecki et al. 2014b).

To enrich our knowledge about the functioning of a digestive tract in ectoparasitic and predatory clitellates we decided to analyze the occurrence and activity of hydrolases, an important group of digestive enzymes, and finally to compare the obtained data among species. In the present study, hydrolase activity was assessed in eight leech species of different food preference (both sanguivorous and carnivorous) as well as in *Branchiobdella astaci* (Branchiobdellida) and *Acanthobdella peledina* (Acanthobdellida), which have not been studied until now, because collecting these unique species is highly problematic due to their occurrence in hardly accessible habitats. For our purposes we analyzed hydrolases within three parts of the digestive system: the foregut (alternatively proboscis, jaws or pharynx – depending on the foregut structure in a given species) and two parts of the midgut – the crop and intestine. Therefore, here we present the first report on types of enzymes synthesized not only in Hirudinida, but also in their relatives: Branchiobdellida and Acanthobdellida.

Materials and methods

Collection of specimens and crude extract preparation

The species studied, their feeding habits and place of collection are listed in Table I. They originated from natural and unpolluted environments. For each species, three specimens were analyzed. All the specimens collected were in a good condition, actively moving and feeding. We assume they were in comparable physiological condition. Predatory species feed in short intervals (every 1–2 days) and had access to large invertebrate prey assemblages at the collection sites. Sanguivores collected directly from their hosts also had direct access to their food sources. Though some sanguivores specializing in fish hosts were collected outside their hosts, we can assume they had free access to their food sources (due to an abundant fish assemblage at the collection sites). It should also be noted that sanguivores (particularly those feeding on tetrapods) are adapted to take food at long time intervals (a few times a year) without any effect on their condition, so it was unlikely that they were captured hungry, taking into account the stage of their life cycle. To collect the material no specific permissions were required for locations/activities, except for *H. medicinalis* (permission WPN.6401.239.2017.MS).

Table I. The list of studied species, their feeding habits and place of collection.

Species (family)	Feeding habit	Place and date of collection
Acanthobdella peledina Grube, 1850 (Acanthobdellidae)	blood	collected from *Thymallus thymallus and Salmo trutta*, Pite River, Sweden, June 2012
Branchiobdella astaci Odier, 1823 (Branchiobdellidae)	omnivorous	collected from *Astacus astacus*, The Kozienicka Forest, June 2009
Theromyzon maculosum (Rathke, 1862) *Placobdella costata* (Fr. Müller, 1846) (Glossiphoniidae)	blood	Turtulski pond, northern Poland, May 2014
Piscicola respirans (Troschel, 1850) (Piscicolidae)	blood	collected from *Thymallus thymallus and Salmo trutta*, Nysa Kłodzka river, April 2015
Piscicola sp. n. (Piscicolidae)	blood	collected from stones in Łupawa river, northern Poland, October 2015
Hirudo medicinalis Linnaeus, 1758 (Hirudinidae)	blood	commercially bought from BIO-GEN company, Namysłów, Poland, June 2012
Erpodbella monostriata (Lindenfeld & Pietruszynski, 1890), *E. nigricollis* (Brandes, 1900) *E. testacea* (Savigny, 1820) (Erpobdellidae)	carnivorous	Ukiel Lake, Olsztyn, Poland, October 2012

To eliminate bacteria and fungi, each collected specimen was kept for two days in an aqueous solution of antibiotics and fungicides (penicillin 100 U ml^{-1}, streptomycin 100 µl ml^{-1}, nystatin 100 U ml^{-1}) (Dziekońska et al. 2009). Then, specimens were frozen at −20°C in vials with physiological saline. After unfreezing, each specimen was dissected and the three parts of the digestive system were carefully isolated: the proboscis, pharynx or jaws depending on the species (see Table II, III, IV), the crop, and the intestine. After isolation, fragments of the digestive system were cut lengthwise, and their contents were washed out with 1 ml of physiological saline. Then the samples were crushed in a glass Potter homogenizer with the addition of 1 ml of physiological saline (0.9% NaCl) and sand until a homogeneous suspension was obtained. The homogenates were centrifuged for 10 min at 3 000 g and supernatants were used for the determination of protein content and for enzymatic activity tests.

It should be noted that field conditions during sampling as well as freezing and unfreezing procedure may affect enzyme activity. For this reason, we treated all specimens in the same way, keeping them under unified laboratory conditions before freezing to standardize samples collected in different conditions and time.

API ZYM analyses

To determine the activity of hydrolases along the digestive system of the analyzed species, we used commercial API ZYM tests (bioMérieux, Lyon, France), which is a laboratory kit for semi-quantitative analysis of production of hydrolytic enzymes. Each strip is composed of 20 microcupules containing dehydrated chromogenic substrates for 19 enzymatic reactions and a control without a substrate. Microcupules contained a buffer with a specific optimum pH value for each enzyme activity as shown in the Tables II, III, IV. Up to now, this type of kit has been used in order to analyze unpurified samples of the digestive systems in invertebrates (Boetius & Felbeck 1995; Martin et al. 2011; Collin & Starr 2013). Nineteen enzymes were identified: peptide hydrolases (leucine, valine, and cystine arylamidases, trypsin, α-chymotrypsin), phosphohydrolases (alkaline

Table II. Activity of hydrolases in the digestive system of *Branchiobdella astaci* and *Hirudo medicinalis*.

Enzyme	pH	Enzyme activities (nmol as mean ± standard deviation)					
		Jaws		Crop		Intestine	
		**B. astaci*	*H. medicinalis*	*B. astaci*	*H. medicinalis*	*B. astaci*	*H. medicinalis*
Peptide hydrolases							
Leucine arylamidase	7.5	0.00	13.33 ± 5.78	0.00	13.33 ± 5.78	0.00	23.33 ± 5.78
Valine arylamidase	7.5	8.33 ± 2.89	0.00	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89
Cystine arylamidase	7.5	8.33 ± 2.89	0.00	8.33 ± 2.89	0.00	6.67 ± 2.89	1.67 ± 2.89
Trypsin	8.5	6.67 ± 2.89	0.00	6.67 ± 2.89	0.00	0.00	0.00
α- Chymotrypsin	7.5	0.00	0.00	0.00	0.00	0.00	0.00
Phosphohydrolases							
Alkaline phosphatase	8.5	0.00	33.33 ± 5.78	0.00	36.67 ± 5.78	0.00	36.67 ± 5.78
Acid phosphatase	5.4	0.00	36.67 ± 5.78	0.00	36.67 ± 5.78	0.00	36.67 ± 5.78
Naphthol-AS-BI-phosphohydrolase	5.4	0.00	6.67 ± 2.89	0.00	16.67 ± 5.78	0.00	8.33 ± 2.89
Ester hydrolases							
Esterase C 4	6.5	0.00	16.67 ± 5.78	0.00	16.67 ± 5.78	0.00	16.67 ± 5.78
Esterase lipase C 8	7.5	0.00	6.67 ± 2.89	0.00	8.33 ± 2.89	0.00	8.33 ± 2.89
Lipase C 14	7.5	6.67 ± 2.89	0.00	6.67 ± 2.89	0.00	0.00	0.00
Glycosidases							
α-Galactosidase	5.4	6.67 ± 2.89	0.00	6.67 ± 2.89	0.00	0.00	0.00
β-Galactosidase	5.4	0.00	6.67 ± 2.89	0.00	6.67 ± 2.89	0.00	8.33 ± 2.89
β-Glucuronidase	5.4	0.00	1.67 ± 2.89	0.00	6.67 ± 2.89	0.00	6.67 ± 2.89
α-Glucosidase	5.4	0.00	0.00	0.00	0.00	0.00	0.00
β-Glucosidase	5.4	0.00	0.00	0.00	0.00	0.00	0.00
N-Acetyl-β-glucosaminidase	5.4	0.00	26.67 ± 5.78	0.00	36.67 ± 5.78	0.00	36.67 ± 5.78
α-Mannosidase	5.4	0.00	0.00	0.00	0.00	0.00	0.00
α-Fucosidase	5.4	0.00	1.67 ± 2.89	0.00	6.67 ± 2.89	0.00	8.33 ± 2.89

**B. astaci - Branchiobdella astaci; H. medicinalis - Hirudo medicinalis*

and acid phosphatases, naphthol-AS-BI-phosphohydrolase), ester hydrolases (esterase C 4, esterase lipase C 8, lipase C 14), glycosidases (α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase). Prior to application to the strip, protein content of the crude extracts was measured according to Bradford (1976) with bovine serum albumin (BSA, Sigma-Aldrich Co., St. Louis, USA) as a standard. Next, the crude extracts were diluted with 0.9% NaCl solution to an average protein content of 1.5 mg ml^{-1}. Following the manufacturer's instructions, 65 µl of the examined solution containing ca 100 µg of protein was added to wells in the provided trays and incubated at 37°C for 4 h. After incubation, the reagents ZymA and ZymB were added to stop the reaction, then exposed to intense fiber-optic light for 5 minutes. The results were expressed using the five-step enzyme activity scale according to the colour chart provided by the manufacturer: 0 – negative reaction; 1 (5 nmol), 2 (10 nmol), 3 (20 nmol), 4 (30 nmol) and 5 (≥ 40 nmol) of suitable colorimetric substrate hydrolyzed during 4 h. The results reported are means of 3 assays.

Data analysis

Principal component analysis and general linear models. Principal component analysis (PCA) (data log-transformed and standardized) was applied to reduce the number of variables for further analyses and to find groups of enzymes with activities correlated with one another. The samples obtained from specific parts of the digestive system of particular species were ordinated along the distinguished principal components to check their similarity with respect to the activities of particular sets of enzymes. Furthermore, species scores for particular principal components were analyzed using general linear models (GLM) with Species as a between-subject factor, Part of the digestive system as a within-subject factor (as three parts were sampled from the same individual), and their interaction. Significant effects of these analyses were further examined using post-hoc Fisher LSD tests with a Dunn-Šidák correction for multiple comparisons.

The PCA was carried out using the Vegan 2.5–5 package for R (Oksanen et al. 2019), whereas GLM were run using SPSS 25.0 (IBM Inc.).

Table III. Activity of hydrolases in the digestive system of predatory leeches of the genus *Erpobdella* and blood feeding *Acanthobdella peledina*.

		Enzyme activities (nmol as mean ± standard deviation)											
		Pharynx				Crop				Intestine			
Enzyme	pH	**E. monostriata*	*E. nigricollis*	*E. testacea*	*A. peledina*	*E. monostriata*	*E. nigricollis*	*E. testacea*	*A. peledina*	*E. monostriata*	*E. nigricollis*	*E. testacea*	*A. peledina*
Peptide hydrolases													
Leucine arylamidase	7.5	23.33 ± 5.78	26.67 ± 5.78	16.67 ± 5.78	23.33 ± 5.78	26.67 ± 5.78	36.67 ± 5.78	36.67 ± 5.78	26.67 ± 5.78	36.67 ± 5.78	36.67 ± 5.78	36.67 ± 5.78	26.67 ± 5.78
Valine arylamidase	7.5	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	0.00	8.33 ± 2.89	16.67 ± 5.78	16.67 ± 5.78	6.67 ± 2.89	23.33 ± 5.78	26.67 ± 5.78	26.67 ± 5.78	6.67 ± 2.89
Cystine arylamidase	7.5	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	0.00	6.67 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	23.33 ± 5.78	8.33 ± 2.89	0.00
Trypsin	8.5	8.33 ± 2.89	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	0.00	8.33 ± 2.89	0.00	6.67 ± 2.89	0.00	8.33 ± 2.89	0.00
α- Chymotrypsin	7.5	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	0.00	6.67 ± 2.89	8.33 ± 2.89	8.33 ± 2.89	0.00	8.33 ± 2.89	8.33 ± 2.89	23.33 ± 5.78	0.00
Phosphohydrolases													
Alkaline phosphatase	8.5	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	16.67 ± 5.78	6.67 ± 2.89	6.67 ± 2.89	0.00	36.67 ± 5.78	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	36.67 ± 5.78
Acid phosphatase	5.4	6.67 ± 2.89	6.67 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	16.67 ± 5.78	16.67 ± 5.78	6.67 ± 2.89	26.67 ± 5.78	16.67 ± 5.78
Naphthol-AS-BI-phosphohydrolase	5.4	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	23.33 ± 5.78	6.67 ± 2.89
Ester hydrolases													
Esterase (C 4)	6.5	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89
Esterase lipase (C 8)	7.5	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	0.00	8.33 ± 2.89	8.33 ± 2.89	16.67 ± 5.78	6.67 ± 2.89	8.33 ± 2.89	16.67 ± 5.78	16.67 ± 5.78	6.67 ± 2.89
Lipase (C 14)	7.5	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	0.00	8.33 ± 2.89	8.33 ± 2.89	8.33 ± 2.89	0.00	8.33 ± 2.89	16.67 ± 5.78	16.67 ± 5.78	0.00
Glycosidases													
α-Galactosidase	5.4	8.33 ± 2.89	0.00	6.67 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	0.00	16.67 ± 5.78	6.67 ± 2.89	16.67 ± 5.78	0.00
β-Galactosidase	5.4	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	0.00	8.33 ± 2.89	8.33 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	16.67 ± 5.78	16.67 ± 5.78	6.67 ± 2.89
β-Glucuronidase	5.4	6.67 ± 2.89	0.00	6.67 ± 2.89	0.00	0.00	0.00	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	0.00
α-Glucosidase	5.4	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	0.00	16.67 ± 5.78	8.33 ± 2.89	26.67 ± 5.78	0.00
β-Glucosidase	5.4	6.67 ± 2.89	0.00	6.67 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	0.00
N-Acetyl-β-glucosaminidase	5.4	8.33 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	16.67 ± 5.78	16.67 ± 5.78	16.67 ± 5.78	16.67 ± 5.78	26.67 ± 5.78	16.67 ± 5.78
α-Mannosidase	5.4	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00	0.00	8.33 ± 2.89	6.67 ± 2.89	0.00	0.00	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89
α-Fucosidase	5.4	16.67 ± 5.78	0.00	6.67 ± 2.89	0.00	16.67 ± 5.78	8.33 ± 2.89	16.67 ± 5.78	6.67 ± 2.89	16.67 ± 5.78	26.67 ± 5.78	26.67 ± 5.78	6.67 ± 2.89

**E. monostriata – Erpobdella monostriata, E. nigricollis – Erpobdella nigricollis, E. testacea – Erpobdella testacea, A. peledina – Acanthobdella peledina*

Table IV. Activity of hydrolases in the digestive system of rhynchobdellid leeches.

		Enzyme activities (nmol as mean ± standard deviation)											
		Probioscis				Crop				Intestine			
Enzyme	pH	*P. sp. nov.*	*P. respirans*	*P. costata*	*T. maculosum*	*P sp. nov.*	*P. respirans*	*P. costata*	*T. maculosum*	*P sp. nov.*	*P. respirans*	*P. costata*	*T. maculosum*
Peptide hydrolases													
Leucine arylamidase	7.5	6.67 ± 2.89	26.67 ± 5.78	6.67 ± 2.89	8.33 ± 2.89	16.67 ± 5.78	26.67 ± 5.78	8.33 ± 2.89	36.67 ± 5.78	36.67 ± 5.78	16.67 ± 5.78	16.67 ± 5.78	36.67 ± 5.78
Valine arylamidase	7.5	16.67 ± 5.78	6.67 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	16.67 ± 5.78	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	8.33 ± 2.89	36.67 ± 5.78
Cystine arylamidase	7.5	6.67 ± 2.89	6.67 ± 2.89	1.67 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	0.00	6.67 ± 2.89	8.33 ± 2.89	8.33 ± 2.89	16.67 ± 5.78
Trypsin	8.5	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00	8.33 ± 2.89	6.67 ± 2.89	0.00	0.00	8.33 ± 2.89	6.67 ± 2.89	0.00	16.67 ± 5.78
α- Chymotrypsin	7.5	0.00	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	8.33 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	0.00	0.00
Phosphohydrolases													
Alkaline phosphatase	8.5	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	16.67 ± 5.78	6.67 ± 2.89	6.67 ± 2.89	16.67 ± 5.78	26.67 ± 5.78	6.67 ± 2.89	6.67 ± 2.89	36.67 ± 5.78	36.67 ± 5.78
Acid phosphatase	5.4	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	26.67 ± 5.78	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	36.67 ± 5.78
Naphthol-AS-BI-phosphohydrolase	5.4	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	36.67 ± 5.78
Ester hydrolases													
Esterase C 4	6.5	0.00	0.00	1.67 ± 2.89	6.67 ± 2.89	0.00	6.67 ± 2.89	16.67 ± 5.78	8.33 ± 2.89	0.00	6.67 ± 2.89	8.33 ± 2.89	26.67 ± 5.78
Esterase lipase C 8	7.5	6.67 ± 2.89	16.67 ± 5.78	1.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	26.67 ± 5.78	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	36.67 ± 5.78	6.67 ± 2.89	36.67 ± 5.78
Lipase C 14	7.5	6.67 ± 2.89	8.33 ± 2.89	0.00	0.00	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	16.67 ± 5.78	0.00	0.00
Glycosidases													
α-Galactosidase	5.4	0.00	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	0.00	0.00	0.00	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00
β-Galactosidase	5.4	6.67 ± 2.89	6.67 ± 2.89	0.00	6.67 ± 2.89	6.67 ± 2.89	16.67 ± 5.78	1.67 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	36.67 ± 5.78
β-Glucuronidase	5.4	0.00	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	8.33 ± 2.89	0.00	0.00	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00
α-Glucosidase	5.4	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	16.67 ± 5.78	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	16.67 ± 5.78
β-Glucosidase	5.4	0.00	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00
N-Acetyl-β-glucosaminidase	5.4	6.67 ± 2.89	8.33 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	26.67 ± 5.78	36.67 ± 5.78	8.33 ± 2.89	6.67 ± 2.89	16.67 ± 5.78	36.67 ± 5.78	36.67 ± 5.78
α-Mannosidase	5.4	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	6.67 ± 2.89	0.00	0.00	6.67 ± 2.89	6.67 ± 2.89	0.00	26.67 ± 5.78
α-Fucosidase	5.4	6.67 ± 2.89	6.67 ± 2.89	1.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	6.67 ± 2.89	8.33 ± 2.89	16.67 ± 5.78	36.67 ± 5.78

**P. sp. n. – Piscicola sp. n., P. respirans – Piscicola respirans, P. costata – Placobdella costata, T. maculosum – Theromyzon maculosum*

Phylogenetic analysis. The values of enzyme activity were coded with the gap-weighting method (Thiele 1993). A new character state (xnew) was calculated according to the following formula:

xnew = n*[(x − min)/(max − min)],

where "max" and "min" are the maximum and minimum mean values of the character across all operational taxonomic units (OTUs), "x" is the mean value of the current taxon, and "n" is the number of allowed character states (in the present study n = 10). Codes for all taxa are shown in Table V.

The analyses based on codes for enzyme activities were carried using PAUP* 4.0b (Swofford 2002) under the branch-and-bound option. Statistical support for clades was assessed using a bootstrap analysis (BS) for 1000 replicates in PAUP. Additional statistics used here were total tree length (L), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) (Table VI). Obtained cladograms were explored in FigTree version 1.4.2 (Rambaut 2014).

Results

Activity of hydrolases

Hydrolytic enzyme activities in different parts of the digestive system in the species examined here (means and standard deviations) are presented in Tables II, III and IV.

In the anterior region of the digestive system and in the crop of *B. astaci*, similar activities of 5 enzymes were detected (valine and cystine arylamidases, trypsin, lipase C 14 and α-galactosidase). In the intestine of this species only valine and cystine arylamidases were active (Table II).

In *H. medicinalis*, 12 enzymes were detected (10 of them in the anterior region of the digestive system, 11 in the crop and 12 in the intestine). The highest activity in the digestive system of this leech was exhibited by both analyzed phosphatases and N-acetyl-β-glucosamidase. Two glycosidases (β-glucuronidase and α-fucosidase) had residual activity, especially in the anterior region (Table II).

In *E. monostriata*, the activity of all the analyzed enzymes was observed. This is especially true for the anterior region where 19 hydrolases were active. Two glycosidases (β-glucuronidase and α-mannosidase) were inactive in the crop and intestine, and esterase C 4 was absent from the intestine. Leucine arylamidase activity was the highest of the tested hydrolases in this species (Table III).

All the tested enzymes were active in *E. nigricollis* except β-glucuronidase (Table III). α-Galactosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-fucosidase were not detected in the anterior region of this species, whereas trypsin was missing in the crop and intestine (Table III).

All the examined hydrolases were detected in *E. testacea*. Sixteen enzymes were active in the anterior region of its digestive system, with trypsin, acid phosphatase and α-mannosidase absent from this part. In the crop, the activity of 18 enzymes (all except alkaline phosphatase) was detected. In the intestine of *E. testacea*, all the examined hydrolases were active (Table III).

In *A. peledina*, only 13 enzymes were detected. The activity of trypsin, α-chymotrypsin, lipase C 14, α-galactosidase, α- and β-glucosidases was not identified. Only 6 enzymes were active in the anterior region, 12 in the crop and 11 in the intestine of *A. peledina*. In this species, the highest activity was exhibited by leucine arylamidase and alkaline phosphatase (Table III).

In *Piscicola* sp. n., 18 hydrolases were active (all except esterase C4). There was no α -chymotrypsin, α -galactosidase, β-glucuronidase and β-glucosidase activity in the proboscis of this species (Table IV).

In the digestive system of *P. respirans*, all the analyzed enzymes were active. Esterase C 4 was missing in the anterior part of the digestive system, and α-galactosidase did not appear in the crop of this species, whereas its intestine contained all the examined enzymes. In particular, distinctly high esterase lipase C 8 activity was noted in the final section of the digestive system of *P. respirans* (Table IV).

Twelve hydrolases were active in *P. costata*. All of them were active in the crop and in the intestine. 10 enzymes occurred in the anterior region of the digestive system of this leech (without valine arylamidase and β-galactosidase). Particularly high activity of N-acetyl-β-glucosaminidase was noted in the crop and intestine, whereas alkaline phosphatase was highly active in the intestine of this species. In the anterior part of the digestive system of *P. costata*, cysteine arylamidase, esterases C4 and C8 and α -fucosidase had very low activities (Table IV).

In *T. maculosum*, the presence of 15 enzymes was confirmed, with lipase C 14, α-galactosidase, β-glucuronidase and β-glucosidase missing. In the anterior region of its digestive system, 11 active hydrolases were observed. Cystine arylamidase, trypsin, α-chymotrypsin, lipase C 14, α-galactosidase, β-glucuronidase, β-glucosidase, and α-mannosidase were not detected in this part. In the crop, activity of α-chymotrypsin was additionally

Table V. Matrix of coded enzyme activities in the digestive system in 8 leech species and *Acanthobdella peledina* and *Branchiobdella astaci*.

Species	Part of digestive system*	Enzyme																		
		Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	α-chymo-trypsin	Alkaline phosphatase	Acid phosphatase	Naphthol-AS-BIphospho-hydrolase	Esterase (C 4)	Esterase lipase (C 8)	Lipase (C 14)	α-galacto-sidase	β-galacto-sidase	β-glucuro-nidase	α-gluco-sidase	β-glucosidase	N-acetyl-βglucos-aminidase	α-manno-sidase	α-fucosidase
A. peledina	A	9	0	0	0	0	5	2	<	4	0	0	0	0	0	0	0	3	0	0
	C	7	0	8	0	0	<	5	5	5	3	0	0	4	8	0	0	5	0	4
	I	7	0	0	0	0	<	5	2	3	2	0	0	2	0	0	0	5	0	2
B. astaci	A	0	5	<	8	0	0	0	0	0	0	8	8	0	0	0	0	0	0	0
	C	0	2	<	8	0	0	0	0	0	0	8	<	0	0	0	0	0	0	0
	I	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T. maculosum	A	3	4	0	0	0	5	2	<	4	4	0	0	<	0	<	0	3	0	4
	C	<	0	0	0	8	7	7	5	5	3	0	0	5	0	<	0	2	0	4
	I	<	<	7	<	0	<	<	<	<	<	0	0	<	0	3	0	<	0	<
P. costata	A	3	0	2	0	0	2	2	<	1	1	0	0	0	0	8	0	3	0	1
	C	2	2	<	0	0	5	2	5	<	3	0	0	1	0	4	0	<	0	4
	I	5	1	4	0	0	<	2	2	3	2	0	0	2	0	3	0	<	0	5
P. respirans	A	<	4	8	8	<	2	2	<	0	<	<	8	<	<	8	<	3	<	4
	C	7	<	8	8	<	2	2	4	4	<	8	0	<	<	4	<	7	8	4
	I	5	1	4	4	4	2	2	2	3	<	<	4	2	<	3	<	5	8	2
P. sp. n.	A	3	<	8	8	0	2	2	<	0	4	8	0	<	0	8	0	3	<	4
	C	5	0	8	<	8	2	2	4	0	3	8	<	4	8	5	<	2	8	4
	I	<	0	3	5	3	2	2	2	0	2	4	4	2	<	3	<	2	8	2
H. medicinalis	A	5	0	0	0	0	<	<	<	<	4	0	0	<	3	0	0	<	0	1
	C	4	0	0	0	0	<	<	<	<	3	0	0	4	8	0	0	<	0	4
	I	6	0	1	0	0	<	<	2	6	2	0	0	2	<	0	0	<	0	2
E. monostriata	A	9	4	8	<	<	2	2	<	4	4	8	<	<	<	8	<	3	<	<
	C	7	2	8	8	8	2	2	5	4	3	<	<	5	0	4	<	2	0	<
	I	<	6	3	4	4	2	5	2	0	2	5	<	2	0	6	<	5	0	5
E. nigricollis	A	<	4	8	8	<	2	2	<	4	4	8	0	<	0	8	0	0	<	0
	C	<	<	<	0	<	2	2	4	4	3	<	<	5	0	4	<	2	<	5
	I	<	7	<	0	4	2	2	2	3	5	<	4	5	0	3	<	5	<	7
E. testacea	A	6	5	<	0	<	2	0	<	4	4	8	8	<	<	8	<	3	0	4
	C	<	<	8	<	<	0	2	4	4	6	<	<	5	8	4	<	5	8	<
	I	<	7	4	5	<	2	7	6	3	5	<	<	5	<	<	<	7	8	7

*A – anterior part; C – crop; I–intestine

Table VI. Statistics of obtained phylogenetic trees.

Parameter	Part of alimentary tract			
	Anterior	Crop	Intestine	Entire tract
Numer of trees	3	2	2	1
Length	290	312	320	959
CI	0.655	0.609	0.594	0.594
RI	0.684	0.664	0.600	0.613
RC	0.448	0.404	0.356	0.364
HI	0.345	0.391	0.406	0.406

detected. Fourteen enzymes were found in the intestine of *T. maculosum*, and their activity was relatively high compared to other parts of its digestive system (Table IV).

Comparisons among species and parts of the digestive system

The two first principal components (PC1 and PC2) resulting from the PCA analysis explained 92% of the variability in enzyme activities (Figure 1(a)). Moreover, the GLM conducted on PC1 and PC2 species scores revealed significant species × part of the digestive system interactions ($F_{2,\ 18}$ = 63.0, $P < 0.001$ and $F_{2,\ 18}$ = 45.0, $P < 0.001$, for PC1 and PC2 respectively), indicating that differences among species varied with specific parts of the digestive system.

PC1 was positively correlated with the activities of all enzymes, with the highest positive correlations shown for the proteases: leucine arylamidase, valine

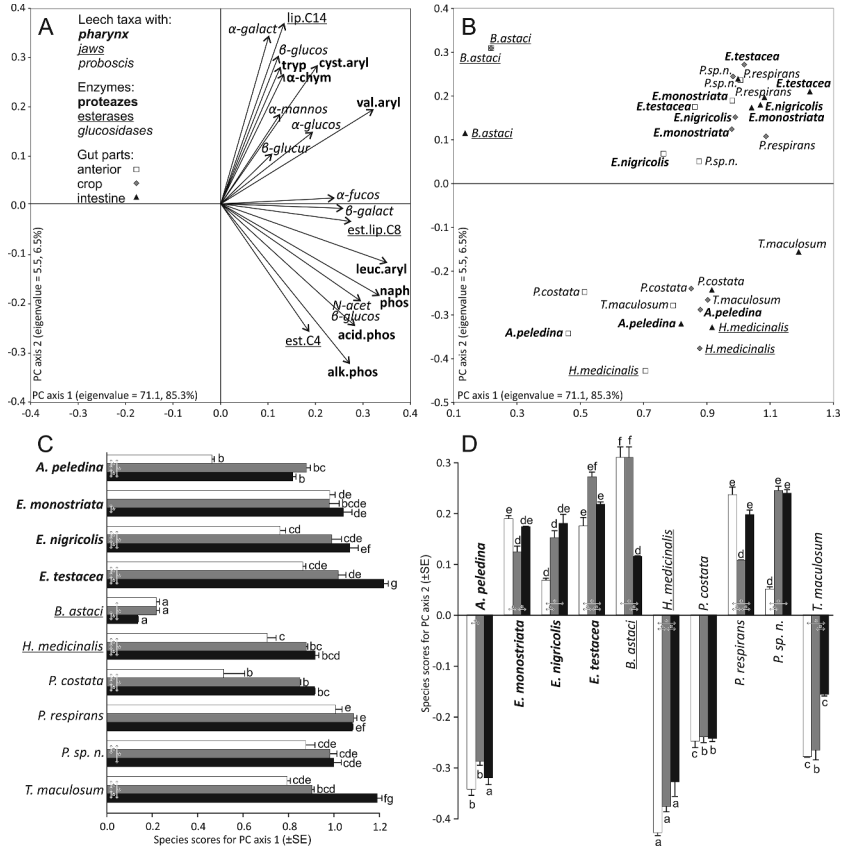

Figure 1. Principal component analysis (PCA) of log-transformed and standardized enzyme activities. (a) Correlations of particular enzyme activities with principal components 1 and 2 determined by the PCA. (b) Ordination of leech species along Principal Components 1 and 2. (c, d). Species scores assigned by the PCA for principal component 1 (the horizontal axis in panel b) principal component 2 (the vertical axis in panel b). Enzyme names in panel a: leuc.aryl – leucine arylamidase, val.aryl – valine arylamidase, cyst.aryl – cystine arylamidase, tryp – trypsin, α-chym – α- chymotrypsin, alk.phos – alkaline phosphatase, acid.phos – acid phosphatase, naphphos – naphthol-AS-BI-phosphohydrolase, est.C4 – esterase (C 4), est.lip.C8 – esterase lipase (C 8), lip.C14 – lipase (C 14), α-galact – α-galactosidase, β-galact - β-galactosidase, α-glucos – α-glucosidase, β-glucos – β-glucosidase, N-acet β-glucos – N-acetyl-β-glucosaminidase, α-mannos – α-mannosidase, α-fucos – α-fucosidase, β -glucur – β-glucuronidase. Values for the anterior part of the digestive system, crop and intestine are shown in white, grey and black, respectively. The same lowercase letters labeling the bars in panels c and d denote the lack of significant differences between particular species for a given part of the digestive system. Arrows with asterisks indicate significant differences between particular parts of the digestive system for a given species. All significances are Dunn-Šidák corrected for multiple comparisons.

arylamidase, phosphatases and naphthol AS-BI-phosphohydrolase, as well as with esterase lipase (C8), β-galactosidase and N-acetyl-β-glucosaminidase (Figure 1(a)). Thus, PC1 discriminated between the species with generally low and high enzyme numbers and activities. Activities of enzymes associated with PC1 were the lowest in *B. astaci* (Figure 1(b)). The PC1 score for this species differed significantly from those for all other species, irrespective of the part of the digestive system (see Table S1 for the full results of post-hoc analyses). Moreover, enzyme activities in the anterior parts of the digestive system of *P. costata* and *A. peledina*, as well as in the crop of *P. costata* and in the intestine of *A. peledina*, were significantly lower than in the remaining species (Figure 1(c-d)). The highest enzyme activities were observed in the intestine of *E. testacea* and *T. maculosum* (Figure 1(c-d)). They were significantly different from those from the other parts of the digestive system and from the other species. In the anterior part of the digestive system of all species except *B. astaci*, *E. monostriata* and *P. respirans*, enzyme activity was lower than in the subsequent parts of the digestive system. Also, enzyme activity in the crop was lower than in the intestine of all species but *A. peledina* and *B. astaci* (where the opposite pattern was found), as well as *P. respirans* and *Piscicola* sp. n. (where no differences between these parts were found).

PC2 was correlated with high activities of valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin and lipase C14 as well as with low activities of phosphatases, esterase C4 and N-acetyl-β-glucosaminidase (Figure 1(a)), (see Table S2 for the full results of post-hoc analyses). In general, this component distinguished between two distinct leech species groups, differing from each other in the activities of the above-mentioned enzyme sets. One group included *B. astaci*, Erpobdellidae, *Piscicola* sp. n. and *P. respirans*, which had high PC2 scores (Figure 1(b)). Among them, *B. astaci* had the highest scores for the anterior digestive system part and for the crop, whereas *Piscicola* sp. n., *P. respirans* and *E. testacea* had the highest scores for the intestine. The other group contained *T. maculosum*, *P. costata*, *A. peledina* and *H. medicinalis*, having low PC2 scores (Figure 1(a)). The two latter species had the lowest scores for PC2 and differed significantly with this respect from the other leeches from the second group (Figure 1(c- d)).

Phylogenetic analysis

The phylogenetic analysis based on enzyme activities in particular parts of the digestive system resulted in trees presenting a lack of monophyly both in the traditionally considered groups of rhynchobdellid and arhynchobdellid leeches and at the family level (Figure 2(a-d)). The representatives of the genera *Piscicola* and *Erpobdella* formed a clade, but within the clade none of the genera was monophyletic. *Branchiobdella astaci* appeared to be sister to the clade of *Piscicola* + *Erpobdella*. The representatives of the family Glossiphoniidae (*P. costata* and *T. maculosum*) formed a clade only when enzymes in the intestine were considered (Figure 2(c)). In other cases, *P. costata* formed a sister branch to the clade of *Piscicola* + *Erpobdella* + *B. astaci* (Figure 2(a,b,d)). *Theromyzon maculosum* appeared to be sister to *H. medicinalis* in the cladogram based on the enzyme activities in the entire digestive system and its anterior part (Figure 2(a,d)). The leech-like annelid *A. peledina*, was placed in the outer position to Hirudinida and *B. astaci* (Figure 2(a-d)). The highest value of the consistency index (CI = 0.6552) and the lowest tree length (TL = 290) were obtained for the cladogram generated using data of enzyme activities in the anterior part of the digestive system (Table VI). Additionally, the analyses setting *B. astaci* in the outgroup were performed (Fig. S1). The statistics for the cladograms were very similar to these obtained for the cladograms with *A. peledina* as an outgroup (TL = 959, CI = 0.5944, HI = 0.4056, RI = 0.6125, compare with the Table VI). However, the topology of the cladograms appeared to by slightly different. There are two clades distinguished: one containing sanguivory leeches and *A. peledina* sister to *T. maculosum* and *H. medicinalis*, and the second including mainly sanguivory piscolids and predatory erpobdellids (Fig. S1).

Discussion

Differences in activity of hydrolases in the digestive systems of the studied taxa

Most likely, bloodsucking in leeches (Hirudinida) derived from predatory Annelida, which can be seen in the feeding strategy of leech relatives, the acanthobdellidans *A. peledina* and *Paracanthobdella livanowi* (Siddall et al. 2001; Erséus & Källersjö 2004; Cichocka & Bielecki 2015). It should be pointed out here that *A. peledina* feeds, to some extent, as predatory leeches do, as its pharynx is constructed similarly to that of these annelids (Epshtein 1987; Bielecki et al. 2014b). Bigger specimens drill holes beneath a dorsal fin to suck blood and often consume large parts of tissue (Andersson 1988). In our study, the level of enzyme activity in

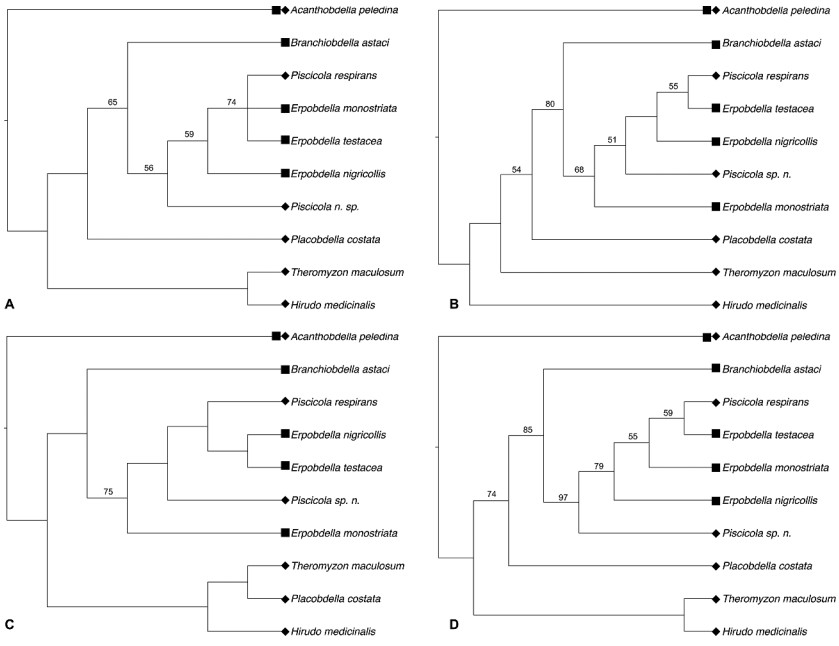

Figure 2. The most parsimonious trees for 8 leech species, as well as *Acanthobdella peledina* and *Branchiobdella astaci* based on hydrolytic enzyme activities coded with gap-weighting method in particular parts of the digestive system: (a) – anterior part, (b) – crop, (c) – intestine, (d) – entire digestive system. ■ – species feeding on tissues, ♦ – species feeding on body fluids. Numbers at nodes indicate bootstrap values > 50.

A. peledina appeared to be similar to bloodsucking leeches (Figure 1), but the outer position of *A. peledina* in the cladograms (Figure 2(a-d)) might result from this mixed feeding strategy, consisting in bloodsucking and taking "bites" of solid tissues. Additionally, *A. peledina*, in contrast to Piscicolidae and similarly to the typical bloodsucking leeches, takes blood and lymph from the deeper layers of the skin. This may explain the result of the PCA and GLM analyses that grouped *A. peledina* together with *H. medicinalis*, *T. maculosum* and *P. costata* (Figure 1).

One of the most striking results of the current study was the low number (five) of active enzymes in the digestive system of *Branchiobdella astaci*. It suggests the high individuality of the digestive physiology of Branchiobdellida in comparison with Acanthobdellida and Hirudinida. It may be connected with the uniqueness of arthropods (crayfish), which are branchiobdellidan hosts (Sawyer 1986). The differences may be caused by chemical properties of their food, i.e. arthropod haemolymph (the basic food type), which has already been suggested by other authors (Small et al. 2007). Arthropods are prey for predatory erpobdellids, glossiphoniids and a few species of fish leeches (Bielecki et al. 2014b). The results of the current study strongly suggest that, contrary to acanthobdellids and true leeches, *B. astaci* digests food mainly in its foregut and crop (anterior and mid parts of the digestive system), because only two peptide hydrolases (valine arylamidase and cystine arylamidase) were found in the intestine (Table II). Notwithstanding all these differences, in our study *B. astaci* was placed as a sister branch to the clade of Piscicolidae and Erpobdellidae (Figure 2 (a-d)). However, the monophyly of the clade comprising true leeches (Hirudinida), leech-like acanthobdellidans (Acanthobdellida) and crayfish worms (Branchiobdellida) seems to be evident, as has been shown in numerous morphological and molecular analyses (e.g. Purschke et al. 1993; Martin 2001; Siddall et al. 2001; Erséus & Källersjö 2004; Marotta et al. 2008; Rousset et al. 2008; Martinez-Ansemil et al. 2012; Tessler et al. 2018). The phylogenetic relationships between those taxa are still unresolved (the history of this debate has been summarized by e.g. Bielecki et al. 2014a; Tessler et al. 2018; Phillips et al. 2019). Most morphological analyses have shown that acanthobdellids are sister to true leeches (e.g. Purschke et al. 1993; Świątek et al. 2012; Urbisz & Świątek 2013) and from the early morphological descriptions *A. peledina* was regarded as an intermediate form between oligochaetous annelids and true leeches (an ancient leech with chaetae) (Livanow 1906,; Sawyer 1986). On the other hand, the use of such molecular markers as COI and 18S DNA sequences revealed that branchiobdellidans are sister to true leeches with

A. peledina as an outgroup (Apakupakul et al. 1999; Erséus & Källersjö 2004; Siddall et al. 2001; Rousset et al. 2008). To complicate matters, combined morpho-molecular studies (18S sequences and sperm characters) performed by Marotta et al. (2008) suggested that Acanthobdellida was sister to true leeches.

The latest molecular investigations using new sequences obtained from the freshly collected *A. peledina* specimens (the formerly used sequences were found to be contaminated) also recovered *A. peledina* as sister to Hirudinida with Branchiobdellida as an outgroup (Tessler et al. 2018; Phillips et al. 2019). Phylogenomic analysis performed by Phillips et al. (2019) showed Branchiobdellida as sister to a clade including Hirudinida and *Acanthobdella* and questioned the hypothesis about *Acanthobdella* as a "missing link" between true leeches and other clitellates. Being suggested by the results of Tessler et al. (2018) and Phillips et al. (2019) additional analyses using *B. astaci* as an outgroup were performed in our study (Fig. S1). The outcome, however, did confirm neither the hypothesis about piscicolid/erpobdellid-like ancestor of leeches nor the hypothesis about Acanthobdellida being sister to true leeches. Nevertheless, the branch support values in our cladograms are not satisfactorily high and this probably suggests that enzymatic data used in the analysis are not suitable to be included in the phylogenetic inference alone.

The occurrence of hydrolases and their activity show strong physiological specializations of *B. astaci* when compared to leeches. The branchiobdellid is clearly separated from other studied taxa considering both number of active enzymes and the place of the highest enzymatic activity which is pictured in the PCA and GLM analysis in this study (Figure 1). The outcome is also coherent with the latest phylogenomic studies by Phillips et al. (2019). To receive wider picture of relations based on hydrolase activity, enzymatic spectra of other Oligochaeta, such as megadriles, lumbriculids, naidids, etc., should be examined. It would be especially valuable to obtain data about hydrolase activity in lumbriculids, considered as close relatives of leech-like annelids (Martin 2001; Siddall et al. 2001; Erséus & Källersjö 2004; Marotta et al. 2008; Rousset et al. 2008; Tessler et al. 2018). It would also be useful to extend the spectrum of analyzed ectoparasitic clitellates to further species of predatory and sanguivory leeches as well as branchiobdellidans.

Deliberating the evolutionary relationships between *B. astaci* and leeches, it should be mentioned that a similar picture of the species placed in a sister grouping with Piscicolidae was obtained in the phylogenetic analysis based on morphometric data (Cichocka & Bielecki 2015; Thorp et al. 2019). In their study, Cichocka and Bielecki (2015) presented some suggestions to explain the similarity and affinity of branchiobdellidans to Piscicolidae fish leeches, e.g. variety in body forms and feeding on crustacean body fluids. All in all, our results suggest some physiological specialization and adaptations of the branchiobdellid species studied, and this seems to be an interesting issue in and of itself. However, as it was mentioned above, the methodological aspect of using enzyme activity levels as data for phylogenetic inference needs to be reconsidered and improved to give more reliable outcome.

The phylogenetic analysis based on the enzymatic activity did not confirm the monophyly of either "Rhynchobdellida" (here considered as Piscicolidae and Glossiphoniidae) or Arhynchobdellida (Erpobdelliformes and Hirudiniformes) (Figure 2). In our study, Erpobdellidae and Piscicolidae formed one group or clade (Figure 1–2). These results seem to be consistent with the hypothesis of the origin of Hirudinida and their ancestor, which most likely was sanguivorous and erpobdellid- or piscicolid-like (Borda & Siddall 2004a, 2004b; Trontelj et al. 1999). Moreover, molecular analyses have shown a close evolutionary relationship between Erpobdellidae and Piscicolidae (Bielecki & Polok 2012). Admittedly, Erpobdellidae are considered as predators and Piscicolidae as bloodsuckers, but if we take a closer look at the type of substance they actually feed on, some similarities can be observed. Erpobdellids consume arthropod haemolymph, whereas piscicolids, although defined as bloodsuckers, actually suck lymph, as the amount of blood in the fish body is small. They were usually collected from fins where numerous lymphatic vessels occur, and the digestive system of these leeches was filled with yellowish or amberish fluid. Blood is mainly taken by piscicolids just before the time of reproduction (Bielecki et al. 1997, 2011b; Cichocka et al. 2018). As fish and mammalian lymphatic systems share many molecular and morphological features (Hedrick et al. 2013), it can be assumed that the products of fat digestion are transported via lymphatic vessels (Dixon 2010). Piscicolids may suck lymph to get these nutrients. Furthermore, as mentioned above, some piscicolids feed on crustaceans by sucking their haemolymph, e.g. *Mysidobdella borealis* on Mysidae, as well as *Baicalobdella torquata*

and *Codonobdella truncata* on Gammaridae (Sawyer 1986). On the other hand, there are some reports on erpobdellid leeches, e.g. *E. octoculata* and *E. vilnensis* found on injured fish, aggregated next to their wounds and probably feeding on the surrounding tissues (Bielecki 1977, 1997; Cichocka et al. 2015; Jabłońska-Barna et al. 2017). This may suggest that Erpobdellidae, similar to fish leeches, can also include lymph in their diet.

The last group of taxa considered here shows enzymatic activity of typical bloodsucking leeches, *H. medicinalis, T. maculosum* and *P. costata*, grouped along the PC2 axis (Figure 1) whereas phylogenetic analysis placed them in different arrangements depending on the part of the digestive system (Figure 2). *Hirudo medicinalis* can suck blood from a wide variety of vertebrates, mainly mammals, but it can also feed on fish and amphibians (Elliot & Dobson 2015). Recently it has been suggested that this medicinal leech also attacks birds (Buczyński et al. 2014). *Placobdella costata* drinks blood from turtles, but it was recently shown to attack birds (Bielecki et al. 2012a) and humans (Wilkialis 1973) as well. *Theromyzon maculosum*, similar to other species of the genus, is monozoic and stenotrophic, preferring bird blood. The similarity in enzyme activity between *T. maculosum, H. medicinalis* and *P. costata* may be explained by their feeding on vertebrate blood.

However, it should be noted that *Theromyzon* leeches bear some primeval characters (e.g. penis and vagina in the reproductive system, structures of the digestive system divided into several diverticuli) and were formerly named *Protoclepsis* (Lukin 1976; Sawyer 1986; Bielecki et al. 2009). It is possible that a blood feeding leech ancestor had similar segmentation in the digestive system, as it is known from other polymerized systems in other animals. It should be noted here that Glossiphoniidae, as the most specious group of leeches (Bielecki et al. 1999), have the highest number of prey and hosts, including both invertebrates and vertebrates. It seems that a wide variety of feeding habits and unique parental care were the basis for the evolutionary success of these leeches.

Differences in enzyme activity among various regions of the digestive system of the studied taxa

The comparison of enzyme activity among different regions of the digestive system suggests that the digestion and potentially absorption in some species take place in the crop, while in others they are carried out in the intestine (Jenings & Van der Lande 1967). Leeches feeding on blood synthesize many substances which are secreted into the host's body, where they inhibit blood coagulation, reduce blood viscosity or dilate blood vessels (Sawyer 1986; Whitaker et al. 2005). Moreover, blood-feeding leeches have their midgut (crop and intestine) differentiated into numerous caeca, enlarging its absorbent surface (Sawyer 1986; Rost-Roszkowska et al. 2012). In the salivary glands of *P. geometra*, the high activity of esterase, as well as acid and alkaline phosphatases, is combined with the fact that they participate in dissolving the mucus covering the host skin and tissues (Jennings & van der Lande 1967; Van der Lande 1968). Van der Lande (1968) and Hovingh and Linker (1999) have also described the presence of esterases in the anterior region of the digestive system of *P. geometra*. Our study has revealed the activity of esterases and peptide hydrolases in the majority of the species examined here. It confirms that the salivary glands participate in the digestion of host tissues, but the differences in the level of enzyme activity result from the different structure of the host's skin. In many invertebrate parasites, hydrolases can be secreted into the lumen of the digestive system or to the external environment (Hinck & Ivey 1976; Matthews 1984; Knox & Kennedy 1988; Moczoń & Wranicz 1999; Jefferies et al. 2001; Irwin et al. 2004). These enzymes (e.g. phosphatases, aminopeptidases, esterases, glycosidases, etc.) fulfill many functions: the digestion of connective tissues, inhibition of blood coagulation, or protecting the parasite against the response of the host immune system. Moreover, they enable the parasites to penetrate the host body. Such a relationship was observed by Żółtowska et al. (2007), showing a significant role of α -fucosidase of the nematode during penetration of the host tissues.

Generally, in our study the enzyme activities in the anterior part of the digestive system in parasitic leeches appeared to be lower than in predators (Figure 1). According to Dziekońska et al. (2009), enzymes synthesized in the anterior region of the digestive system in predatory leeches feeding on large invertebrates are responsible for the beginning of digestion. Our study confirmed this statement, because as many as 16 to 19 enzymes have been found to be active in the pharynx of predatory leeches. They possess the whole spectrum of enzymes including those which take part in blood digestion. It reinforces the hypothesis about a blood-feeding ancestor of leeches. However, it should be mentioned that predatory leeches feed on invertebrates which also contain blood or haemolymph in their body.

According to Jenings and Van der Lande (1967), digestion takes part in the crop of *P. geometra*, where ingested erythrocytes are digested by enzymes, e.g. esterase, for 10 days. Additionally, water and all water-soluble compounds (e.g. glucose) are absorbed in the crop of *P. geometra*. Moreover, enzymatic (Dziekońska et al. 2009) and ultrastructural studies (Rost-Roszkowska et al. 2012) showed that digestion and absorption occur in the crop of these leeches. This statement was confirmed in our study as in the crop of the analyzed species (except *B. astaci*) the activity of acid and alkaline phosphatases was detected. These enzymes are involved in the digestion of numerous nourishments. Our study revealed that the number of active enzymes in the crop of leeches that parasitize fish and predatory leeches is similar (17 to 18 enzymes), as reported by Dziekońska et al. (2009). Moreover, we observed a very low level or absence of trypsin and α-chymotrypsin activity in the crop of all the leeches analyzed during our study. According to Roters and Zebe (1992a, 1992b) and Baskova and Zavalova (2001), low concentration or lack of these hydrolases in the crop of *H. medicinalis* can be caused by the presence of their inhibitors. They inhibit the activity of proteases which are present in leukocytes, protecting the accumulated blood against its digestion. Inhibitors belong to two types: bdellins, which inhibit trypsin, plasmin and acrosine; and eglins, which inhabit chymotrypsin, elastin, cathepsin D and subtilisin (Baskova & Zavalova 2001). It has been suggested that, although not all leeches feed on blood, they all have the same pattern of enzymatic activity (Borda & Siddall 2004a, 2004b). According to Borda and Siddall (2004a), the ancestor of leeches had a similar body to that of the representatives of the family Erpobdellidae or Piscicolidae and was a blood feeder. This hypothesis was also confirmed by further analyses of morphometric characters (Cichocka & Bielecki 2015). It can be justified by our current study, as the numbers of active enzymes in Piscicolidae and Erpobdellidae were similar to each other (17–18) and rather different from the number found in *H. medicinalis* (11) and even *A. peledina* (12) and *B. astaci* (5) (Tables II, III).

In Piscicolidae leeches, alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase had stable activity levels in the entire digestive system. These enzymes are responsible for absorption of food masses. Our results suggest that the digestion takes place along the entire digestive system at a similar level. In the crop and intestine of Piscicolidae, high activity of esterases has been detected, confirming the results of Jennings and van der Lande (1967) and Dziekońska et al. (2009). Similar to other invertebrates (e.g., *Fasciola hepatica*) these enzymes are associated with intramembranous transport (Humiczewska 2002).

In our study, the activity of lipase C14 was not found in the intestine of *H. medicinalis, A. peledina, B. astaci, T. maculosum* and *P. costata*. Lipase, as the enzyme responsible for hydrolysis of esters of glycerol and fatty acids, has not been described in the *T. tessulatum* (Jennings & van der Lande 1967), *Haemopis sanguisuga, E. octoculata, G. complanata, P. geometra* (Dziekońska et al. 2009). It has been suggested that endosymbionts participate in the digestion of lipids, so lipase does not have to be synthesized (Jennings & van der Lande 1967). In leeches from the genus *Theromyzon*, endosymbionts (e.g., *Aeromonas, Klebsiella, Proteobacteria, Pseudomonas* including *P. hirudinis, Xanthomonas*) accumulate in the cytoplasm of intestinal cells (Büsing et al. 1953; Graf 1998; Kikuchi & Fukatsu 2002; Kunicki–Goldfinger 2008). However, the lack of lipase may also be due to the small amount of lipids in their diet.

In the majority of leeches analyzed here, except Piscicolidae, high enzyme activity was detected in the intestine. The high activity of N-acetyl-β-glucosaminidase in the intestine and crop in all of the leeches examined here (with the exception of *B. astaci*) is connected with the digestion of bacterial cell walls and chitin (Boetius & Felbeck 1995; Dziekońska et al. 2009) as well as with the hydrolysis of products of digestion of hyaluronic acid, which is a component of connective tissues.

Conclusions

Our studies showed that: (1) the intestine is the main region of the digestive system which is responsible for digestion and possible absorption in Hirudinida and Acanthobdellida, while in Branchiobdellida these processes start in the foregut and crop and only five enzymes was active; (2) the level of enzyme activity and their composition in the anterior region of the digestive system (proboscis, jaws or pharynx) is connected with different modes of feeding; (3) the types of active enzymes are associated with the type of food (the different structure of epidermis/dermis covering the host body) and its chemical character (blood, haemolymph, tissue fluid); (4) the lack or low activity level of trypsin and chymotrypsin in all Hirudinida supports the hypothesis about a blood-feeding ancestor of the

leeches; (5) using the activity of enzymes for evolutionary inference, our outcome confirms the previous results, based on molecular phylogenetics, showing that "Rhynchobdellida" constitute a paraphyletic group; (6) Arhynchobdellida appear not to be a monophyletic group in contrast to the earlier studies based on molecular and morphological data; (7) Using enzyme activities levels as data for phylogenetic analysis seems to be highly problematic and requires further considerations for optimization of methodology

Future studies should focus on the precise enzymatic activity in all regions of the digestive system in the remaining taxa of Clitellata, first of all in Lumbriculida, which should enable us to draw conclusions about the evolutionary history of food preferences among ectoparasitic and predatory clitellates. The results presented in this paper form the basis for further research on the relationship between phylogeny and enzymology within the Clitellata.

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Supplementary material

Supplemental data for this article can be accessed here.

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