

## Recent evolution of ancient Arctic leech relatives: systematics of Acanthobdellida

DANIELLE BOŽENA DE CARLE<sup>1,2,†</sup>, ŁUKASZ GAJDA<sup>3,†</sup>, ALEKSANDER BIELECKI<sup>4</sup>, STANISŁAW CIOS<sup>5</sup>, JOANNA M. CICHOCKA<sup>4,6</sup>, HEIDI E. GOLDEN<sup>7,◉</sup>, ANDREW D. GRYSKA<sup>8</sup>, SERGEY SOKOLOV<sup>9</sup>, MARINA BORISOWNA SHEDKO<sup>10</sup>, RUNE KNUDSEN<sup>11</sup>, SERGE UTEVSKY<sup>12,\*</sup>, PIOTR ŚWIĄTEK<sup>3,\*</sup> and MICHAEL TESSLER<sup>13,14,15,\*</sup>

<sup>1</sup>*Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario M5S 2B4, Canada*

<sup>2</sup>*Department of Natural History, Royal Ontario Museum, Toronto, Ontario M5S 2C6, Canada*

<sup>3</sup>*Faculty of Natural Sciences, Institute of Biology, Biotechnology and Environmental Protection, University of Silesia in Katowice, Katowice 40-007, Poland*

<sup>4</sup>*Department of Zoology, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Olsztyn 10-719, Poland*

<sup>5</sup>*Stryjeńskich Street 6/4, 02-791 Warsaw, Poland*

<sup>6</sup>*XI High School, Kołobrzeska Street 9, Olsztyn 10-444, Poland*

<sup>7</sup>*Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269, USA*

<sup>8</sup>*Sport Fish Division, Alaska Department of Fish and Game, Fairbanks, AK 99701, USA*

<sup>9</sup>*A. N. Severtsov Institute of Ecology and Evolution, Moscow, Russia*

<sup>10</sup>*Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia 690022*

<sup>11</sup>*Department of Arctic and Marine Biology, The Arctic University of Norway, PO Box 6050 Langnes, N-9037 Tromsø, Norway*

<sup>12</sup>*Department of Zoology and Animal Ecology, V. N. Karazin Kharkiv National University, Kharkiv 61022, Ukraine*

<sup>13</sup>*Department of Biology, St. Francis College, Brooklyn, NY, USA*

<sup>14</sup>*Sackler Institute for Comparative Genomics, American Museum of Natural History, New York, NY 10024, USA*

<sup>15</sup>*Division of Invertebrate Zoology, American Museum of Natural History, New York, NY 10024, USA*

Received 1 July 2021; revised 17 January 2022; accepted for publication 20 January 2022

Acanthobdellida gnaw into the sides of salmonid fishes in frigid Arctic lakes and rivers, latching on with fearsome facial hooks. Sister to leeches, they are an ancient lineage with two described species. Unfortunately, Acanthobdellida are rarely collected, leading to a paucity of literature despite their unique morphology. Populations range from Eurasia to Alaska (USA), but few specimens of *Acanthobdella peledina* are represented in molecular studies, and no molecular data exist for *Paracanthobdella livanowi*, making their taxonomic position difficult to assess. We use phylogenetics and morphology to determine whether allopatric populations of *A. peledina* are distinct species and assess the current classification scheme used for Acanthobdellida. We produce a new suborder, **Acanthobdelliformes**, to match the

\*Corresponding authors. E-mail: [serge.utevsky@karazin.ua](mailto:serge.utevsky@karazin.ua); [piotr.swiatek@us.edu.pl](mailto:piotr.swiatek@us.edu.pl); [mtessler@sfc.edu](mailto:mtessler@sfc.edu)

†D. B. de Carle and Ł. Gajda are co-first authors for this publication.

[Version of record, published online 16 July 2022; <http://zoobank.org/urn:lsid:zoobank.org:pub:A02EF0F4-008F-4974-9C87-9D738CD1B6E8>]

taxonomy within Hirudinea. Scanning electron micrographs indicate species-level differences in the anterior sucker and facial hooks; molecular phylogenetics mirrors this divergence between species. We assign both species to the family Acanthobdellidae and abandon the family Paracanthobdellidae. Alaskan and European *A. peledina* populations are morphologically similar, but appear phylogenetically divergent. Our data strongly suggest that members of the order Acanthobdellida diverged relatively recently in their ancient history, but based on genetic distance, this divergence appears to pre-date the most recent cycles of glaciation.

**ADDITIONAL KEYWORDS:** Annelida – hook-faced fish worms – phylogenetics – scanning electron microscopy – taxonomy.

## INTRODUCTION

### OVERVIEW

Determining the evolutionary relationships between animals illuminates fundamental shifts in morphology, ecology and behaviour. Accordingly, a foundational question in annelid systematics has been: what is the sister group to leeches? Mounting evidence points to Acanthobdellida Livanow, 1905 as the answer to this question (reviewed by [Kutschera & Epshtein, 2006](#); [Tessler \*et al.\*, 2018a](#)). These are ectoparasites, primarily of salmonid fishes, and are Arctic and sub-Arctic in distribution. Found primarily in remote boreal locations, they are rarely collected. Therefore, these ancient annelids are understudied, belying their importance in understanding the early evolution of leeches and their unique place on the tree of life, let alone their own diverse suite of unique traits.

Acanthobdellida comprise two known species: *Acanthobdella peledina* Grube, 1851 and *Paracanthobdella livanowi* (Epstein, 1966) (see [Fig. 1](#); [Table 1](#)). These are each placed in their own monotypic family: Acanthobdellidae Livanow, 1905 and Paracanthobdellidae [Epstein, 1987](#), respectively. The former species is the better studied of the two, appearing in a few molecular studies and a number of studies on European populations in particular. However, it has a notable disjunct population in Alaska, USA, which has not been studied since the first records in the 1970s ([Holmquist, 1974](#); [Hauck \*et al.\*, 1979](#)). The second species, *P. livanowi*, appears in few studies and has yet to be incorporated into molecular phylogenetic analyses. In the present study, we used new samples from a broad set of localities ([Fig. 2](#); [Table 2](#)) and sought to determine the evolutionary relationships between populations and species of Acanthobdellida, and the true extent of molecular and morphological diversity of this order.

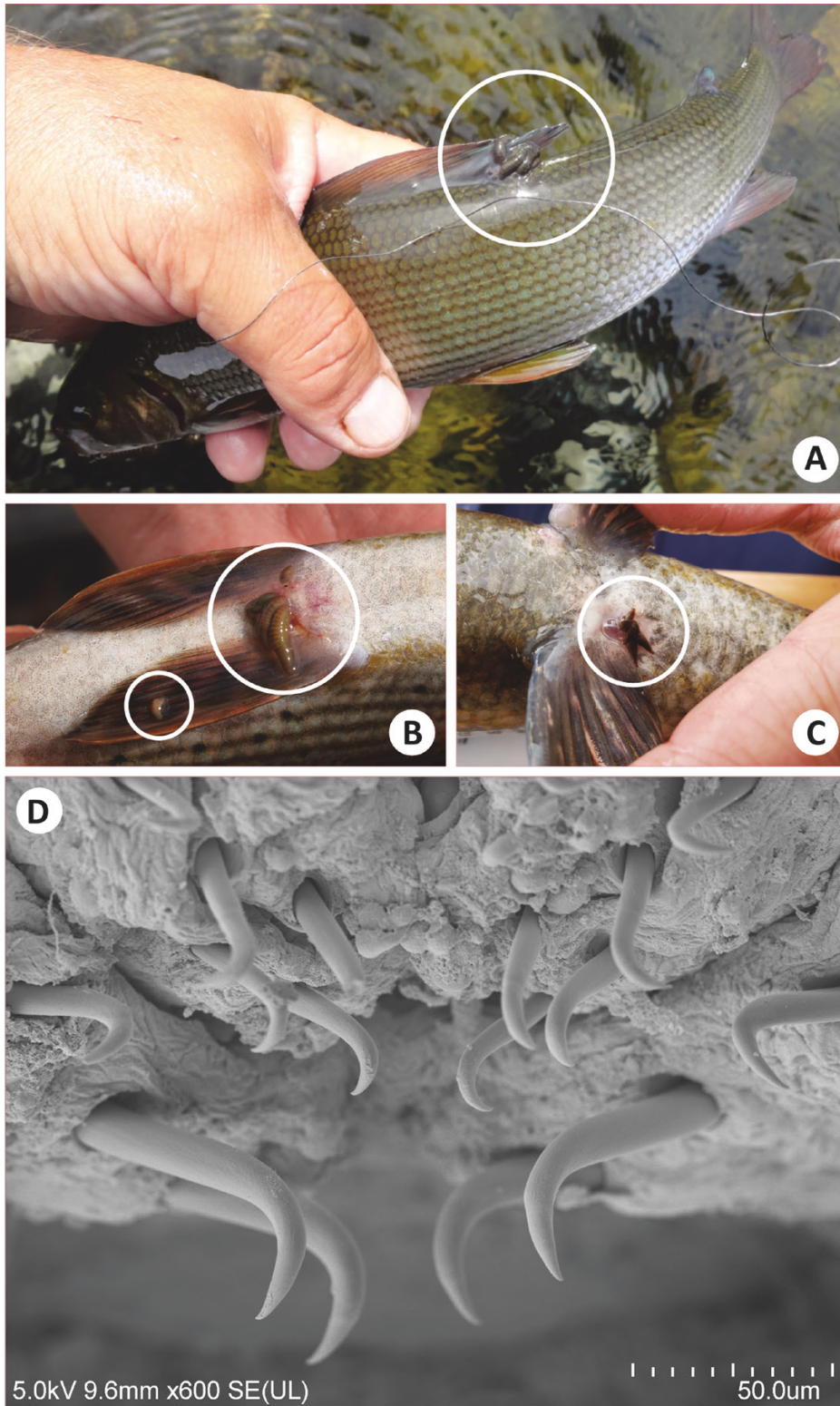
We decided to confer upon them the common name ‘hook-faced fish worms’ (this charismatic feature is illustrated in the scanning electron micrograph in [Fig. 1D](#)). They have been called ‘fish-worms’ and ‘fish-lice’ in Lapland ([Dahm, 1962](#)), but the former is not especially descriptive (e.g. various nematodes and trematodes also fit this name) and the latter is

misleading (e.g. parasitic arthropods also bear this moniker). Accordingly, we have added specificity to the common name by referencing the hook-like chaetae on their anterior region.

### DISTRIBUTION AND ECOLOGY

Of the two acanthobdellidans, *A. peledina* is clearly the more widespread species. We present a distribution map in [Figure 2](#), which details the largely northern boreal range, often above the Arctic Circle. Its distribution is best studied in Eurasia, where it is found in a variety of countries to the west and extends to Siberia and the Russian Far East ([Kaygorodova \*et al.\*, 2012](#)). In the 1970s, a notable range extension was documented as specimens were discovered in Alaska ([Holmquist, 1974](#); [Hauck \*et al.\*, 1979](#)). It is unclear whether the Alaskan population is genetically distinct or perhaps a more recent transplant from Eurasia. There is at least one study documenting the distribution of *A. peledina* in the Lake Baikal area, eastern Siberia, Russia, which is more moderate in climate than most other localities where this species is found ([Kaygorodova & Dzyuba, 2018](#)). *Paracanthobdella livanowi* is restricted to the Russian Far East. Specifically, it is best documented in the Kamchatka Peninsula, but is also known from the Chukchi Peninsula and around Taiu Bay ([Utevsky \*et al.\*, 2013](#)). The ranges of both species likely overlap ([Fig. 2](#); [Table 1](#)) in the Kamchatka region ([Kaygorodova \*et al.\*, 2012](#)).

Acanthobdellida species parasitize freshwater fishes, most of which are from the family Salmonidae ([Table 1](#)). They are generally found around the base of fins (especially the dorsal fin), but they are known to latch onto a variety of places on the body of a fish. They feed on blood and tissue of the host ([Kutschera & Epshtein, 2006](#); [Bielecki \*et al.\*, 2014](#)). A few specimens of *P. livanowi* have been found with insect larvae (Diptera and Odonata) in their stomach contents, suggesting that they have a broader dietary range than fish alone ([Bielecki \*et al.\*, 2014](#)). The fact that both acanthobdellidan species are, at least in part, sanguivorous has important implications for the evolution of blood-feeding within Hirudinea. Given that Acanthobdellida are known to be the sister group

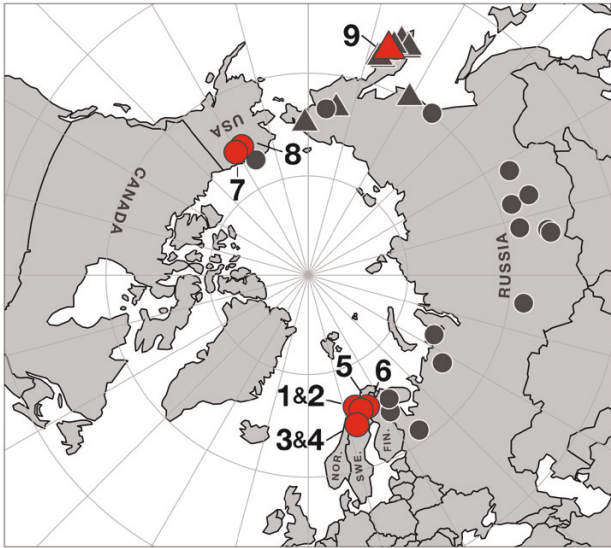


**Figure 1.** Photographs of Acanthobdellida species. A, B, *Acanthobdella peledina* from two separate grayling [*Thymallus thymallus* (Linnaeus, 1758)] individuals in Scandinavia. C, *A. peledina* on Arctic grayling [*Thymallus arcticus* (Pallas, 1776)] from Alaska. Circles highlight *A. peledina* individuals on their hosts. D, a scanning electron micrograph of *Paracanthobdella livanowi* emphasizes the eponymous hooks we commemorate in the new common name: hook-faced fish worms.

**Table 1.** Comparison of important morphological features, distribution, ecology and *COI* distances between *Acanthobdellida* species

Category	<i>Acanthobdella peledina</i>	<i>Paracanthobdella livanowi</i>
Anterior sucker <sup>1</sup>	Absent	Present (first five segments)
Chaetae (setae) <sup>1,2</sup> (Table 3)	One type: similar for first five rows; angle of flexion (hooks) ~90°	Two types: broader for fourth and fifth rows; angle of flexion ~150° (apart from chaetae on segment 3)
Crop and oesophagus <sup>2</sup>	Not distinct from one another; crop undivided	Distinct from one another; crop divided
Segments (somites) <sup>2</sup>	31	31
Ovisac shape <sup>2</sup>	Twisted; 3.0–4.5 segments long; extends posteriorly to ½ of testisac	Elongated with 'horseshoe-shaped' ends; 7.5–9.0 segments long; extends posteriorly to full length of testisac or farther
Testisacs <sup>2</sup>	Posterior end rounded; seven segments long	Posterior end curved; eight segments long
Distance between male and female gonopores <sup>5</sup>	Three complete annuli; however, male and female gonopores can be slightly anterior or posterior to their respective furrows	Three complete annuli; however, male and female gonopores can be slightly anterior or posterior to their respective furrows
Geography <sup>3,4</sup> (Fig. 2)	Broadly across northern Eurasia and Alaska	Russian Far East
Free-living status <sup>2,5</sup>	Only to reproduce (sometime between September and April)	Commonly observed in this state
Host records <sup>2,3,4,6,*</sup> (parasitic feeding mode)	Salmonidae: <i>Brachymystax lenok</i> (Pallas, 1773); <i>Coregonus autumnalis</i> (Pallas, 1776); <i>Coregonus lavaretus</i> (Linnaeus, 1758); <i>Coregonus muksun</i> (Pallas, 1814); <i>Coregonus nasus</i> (Pallas, 1776); <i>Coregonus peled</i> (Gmelin, 1789); <i>Coregonus pidschian</i> (Gmelin, 1789); <i>Coregonus sardinella</i> Valenciennes, 1848; <i>Coregonus tugun</i> (Pallas, 1814); <i>Esox lucius</i> Linnaeus, 1758; <i>Hucho taimen</i> (Pallas, 1773); <i>Prosopium cylindraceum</i> (Pennant, 1784); <i>Salmo salar</i> Linnaeus, 1758; <i>Salmo trutta</i> Linnaeus, 1758; <i>Salvelinus alpinus</i> (Linnaeus, 1758); <i>Salvelinus neiva</i> Taranetz, 1933; <i>Stenodus leucichthys</i> (Güldenstädt, 1772); <i>Stenodus nelma</i> (Pallas, 1773); <i>Thymallus arcticus</i> (Pallas, 1776); <i>Thymallus baicalensis</i> Dybowski, 1874; <i>Thymallus pallasi</i> Valenciennes, 1848; and <i>Thymallus thymallus</i> (Linnaeus, 1758)	Salmonidae: <i>Oncorhynchus mykiss</i> (Walbaum, 1792); <i>Salvelinus leucomaenis</i> (Pallas, 1814); <i>Salvelinus malma</i> <sup>‡</sup> (Walbaum, 1792); and <i>Salvelinus taranetzi</i> Kaganowsky, 1955 Gasterosteidae: <i>Gasterosteus aculeatus</i> Linnaeus, 1758
Alternative prey species <sup>2</sup> (predatory feeding mode)	Lotidae: <i>Lota lota</i> (Linnaeus, 1758)	Odonata and Chironomidae larvae
<i>COI</i> distance within species <sup>7</sup>	Average = 0.42%; range = 0.00–1.52% (please note that this does not include Alaskan samples)	0.11%
<i>COI</i> distance between species <sup>7</sup>	Average = 13.20%; range = 13.17–13.49%	

<sup>1</sup>Sawyer (1986);<sup>2</sup>Bielecki *et al.* (2014);<sup>3</sup>Utevsky *et al.* (2013);<sup>4</sup>Kaygorodova *et al.* (2012);<sup>5</sup>Epstein (1987);<sup>6</sup>Mitenev & Sul'man (1999);<sup>7</sup>present study and citations therein.\*Multiple papers reference another paper that has a record of *A. peledina* being found on the marine fish *Scophthalmus maximus* (Linnaeus, 1758); however, the original source paper does not seem to reference this, and this seems highly unlikely because *A. peledina* is found in freshwater.<sup>‡</sup>*Acanthobdella peledina* has also been reported from *Salvelinus lepechini* (Glelin, 1789), which has been synonymized with *S. alpinus* (Makhrov *et al.*, 2019).<sup>§</sup>*Paracanthobdella livanowi* has been reported from *Salvelinus albus* Glubokovsky, 1977, *Salvelinus kronocius* Viktorovsky, 1978 and *Salvelinus schmidtii* Viktorovsky, 1978, all of which are now recognized as junior synonyms of *S. malma* (Esin & Markevich, 2017), and *Salvelinus krogiusae* Glubokovsky, Frolov, Efremov, Ribnikova, & Katugin, 1993, which has been synonymized with *S. taranetzi* by these authors.



**Figure 2.** Map of Acanthobdellida collecting records across their known distribution. Circles are records for *Acanthobdella peledina*, triangles are for *Paracanthobdella livanowi*. Red triangles and circles represent localities from the present study, with numbers corresponding to those in Table 2. Dark grey triangles and circles represent previously published records (from Kaygorodova *et al.*, 2012; Utevsky *et al.*, 2013; Kaygorodova & Dzyuba, 2018 and references therein).

to leeches (Hirudinida), it has been posited that the ancestral leech was sanguivorous (Trontelj *et al.*, 1999; Tessler *et al.*, 2018a). It follows that the most recent common ancestor of leeches and acanthobdellidans might also have been blood-feeding.

In our experience in the field, *A. peledina* is highly variable in abundance, which local fishermen have confirmed. This is also confirmed by other empirical work where worms could be found attached to hosts in some seasons, but not in others, and they ranged in prevalence from exceedingly low numbers to more than two-thirds of all examined graylings (Kaygorodova *et al.*, 2012).

The species are sometimes collected apart from their hosts. This type of free-living habit appears to be restricted to breeding individuals and juveniles in *A. peledina* (Andersson, 1988; Kaygorodova *et al.*, 2012). *Paracanthobdella livanowi* is more frequently found free-living (Utevsky *et al.*, 2013), which might have to do with its ability to feed on insect larvae in addition to fish.

#### PRIOR PHYLOGENETICS RESEARCH

The exact placement of *A. peledina* among annelids was controversial for more than a century (Brinkhurst

& Gelder, 1989; Purschke *et al.*, 1993). This controversy continued for ~20 years after the advent of molecular phylogenetic analysis, because DNA sequences attributed to this taxon (AY040701, AF115978 and AF003264), and used in a number of papers (Siddall & Bureson, 1998; Apakupakul *et al.*, 1999; Gelder & Siddall, 2001; Siddall *et al.*, 2001), turned out to be sequences of contaminants (see Tessler *et al.*, 2018a: table 1). We discovered and reconciled this error in a study that produced a large multilocus phylogeny using a broad suite of taxa (Tessler *et al.*, 2018a). Those results showed that *A. peledina* seems to be sister to leeches, which reflects the early work on this taxon that was either done with morphology or did not have molecular contamination issues (Purschke *et al.*, 1993; Brinkhurst, 1999; Martin, 2001; Rota *et al.*, 2001; Kaygorodova & Sherbakov, 2006; Marotta *et al.*, 2008; Świątek *et al.*, 2012). The placement of *A. peledina* as sister to leeches further supports a single origin of vertebrate parasitism within clitellates (Tessler *et al.*, 2018a). A number of morphological synapomorphies also support the phylogeny and link leeches with *A. peledina* (Purschke *et al.*, 1993). Unfortunately, before the present study, *P. livanowi* had not been examined in a molecular or phylogenetic context.

Most prior molecular phylogenetic work on *A. peledina* has focused on *COI* and 18S sequence data, but more recent studies have incorporated other loci, such as 12S, 16S and 28S (Tessler *et al.*, 2018a; Bolbat *et al.*, 2019). In addition to the multilocus sequencing data, a mitochondrial genome (Bolbat *et al.*, 2020), ultraconserved element (UCE) data (Phillips *et al.*, 2019a, b) and a transcriptome (Iwama *et al.*, 2021) have been sequenced. The transcriptome has been used subsequently to study the presence and absence of anticoagulants in this species (Iwama *et al.*, 2022).

The results of Tessler *et al.* (2018a) have been confirmed by a corrected version of the next generation UCE dataset on a limited taxon set (Phillips *et al.*, 2019a, b). The mitogenome was incorporated into a phylogenetic tree when it was published, but the study was unclear about the exact data sources used, and it did not include any branchiobdellidans. Nevertheless, it found *A. peledina* sister to leeches (Bolbat *et al.*, 2020). Not every study has recovered *A. peledina* as the sister group to leeches, but studies that found alternative relationships have tended to use smaller datasets, have different foci, or are poorly supported or unresolved (e.g. Erseus & Kallersjo, 2004; Rousset *et al.*, 2008; James & Davidson, 2012; Bolbat *et al.*, 2019; see also Tessler *et al.*, 2018a: table 1).

Only a few specimens of *A. peledina* from a limited number of localities have been sampled for molecular studies. Although the documented range of *Acanthobdella* extends across northern Eurasia and into western North America, the vast majority of

**Table 2.** Localities and GenBank accession numbers for Acanthobdellida individuals used in this study

Identification	Country	Locality	COI (mtDNA)	12S (mtDNA)	16S (mtDNA)	18S (nuclear)	28S (nuclear)
<b><i>Acanthobdella peledina</i></b>							
AN1	Norway	1. Lille Rostavatn Lake, 68°59'27.60"N, 019°38'25.08"E	OL964360	OM060313	–	OM060279	–
AN2	Norway	1. Lille Rostavatn Lake, 68°59'27.60"N, 019°38'25.08"E	OL964359	OM060312	–	OM060280	–
AN3	Norway	1. Lille Rostavatn Lake, 68°59'27.60"N, 019°38'25.08"E	OL964348	OM060301	–	OM060291	OM060262
AN4‡	Norway	2. Moskánjávri Lake, 68°55'18.33"N, 020°11'56.65"E	OL964346	OM060299	–	OM060292	OM060260
AN5‡	Norway	2. Moskánjávri Lake, 68°55'18.33"N, 020°11'56.65"E	OL964347	OM060300	–	OM060293	OM060261
AN6†	Norway	2. Moskánjávri Lake, 68°55'18.33"N, 020°11'56.65"E	OL964362	OM060315	–	OM060278	OM060270
as1	Sweden	3. Skuppe, Pite River, 66°26'59.2"N, 18°20'23.2"E	MH351651	–	MH351635	MH351628	MH351642
as2	Sweden	3. Skuppe, Pite River, 66°26'59.2"N, 18°20'23.2"E	MH351652	–	MH351636	MH351629	MH351643
Mitogenome	Sweden	3. Skuppe, Pite River, 66°26'59.2"N, 18°20'23.2"E	MT741802	–	–	–	–
AP1§	Sweden	4. Pite River, 65°21'52"N, 21°19'22"E	OL964352	OM060305	–	OM060286	–
AP2	Sweden	4. Pite River, 65°21'52"N, 21°19'22"E	OL964349	OM060302	–	OM060290	OM060263
AP3	Sweden	4. Pite River, 65°21'52"N, 21°19'22"E	OL964358	OM060311	–	OM060281	OM060268
AP4§	Sweden	4. Pite River, 65°21'52"N, 21°19'22"E	OL964353	OM060306	–	OM060287	–
AP5§	Sweden	4. Pite River, 65°21'52"N, 21°19'22"E	OL964354	OM060307	–	OM060288	–
AL1*	Sweden	5. Lainio River, 68°17'02.5224"N, 21°26'19.7412"E	OL964363	OM060316	–	OM060274	OM060271
AL3*	Sweden	5. Lainio River, 68°17'02.5224"N, 21°26'19.7412"E	OL964364	OM060317	–	OM060275	OM060272
AL4†	Sweden	5. Lainio River, 68°17'02.5224"N, 21°26'19.7412"E	OL964361	OM060314	–	OM060277	OM060269
AL5*	Sweden	5. Lainio River, 68°17'02.5224"N, 21°26'19.7412"E	OL964365	OM060318	–	OM060276	OM060273
F1	Finland	6. Lapland, Peltojoki, 68°23'6.72"N, 24°9'10.08"E	OL964357	OM060310	–	OM060282	OM060267
F2¶	Finland	6. Lapland, Peltojoki, 68°23'6.72"N, 24°9'10.08"E	OL964355	OM060308	–	OM060283	OM060265
F3§	Finland	6. Lapland, Peltojoki, 68°23'6.72"N, 24°9'10.08"E	OL964351	OM060304	–	OM060285	–
F4¶	Finland	6. Lapland, Peltojoki, 68°23'6.72"N, 24°9'10.08"E	OL964356	OM060309	–	OM060284	OM060266
F5	Finland	6. Lapland, Peltojoki, 68°23'6.72"N, 24°9'10.08"E	OL964350	OM060303	–	OM060289	OM060264
IMO33	USA	7. Alaska, I-Minus Lake Outlet, 68°33'24.00"N, 149°34'29.27"W	–	–	OL953208	–	OM060256
IMO9	USA	7. Alaska, I-Minus Lake Outlet, 68°33'24.00"N, 149°34'29.27"W	–	OM060296	–	–	OM060255
AlaskaB	USA	8. Alaska, Chandler Lake, 68°14'57.94"N, 152°42'40.30"W	–	–	–	OM060295	OM060258
<b><i>Paracanthobdella livanowi</i></b>							
64	Russia	9. Kamchatka, Lake Kronotskoye, 54°43'1.20"N, 160°21'36.00"E	OL964344	OM060297	–	–	OM060257
PL11	Russia	9. Kamchatka, Lake Kronotskoye, 54°43'1.20"N, 160°21'36.00"E	OL964345	OM060298	–	OM060294	OM060259

Specimens that had identical sequences for all loci were treated as a single tip for phylogenetic analyses; these are demarcated as follows: \*, †, ‡, § and ¶.

prior work has been conducted on specimens from the Nordic countries. Fewer specimens, and no molecular data, are available for *P. livanowi*, making its debated taxonomic position difficult to assess based on existing studies.

#### PRIOR MORPHOLOGICAL RESEARCH

The fundamental early works on *A. peledina* were written near the beginning of the 20<sup>th</sup> century (Kowalevsky, 1896; Livanow, 1906, 1931). Members of Acanthobdellida have been referred to as a ‘missing link’ between leeches and other clitellates. Certain plesiomorphic features of Acanthobdellida hint at a relationship with non-hirudinid clitellates, such as the presence of chaetae (also referred to as setae or bristles) and an oligochaete-like male reproductive system (Purschke *et al.*, 1993). They have less developed suckers (Bielecki *et al.*, 2014); however, they also exhibit features that link them to leeches, such as subdivided segments, eyes, reduced or absent internal segmentation, certain digestive enzymes, no visible clitellum and several reproductive and nervous system characters (Sawyer, 1986; Brinkhurst & Gelder, 1989; Purschke *et al.*, 1993; Westheide, 1997; Cichocka *et al.*, 2021).

The acanthobdellidan feeding apparatus has been described as a rudimentary version of the leech proboscis (Bielecki *et al.*, 2014); however, we also note that their feeding apparatus is similar to the eversible pharynx found in other non-hirudinid clitellates, and the leech proboscis is likely to be a modified pharynx (Brinkhurst, 1982, 1999). This has led others to suggest that probosces are plesiomorphic for leeches, rather than a synapomorphy for the defunct order Rhynchobdellida (Trontelj *et al.*, 1999; Tessler *et al.*, 2018a).

A number of important morphological features have been compared between *A. peledina* and *P. livanowi*. We have highlighted some of the more notable differences in Table 1, many of which are described in greater detail by Bielecki *et al.* (2014). Of the two studies to include specimens of *A. peledina* from Alaska, only one went into detail about the morphology of the specimen found, and it appeared to be a juvenile (Holmquist, 1974).

#### PRESENT RESEARCH GOALS

In this study, we build a framework from molecular phylogeny and scanning electron microscopy-based morphology to fill gaps in the knowledge of hook-faced fish worms. Our specific goals were as follows: (1) to place *P. livanowi* in a phylogenetic tree to determine its relationship to *A. peledina*, including how recently

the two acanthobdellidan species diverged; (2) to determine whether *A. peledina* in general is truly one extremely widespread species or whether it is made up of multiple cryptic species, especially focusing on the allopatric populations of *A. peledina* in Alaska; and (3) to assess whether *P. livanowi* is placed most accurately within its monotypic genus and family.

## MATERIAL AND METHODS

### SPECIMEN ACQUISITION

The acanthobdellidan specimens used in this study were from a variety of countries and localities and are listed in Table 2. Acanthobdellidans preserved in ethanol were used for molecular analysis. Other specimens were fixed in 96% ethanol or in 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for examination using a stereo (dissection) microscope and compound microscope, and for scanning electron microscopy analysis. Alaskan specimens are housed in the Invertebrate Zoology collection at the American Museum of Natural History, European specimens at the Institute of Biology at the University of Silesia in Katowice and Asian specimens at the Department of Zoology and Animal Ecology, V. N. Karazin Kharkiv National University.

### DNA AMPLIFICATION AND SEQUENCING

DNA from Alaskan specimens was amplified according to our prior work (Tessler *et al.*, 2018a) for 28S, 18S, 16S and *COI*; 12S amplification followed protocols from our other prior work (de Carle *et al.*, 2017). Throughout the course of our research, we noticed that acanthobdellidan DNA is difficult to amplify and sequence. These specimens were no exception; amplification of *COI* was attempted for the Alaskan specimens using a primer set that amplifies a larger stretch of *COI* (Tessler *et al.*, 2018b; and used successfully in that study for a Swedish specimen of *A. peledina*), in addition to LCO and HCO (Folmer *et al.*, 1994), but none of the resulting PCR products produced usable sequences for the Alaskan samples. We also endeavoured to amplify additional nuclear loci without success. Despite several concerted efforts, we have never been able to amplify ITS, and repeated attempts to sequence histone H3 yielded only host DNA.

For the European samples, amplification of 12S followed the same methods as above. In order to generate sequences for *COI*, 28S and 18S from Swedish, Finnish and Norwegian specimens of *A. peledina* [particularly those contaminated by material from fishes, *Salmo trutta* Linnaeus, 1758 and *Thymallus thymallus*

(Linnaeus, 1758)], a series of acanthobdellidan-specific primers was designed. In a few cases, these primers were used in combination with primers from other studies (see [Supporting Information, Table S1](#) and citations therein).

Amplification of 18S and *COI* from *P. livanowi* was achieved using the same methods as for the European *A. peledina* specimens. Additional primers [ACA873Rev, ACA940Rev and COI-E (Bely & Wray, 2004)] have also proved suitable for *COI* amplification in this species. In contrast, primers used to amplify *COI* for hirudinids in other studies (Williams *et al.*, 2013; Tessler *et al.*, 2018b) did not produce any bands for *P. livanowi*. To amplify 28S ribosomal DNA (rDNA) from *P. livanowi* contaminated by fish material, we designed another set of acanthobdellidan-specific primers: 28SFrw390 and 28SRev1217 ([Supporting Information, Table S1](#)).

#### PHYLOGENETIC ANALYSES

The phylogenetic matrix included 74 terminals (of which, 20 were acanthobdellidans; see [Table 2](#)), each with sequences for three or more of the five loci. Outgroup sequences included taxa from the remaining hirudinean orders, Branchiobdellida Holt, 1965 ( $N = 24$ ) and Hirudinida Macleay, 1918 ( $N = 28$ ), and two lumbriculid species [*Eremidrilus coyote* Fend & Rodriguez, 2003 and *Lumbriculus variegatus* (Müller, 1774)]. Following the results of previous studies (e.g. Erséus & Källersjö, 2004; Tessler *et al.*, 2018a), the tree was rooted on the branch leading to Lumbriculidae. A complete list of sequences used for phylogenetic analysis is available in the [Supporting Information \(Table S2\)](#). We did not include three sequences that have been used in prior studies and have been found to represent contaminants (AY040701, AF115978 and AF003264). Three other sequences (AY040680, AF099948 and AF099953) were also excluded, because they had peculiarities that indicated possible quality issues and did not meet the minimum matrix occupancy requirement. Specimens that had identical sequences for all loci (*COI*, 12S, 16S, 18S and 28S) were treated as a single tip for the purposes of phylogenetic analyses (see [Table 2](#)).

Sequences were aligned with MAFFT v.7.453 (Katoh & Standley, 2013), using automatic choice of search strategy for *COI*, 12S, 16S and 18S. To account for long gaps caused by the sequencing of varying regions across different studies, the E-INS-i strategy was used for 28S. Uncorrected pairwise distance within and between acanthobdellidan species was calculated using MEGA X (Kumar *et al.*, 2018). Phylogenetic analyses were performed on three datasets: one with only mitochondrial loci (*COI*, 12S and 16S); one with nuclear loci (18S and 28S); and one with all five loci

concatenated. Model testing, maximum likelihood tree inference and bootstrapping (1000 pseudoreplicates) were performed using IQ-TREE 2 (Minh *et al.*, 2020). IQ-TREE was called as follows: 'iqtree2 -s <matrix> -spp <partitions> -m TESTMERGE -mset mrbayes -ninit 10 000 -bb 10 000 -wbt1'. The data matrix and the resulting tree files can be found in the [Supporting Information \(Files S1–S4\)](#).

#### MORPHOLOGICAL METHODS

To compare the two species, we focused on external morphology, especially on the anterior body part bearing chaetae, the clitellar region with gonopores and, to a lesser extent, on the posterior sucker. Additionally, the chaetal dimensions (length, breadth and flexion angle) were measured. All comparisons were made between specimens of similar size. For scanning electron microscopy and stereo microscope analysis, specimens were divided into three size categories: small specimens (3–5 mm long, with a maximum width of 0.8 mm), medium-sized (6–10 mm long, with a maximum width of 2 mm) and larger specimens (11–13 mm long, with a maximum width of 3 mm). Additionally, in the case of *A. peledina*, we analysed two very large specimens (25 mm long, with a maximum width of 9 mm) using a stereo microscope. These specimens were collected during winter; they were found in a fishing net but were not attached to any host. Most probably, they were free-living at the time of collection.

For analyses of the chaetae, fully grown specimens were chosen ([Table 3](#)). The anterior body fragments were incubated with 0.1% trypsin solution to digest the body tissues and release the chaetae. The isolated chaetae were mounted onto microscope slides, covered with coverslips and incubated for ~24 h at 30–40 °C. Then, chaetae were analysed under an Olympus U-DA 1M17005 microscope using CELL^B software. Chaetae from all five segments were measured, with five measurements recorded per chaeta: chaetal length, chaetal breadth at each of three points [(1) in the place where the chaeta is bent (flexion point); (2) at the midpoint of the chaeta (the distal part which extends outside the body); and (3) at the proximal part (the part of the chaeta hidden within the body)] and the angle of chaeta flexion. For the last measurement, two artificial lines were created to measure the angle of chaeta flexion: one along the middle of the chaeta and the second along the chaeta tip. Examples of each measurement are displayed in [Figure 3A–C](#). A total of 640 chaetae were measured: two chaetae from each segment (segments 1–5) in 32 individuals of *A. peledina* and 32 individuals of *P. livanowi* ([Table 3](#)).

For stereo microscope analysis, fixed specimens were washed in phosphate-buffered saline (PBS) buffer and

**Table 3.** Morphometrics of the chaetae in *Acanthobdella peledina* and *Paracanthobdella livanowi*. Values are averaged across all specimens measured. For measurement specifications, see Figure 3A–C.

Segment number	Specimen length (mm)	Chaetal length ( $\mu\text{m}$ )	Breadth at flexion point ( $\mu\text{m}$ )	Breadth at distal midsection ( $\mu\text{m}$ )	Breadth at proximal midsection ( $\mu\text{m}$ )	Flexion angle ( $^{\circ}$ )
<b>A. peledina:</b> 32 specimens examined						
1		128	6	12	8	90
2		146	6	12	8	85
3	11–15	160	6	12	7	90
4		209	6	11	8	90
5		262	6	13	8	90
<b>P. livanowi:</b> 32 specimens examined						
1		227	8	15	9	160
2		254	8	17	9	150
3	15–23	276	8	18	9	97
4		310	14	27	17	140
5		380	15	25	15	138

**Figure 3.** The chaetae of Acanthobdellida. Silhouettes show exemplary measurements of: A, chaetal length; B, chaetal breadth (from top to bottom) at the point of flexion, midsection (distal) and midsection (proximal); and C, flexion angle. Photographs, taken using a compound microscope, show: D, the chaetae of *Acanthobdella peledina* in the first segment; E, the chaetae of *Paracanthobdella livanowi* in the first segment; F, the chaetae of *A. peledina* in the fifth segment; and G, the chaetae of *P. livanowi* in the fifth segment. Scale bars: 35  $\mu\text{m}$  (D–G).

placed on Petri dishes, and an Olympus ZX81 camera and a Leica M205C stereomicroscope were used. For scanning electron microscopy analysis, both ethanol- and glutaraldehyde-fixed specimens were washed in PBS buffer, then postfixed in 1%  $\text{OsO}_4$  in a 0.1 M phosphate buffer (pH 7.4) for 2 h. After osmium postfixation, samples were dehydrated in a series of ethanol washes from 30 to 99.9%. Then, samples were dried in a Leica CPD 300 critical point dryer (Leica

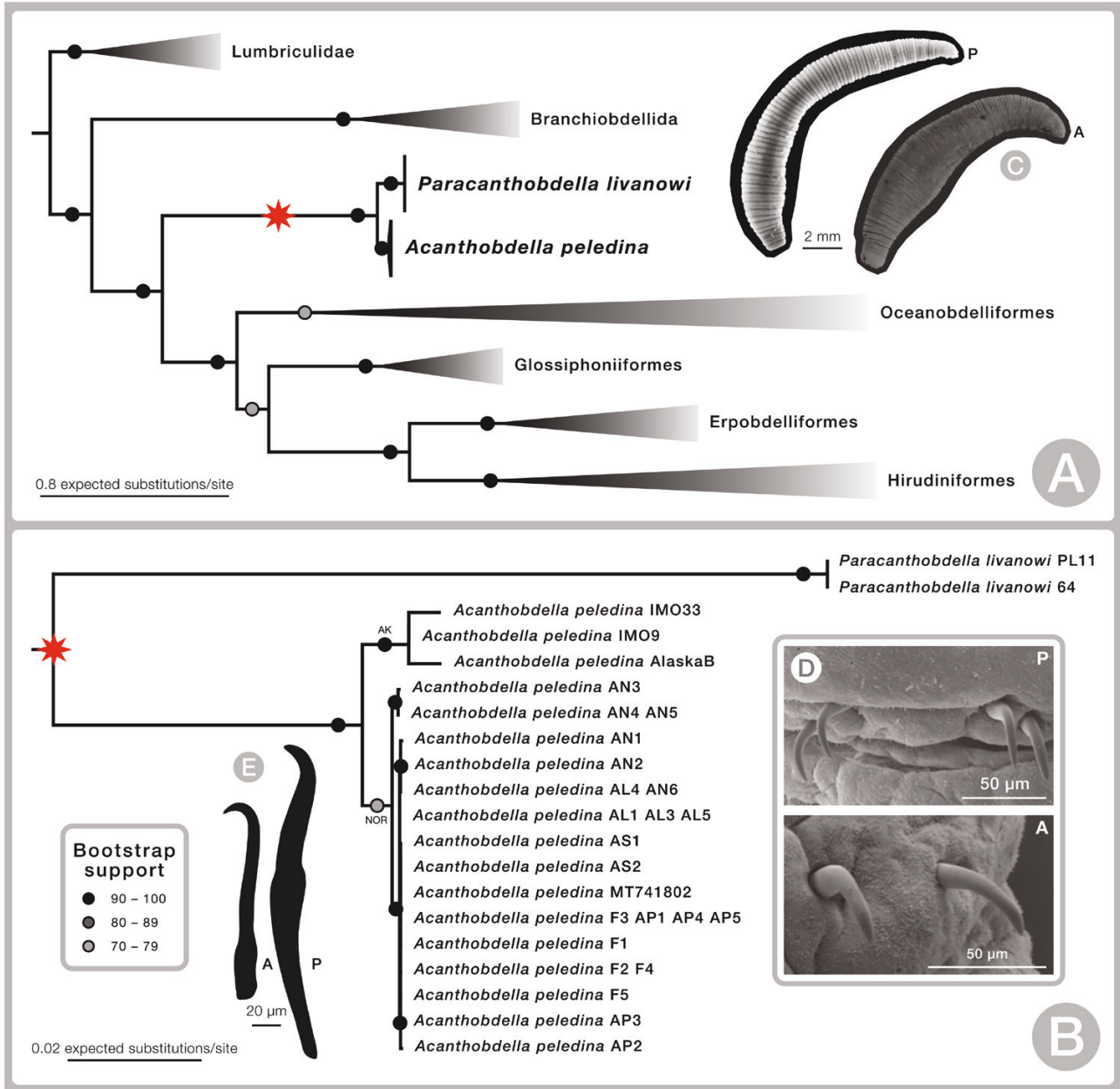
Microsystems, Vienna, Austria) and mounted on aluminium stubs with double-sided adhesive carbon tape and sputter coated with gold in a Pelco SC-6 sputter coater (Ted Pella, Redding, CA, USA) to obtain a layer ~25 nm thick. Specimens were analysed with a Hitachi SU8010 field emission scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) at 5.0 or 10 kV accelerating voltage with a secondary electron detector.

## RESULTS

## MOLECULAR RESULTS

All three phylogenetic analyses (mitochondrial loci, nuclear loci and all loci combined) agree that

Acanthobdellida is monophyletic and sister to leeches (Fig. 4A; Supporting Information, Files S2–S4). *Paracanthobdella livanowi* is sister to *A. peledina* (Fig. 4). These species are genetically distinct, with a



**Figure 4.** Maximum likelihood phylogeny of Acanthobdellida (starred node). A, B, Majority rule consensus of 1000 bootstraps (log likelihood =  $-70\,595.750$ ). The entire phylogeny (A) shows Acanthobdellida as sister to Hirudinida (leeches). Taxa with multiple representative taxa are collapsed to triangles, with the length of the triangle corresponding to the maximum branch length from the base of the clade to the tips. The starred clade from A is expanded to show all Acanthobdellida individuals (B). The Alaskan (AK) and Nordic (NOR) *Acanthobdella peledina* populations form distinct clades. C–E, inset images show photographs of overall morphology of medium-sized specimens (C), scanning electron micrographs of the fifth row of chaetae (D) and silhouettes of chaetae from the first chaetal row (E). For all inset images, species are indicated using ‘A’ for *A. peledina* and ‘P’ for *Paracanthobdella livanowi*.

*COI* distance of > 13% (Table 1), and they reside on a long branch that diverges from leeches.

Within *A. peledina*, the Alaskan specimens appear as sister to the other populations in the concatenated and mitochondrial trees (Fig. 4B; Supporting Information, Files S2–S4). Unfortunately, *COI* was not available for the Alaskan specimens, hence a consistent genetic distance does not seem worth calculating. Furthermore, there is a notable amount of missing data for different samples, and a different region of 28S was sampled for the Alaskan and European specimens. Nevertheless, a number of synapomorphies exist for the Alaskan specimens. 12S, 16S and 28S all appear to have single nucleotide differences for the Alaskan population, but 12S has only one sequence of the proper length to determine this. *ND1*, which was not used in our phylogenetic matrix, was compared between one Alaskan specimen and *ND1* from the only mitogenome of *A. peledina*. This gene appears to have multiple differences between individuals but cannot be generalized without further sampling.

Within the Nordic *A. peledina* samples, genetic variability was limited. Many samples had near-identical genetic information (see Tables 1, 2). The furthest genetic divergence between samples was ~1.5% for *COI*, while most were less divergent or identical.

#### GENERAL MORPHOLOGY, *A. PELEDINA* VS. *P. LIVANOWI*

The preserved specimens of both species are white or yellowish (Fig. 5), except for the large *A. peledina* specimens filled with blood, which are much darker (Fig. 5C). The natural colour pattern and eye pigmentation are not preserved (other work has discussed this colour change; Bielecki *et al.*, 2014), except for the chaetae, which are brownish along their length, darkening to black at the distal end (Fig. 5). We did not find prominent differences in external morphology between populations of *A. peledina* collected in different Nordic localities and in Alaska. Accordingly, the following descriptions of *A. peledina* refer to all analysed *A. peledina* specimens in aggregate.

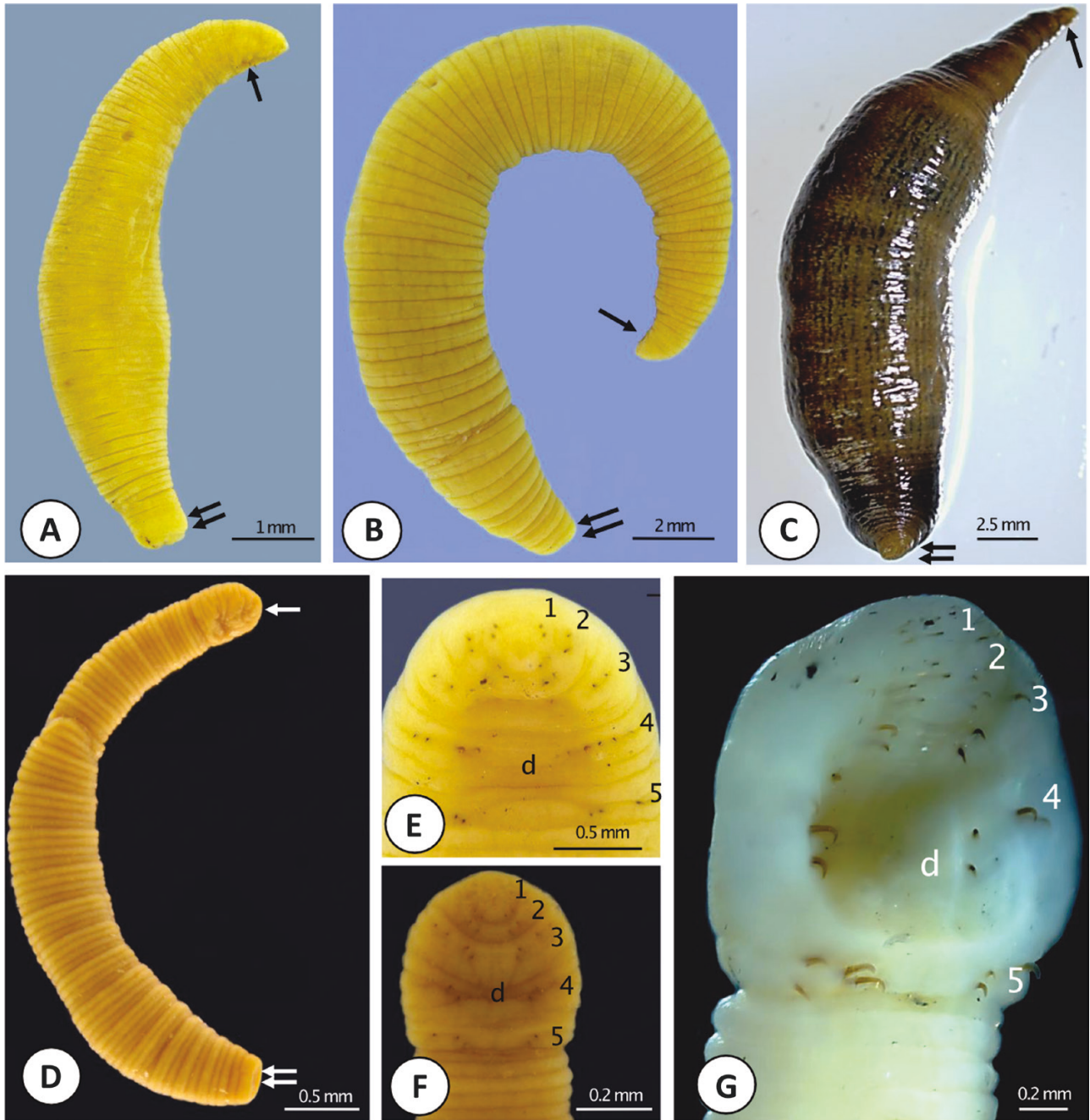
The overall morphology of small specimens representing both species is similar, except for small differences in the shape of the anterior part of the body (see below; Fig. 5). The body is narrow and elongated, worm-like (Fig. 5). In both species, the anterior body bears the mouth opening and five rows of chaetae (Figs 5–8), whereas the posterior end forms an inconspicuous sucker (Figs 5, 6). The number and distribution of chaetae are the same in both species (i.e. five rows of chaetae in five subsequent segments; Figs 5E, F, 7, 8). There are four separate pairs of chaetae in each row; hence, eight chaetae per segment (Figs 5E, F, 7, 8). In total, 40 chaetae are present. The anterior body differs

in shape: in *A. peledina*, it is cone shaped, whereas it is cup shaped in *P. livanowi* (Figs 6, 7). The mouth opening is narrow, cleft-like and surrounded by the first row of chaetae (Figs 7, 8). In close vicinity to the mouth, some receptors (preliminarily identified as chemoreceptors) occur (Fig. 8A, B). Between the third and fifth rows of chaetae, an inconspicuous deepening can be observed, which is better developed in *P. livanowi* (Figs 7, 8). Gonopores in small specimens are hardly visible (Fig. 9A). They form narrow clefts, with the male pore laying in the furrow between segments XI and XII, and the female pore located three complete annuli below, on the last annulus of segment XII. Below the female pore, the entrance to the spermatheca (area copulatrix) occurs in the furrow between segments XII and XIII (Fig. 9A). In both species, the posterior end bears the sucker in the form of a rounded depression (Fig. 6).

In medium-sized and larger specimens of both species, the difference in the form of the anterior part of the body is clear. In *A. peledina*, this region is still cone shaped, but the deepening between rows of chaetae becomes more pronounced (Figs 5A, B, E, 7C, 8C). In *P. livanowi*, the anterior body end is cup shaped, with a conspicuous deepening between the chaetae (Figs 5G, 7D, 8D). In *A. peledina*, there is no clear demarcation between the chaetiferous segments and the rest of the body, whereas in *P. livanowi* this region forms the anterior sucker and is clearly separated from the rest of body by a constriction (Figs 5A, B, G, 7C, D). In larger specimens of *P. livanowi* (in contrast to *A. peledina*), the segment limits in the vicinity of the anterior sucker are hardly visible (Figs 5G, 7D). The posterior sucker in larger specimens of both species is still inconspicuous; it is narrower than the rest of the body and cone-like (Figs 5A–D, 9C, D). The sucker itself is in the form of a shallow, crater-like depression (Fig. 9C, D).

In larger specimens of both species, the distribution of chaetae is the same as in small specimens (Fig. 8). However, the external portions of the chaetae in *P. livanowi* are longer and hook-like (Fig. 8C–F). It should be mentioned that, in a few specimens, some chaetae were damaged or completely absent; most probably, they were damaged during collection of the material. In both species, there is a significant correlation between body size and the chaetal length for each segment (in *A. peledina*,  $r = 0.83–0.97$ ,  $P < 0.001$ ; in *P. livanowi*,  $r = 0.96–0.99$ ,  $P < 0.001$ ).

All chaetae ( $N = 20$ ) were measured for 32 individuals of *A. peledina* and *P. livanowi*, for a total of 640 chaetae per species; the detailed results of this analysis are summarized in Table 3. The average length of chaetae increases from segment 1 to 5 in both species (Table 3). In both species, chaetae located in the first three segments (1–3) are shorter than those from segments 4 and 5. Additionally, the breadth of



**Figure 5.** General morphology of *Acanthobdella peledina* (A–C, E) and *Paracanthobdella livanowi* (D, F, G) visualized by stereo microscope. A–C, E, *A. peledina*: medium-sized specimen (7 mm; Sweden; A), large specimen (12 mm; Norway; B), very large specimen (25 mm; Finland; C); and anterior body region of specimen shown in B (E; note higher magnification). D, F, G, *P. livanowi*: small specimen (5 mm; Kamchatka; D), anterior body region of specimen figured in D (F; note higher magnification) and anterior body region of a large specimen (12 mm; Kamchatka; G). Arrow, anterior sucker; double arrows, posterior sucker; d, deepening between pairs of chaetae; Arabic numerals mark rows of chaetae.

chaetae is maximal in the middle, whereas both ends are thinner (Table 3). The recorded differences between species constituted chaetal breadth and flexion angle (Fig. 3). In *A. peledina*, chaetae from all five segments are of similar breadth, whereas in *P. livanowi* the

chaetae from segments 1–3 are distinctly thinner, with the chaetae in segments 4 and 5 being almost twice as broad (Table 3). In *A. peledina*, chaetae are flexed at a right angle, whereas in *P. livanowi* the angle is usually obtuse and varies from 97 to 160° (Fig. 3; Table 3).

In larger specimens, the unpaired gonopores are clearly visible (Fig. 9B). In *A. peledina*, the male gonopore is located two-thirds of the way down the length of the fourth annulus of segment XI, and the female gonopore is located three complete annuli below, one-third of the way down the length of the last annulus of segment XII (not shown). The spermathecal opening is located on the first annulus of segment XIII, close to the furrow separating it from the previous segment (not shown). In *P. livanowi*, the male gonopore is located two-thirds of the way down the length of the fourth annulus of segment XI; the female gonopore is located three complete annuli below, in the middle of the fourth annulus of segment XII (Fig. 9B). The opening of the spermatheca is located on the next annulus below the female gonopore, which is the first annulus of segment XIII (Fig. 9B).

## TAXONOMY

### TAXONOMIC SCHEME

*Class:* Clitellata Michaelsen, 1919

*Subclass:* Hirudinea Lamarck, 1818

*Order:* Acanthobdellida Livanow, 1905

*Suborder:* **Acanthobdelliformes** Cios, de Carle, Świątek, Tessler & Utevsky **subord. nov.**

*Family:* Acanthobdellidae Livanow, 1905

*Genus:* *Acanthobdella* Grube, 1851

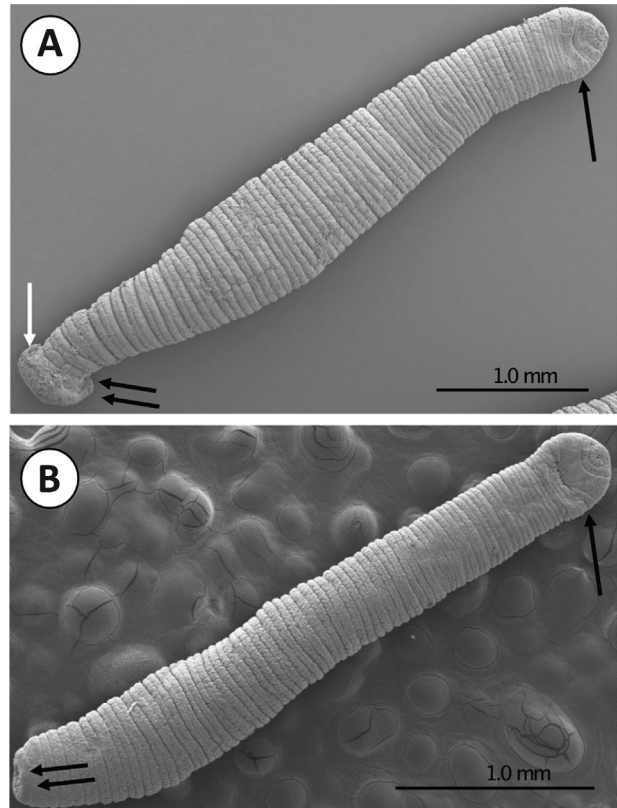
*Species:* *Acanthobdella peledina* Grube, 1851

*Genus:* *Paracanthobdella* Epstein, 1987

*Species:* *Paracanthobdella livanowi* (Epstein, 1966)

### NOTES ON OUR TAXONOMIC SCHEME

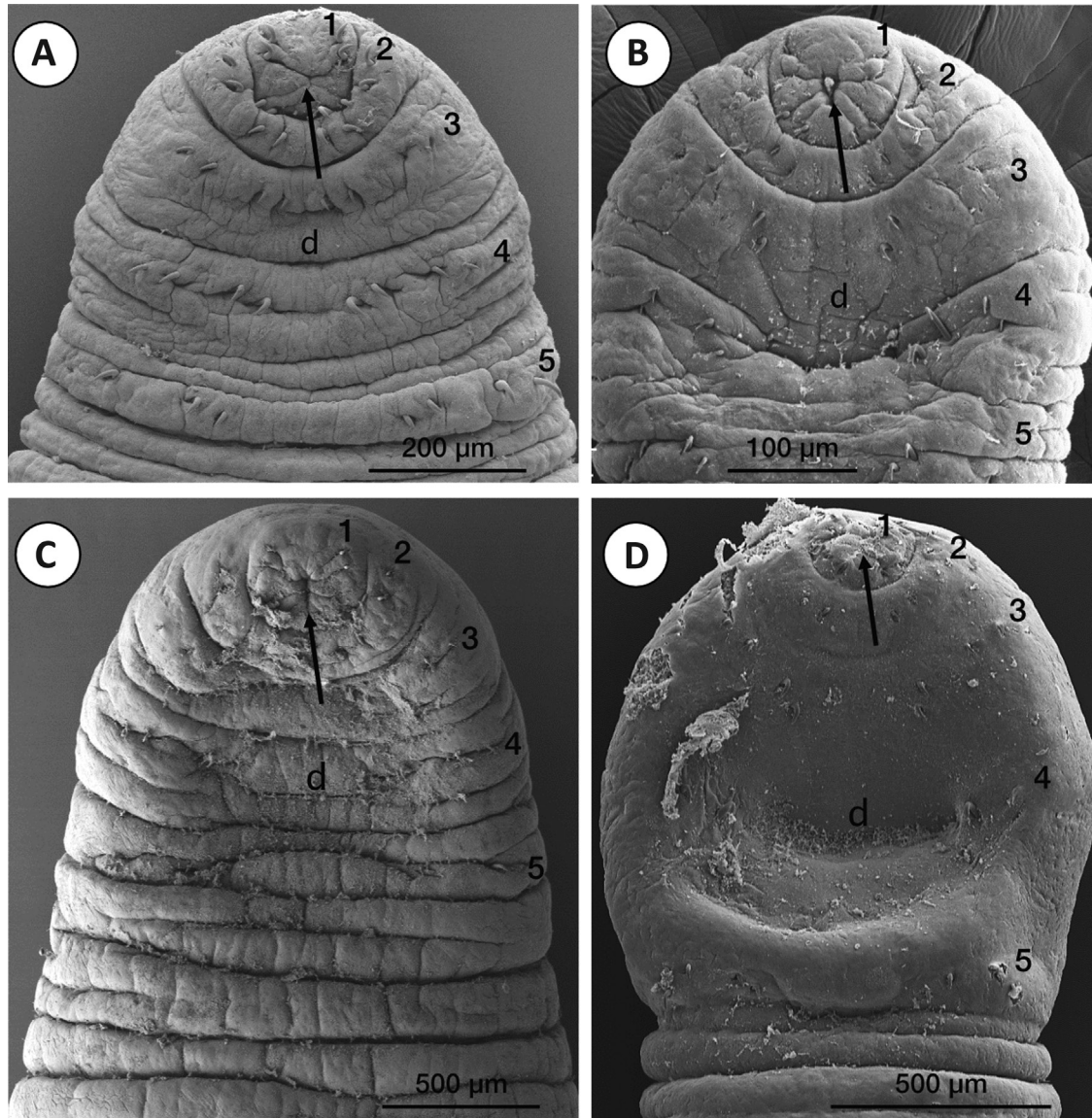
Here, we present a taxonomic scheme for hook-faced fish worms (Acanthobdellida). Despite its historical significance, we do not retain the family Paracanthobdellidae for *P. livanowi*, because both morphological and molecular analyses suggest that the two acanthobdellidan species share many characteristics. Initially, Acanthobdellidae and Paracanthobdellidae were differentiated based on a few morphological differences that were viewed as taxonomically important (Epstein, 1987). Specifically, members of Paracanthobdellidae were said to possess a primitive prostomium and well-developed anterior sucker. Although the two-family system seemed well supported at the time, we have found that the prostomium of *P. livanowi* is not more conspicuous or developed than the anterior region of *A. peledina*. Moreover, the area between rows of chaetae in the cephalic extremity of the latter can be deepened



**Figure 6.** Scanning electron micrographs of general morphology in small specimens. A, *Acanthobdella peledina* (4 mm; Alaska). B, *Paracanthobdella livanowi* (3.5 mm; Kamchatka). Single black arrows, anterior body region; double black arrows, posterior sucker. In the *A. peledina* specimen, some fragments of host tissue (white arrow) are still attached to the sucker.

such that it resembles a shallow sucker. It should also be noted that juvenile individuals of *P. livanowi* do not bear well-developed anterior suckers; this character is only common to large-bodied individuals of the species. The sum of this evidence suggests that the morphological differences between the two acanthobdellidan species, although pronounced, are not sufficient to warrant two families. We therefore classify both *Acanthobdella* and *Paracanthobdella* under the single family Acanthobdellidae.

We establish a new suborder Acanthobdelliformes to match better the taxonomy erected for Hirudinea in prior work (Tessler *et al.*, 2018a), which divided Hirudinida (leeches) into five suborders (Americobdelliformes, Erpobdelliformes, Hirudiniformes, Glossiphoniiformes and Oceanobdelliformes). Acanthobdelliformes is defined by the presence of chaetae on each of five contiguous segments in the anterior body region, and 31 segments (mid-body ones are quadrannulate with annulus a3 being subdivided) (Sawyer, 1986;



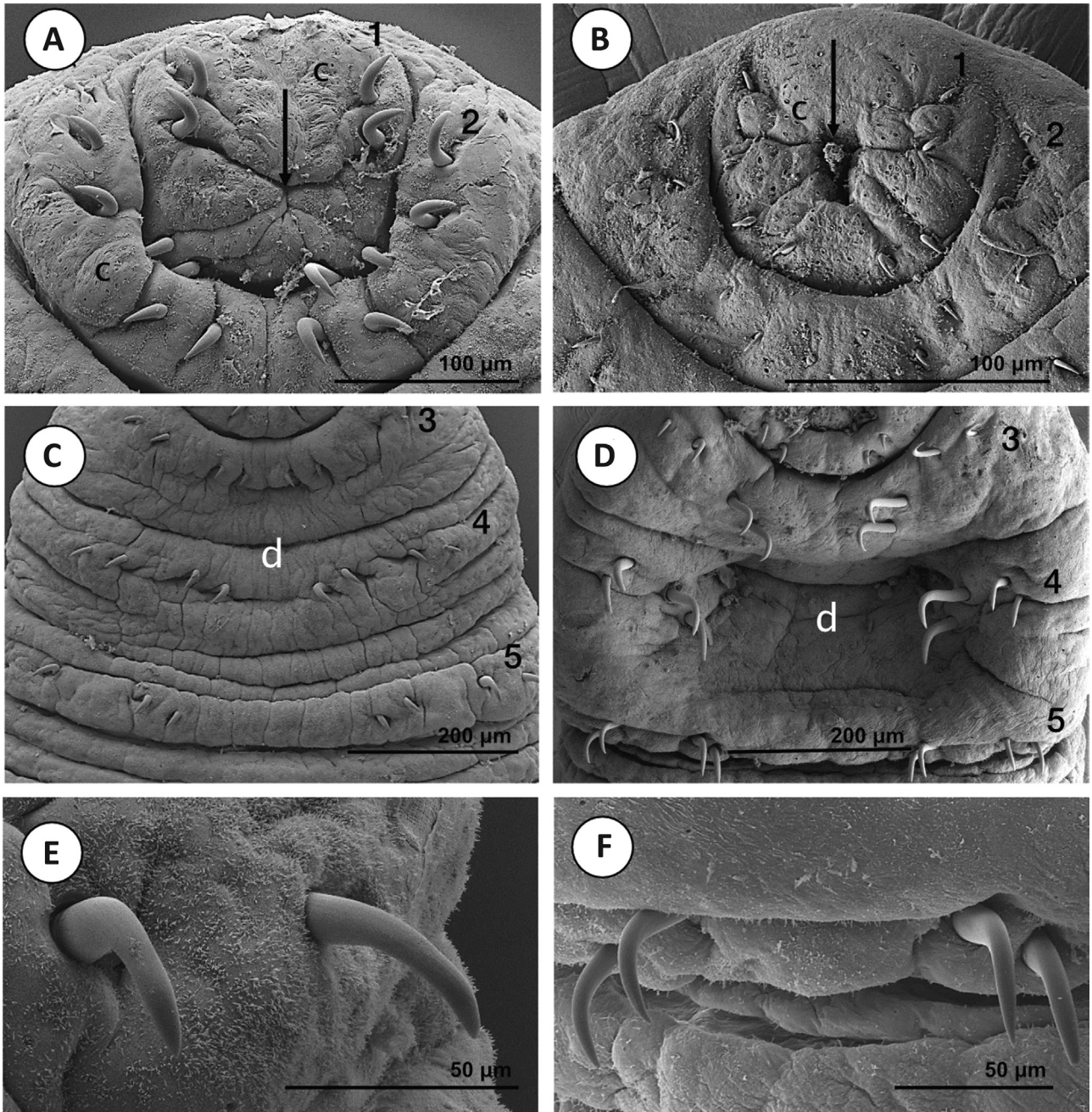
**Figure 7.** Scanning electron micrographs of the anterior body region. A, B, small specimens of *Acanthobdella peledina* (5 mm; Sweden; A) and *Paracanthobdella livanowi* (3.5 mm; B). C, D, large specimens of *A. peledina* (12 mm; Norway; C) and *P. livanowi* (11 mm; Kamchatka; D). In the large *P. livanowi* specimen, body segmentation and chaetae in the anterior sucker are barely visible. Arrow, mouth opening; Arabic numerals 1–5 indicate rows of chaetae; d, deepening between pairs of chaetae.

Purschke *et al.*, 1993; Bielecki *et al.*, 2014). Although previous studies have reported different numbers of segments for each acanthobdellidan species (e.g. 29 for *Acanthobdella* and 30 for *Paracanthobdella*; Bielecki *et al.*, 2014), this discrepancy is attributable to presumed differences in the number of segments that comprise the posterior sucker, which should be substantiated by a careful morphological analysis in the future.

The higher taxonomy (class through order) follows our prior classification scheme (Tessler *et al.*, 2018a). Others have constructed alternative schemes,

but we feel ours to be internally more consistent and phylogenetically appropriate. Alternative classifications include subclass Acanthobdellidea Livanow, 1905 (Archihirudinea Lukin, 1956 is an equivalent synonym) and subclass Acanthobdelliones (Epstein, 1987).

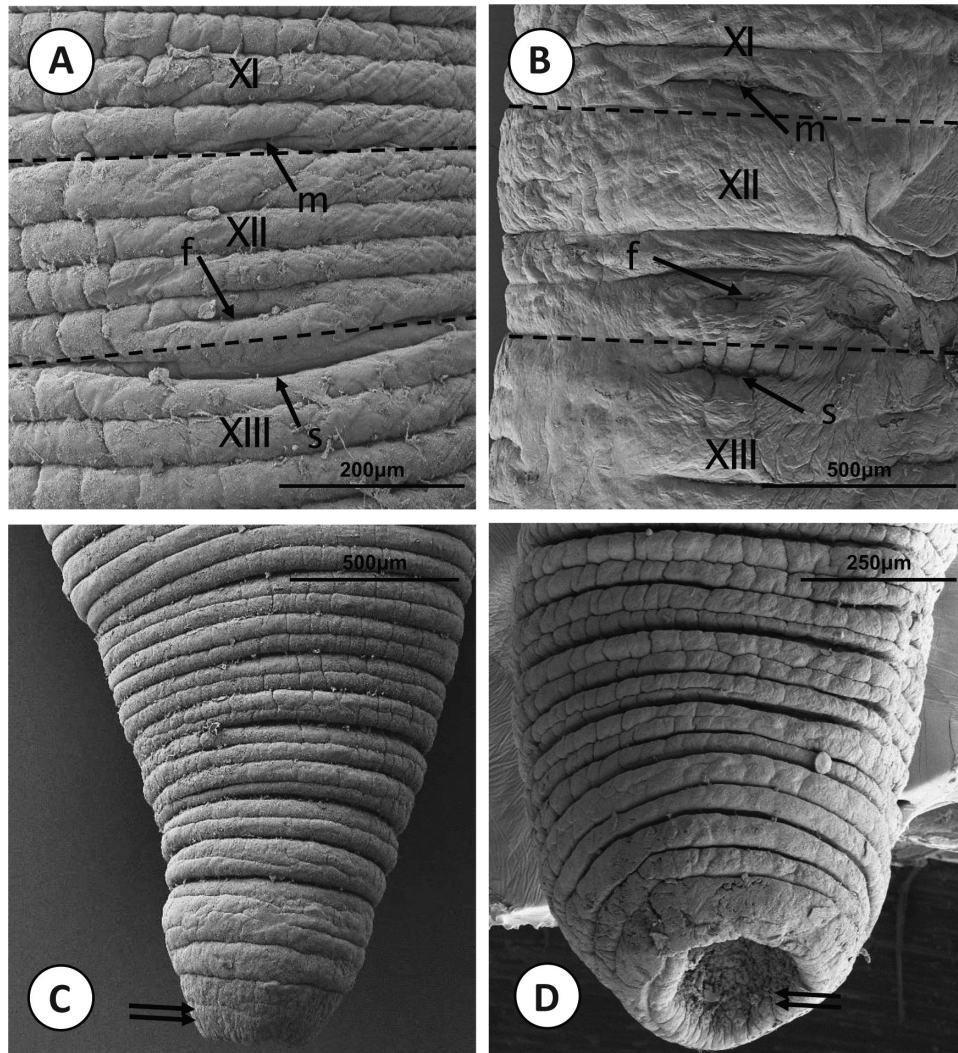
It is important to note that the two acanthobdellidan species were originally in the same genus (Epstein, 1966). However, in the late 1980s, *P. livanowi* was given its own genus and even family (Epstein, 1987). Ultimately, the decision to classify a monophyletic lineage of two species into one vs. two genera and/or



**Figure 8.** Scanning electron micrographs showing details of the anterior body region. A, B, first and second rows of chaetae in small *Acanthobdella peledina* (5 mm; Sweden; A) and small *Paracanthobdella livanowi* (3.5 mm; Kamchatka; B). C, D, third, fourth and fifth rows of chaetae in medium-sized *A. peledina* (7 mm; Sweden; C) and *P. livanowi* (6 mm; Kamchatka; D). E, F, chaetae from the fifth row of medium-sized *A. peledina* (6 mm; Sweden; E) and medium-sized *P. livanowi* (6 mm; Kamchatka; F). Arrow indicates mouth opening; Arabic numerals 1–5 indicate rows of chaetae; c, putative chemoreceptors; d, deepening between pairs of chaetae.

families is subjective. We have decided to retain the genus-level classification proposed by Dr Epstein (rather than lump them) to honour his contributions to the study of Hirudinea and accentuate the differentiation of the anterior sucker, chaetae and

internal anatomy that separates these species. This is also in concordance with the suggestions in the most recent, broad morphological comparison paper (Bielecki *et al.*, 2014); see Table 1 for some of the differences between these species.



**Figure 9.** Scanning electron micrographs of gonopores and posterior body regions. A, B, gonopores in a small *Acanthobdella peledina* (4 mm; Alaska; A) and a large *Paracanthobdella livanowi* (11 mm; Kamchatka; B). C, D, the posterior body region for a medium-sized *A. peledina* (7 mm; Sweden; C) and a medium-sized *P. livanowi* (6 mm; Kamchatka; D). Double arrows indicate the posterior sucker; f, female gonopore; m, male gonopore; s, opening of the spermatheca. Dotted lines and Roman numerals denote segments.

## DISCUSSION

Our results indicate that the hook-faced fish worms (Acanthobdellida) diverged from leeches a long time ago and that this order has many presumably plesiomorphic features, yet the extant species, and populations thereof, diverged in relatively recent times. This is the first time that *P. livanowi* has been included in a molecular phylogenetic study, helping to ascertain the aforementioned patterns. Furthermore, this is the first time that American populations of *A. peledina* have been incorporated into a molecular phylogenetic study, helping to indicate that this population is genetically distinct from the Nordic populations. Nevertheless, these populations appear to be

indistinguishable morphologically based on scanning electron micrographs. Although Acanthobdellida has received more attention over the last decade, our results help to fill in important understanding of the evolution of this fish-parasitizing clade.

## SYSTEMATICS OF HOOK-FACED FISH WORMS (ACANTHOBDELLIDA)

Acanthobdellida are clearly monophyletic and sister to leeches. Prior problems with placement of *A. peledina* based on sequences from contaminants caused a lot of problems for past studies, which we helped resolve recently (Tessler *et al.*, 2018a). These species, in our

experience, are difficult to work with molecularly. It is easy to sequence contaminants from the environment or the host tissue. Even in recent studies, this has been an issue (Phillips *et al.*, 2019a, b). However, the present paper bolsters the claim that Acanthobdellida are sister to leeches, helping to substantiate them as a unique order in Hirudinea. To make this order better match the rankings found in the sister order (leeches), we have erected the new suborder Acanthobdelliformes. Leeches comprise five suborders, in comparison.

*Paracanthobdella livanowi* is sister to *A. peledina* (Fig. 4; Supporting Information, Files S2–S4), as was expected based on morphology. The genetic distance between the two species at the *COI* locus (13.20%) is higher than values for other hirudinean species pairs, which has been reported at ~8% (Oceguera-Figueroa *et al.*, 2010; de Carle *et al.*, 2017; Iwama *et al.*, 2019). Interestingly, although the divergence between *A. peledina* and *P. livanowi* is substantial, the split is relatively recent, especially in comparison to the long branch length for Acanthobdellida and the level of variability found in the closely related branchiobdellidans and leeches. The result is consistent for the mitochondrial, nuclear and concatenated datasets (Fig. 4; Supporting Information, Files S2–S4). Although patterns of glaciation are often invoked to explain recent divergences in northern species, estimates for the rate of *COI* divergence between species pairs of annelids have been < 1%/Myr (Chevaldonné *et al.*, 2002). Therefore, although the divergence between the two acanthobdellidan species is a relatively recent event in the history of the lineage, it is likely to pre-date the most recent glacial cycles. It bears mentioning that the ranges of both species are most likely to overlap in the Kamchatka region (Table 1; Fig. 2) (Kaygorodova *et al.*, 2012) and that the known hosts of both species include salmonid fishes. Unfortunately, without fossils it is difficult to make any sense of this or even to attempt a molecular clock analysis that would provide much confidence.

#### POPULATIONS OF *A. PELEDINA*

The present evidence suggests that *A. peledina* from Alaska is distinct, to some degree, from European samples. However, Siberia and the Russian Far East have not been adequately sampled genetically for *A. peledina*. Accordingly, it is difficult to determine the genetic variability and population structuring of this species. Coupled with increased taxon sampling, additional genetic sampling of Alaskan populations could help to indicate whether they are a unique species or population. Sampling of quickly evolving nuclear loci or, ideally, next generation sequence data (e.g. RADSeq) would be useful for determining whether gene flow exists between the Alaskan and

Nordic localities. Unfortunately, *COI*, the most common marker for determining differences between leech species and populations (de Carle *et al.*, 2017; Tessler *et al.*, 2018c; Mack *et al.*, 2019), and additional nuclear loci did not amplify for these samples, potentially leading to some issues with missing data. Furthermore, given that no external morphological differences were noted between samples of Nordic and Alaskan *A. peledina*, we refrain from formal species or population delimitation analyses at this time. Nevertheless, the fact that the Alaskan population is sister to, and genetically divergent from, the Nordic samples suggests that this is not an invasive or non-native species that was translocated only in recent times by humans, which would have been plausible given that the first records of this species in Alaska came from the 1970s (Holmquist, 1974; Hauck *et al.*, 1979) and that it has not officially been reported since then, despite the clear importance of these American animals.

The Nordic populations are fairly similar genetically, despite being sampled from multiple (albeit geographically close) countries. The maximum genetic distance at the *COI* locus is 1.52%, which is below the average value (~2.4%) typically reported for species of Hirudinea (Kvist, 2015; de Carle *et al.*, 2017; Anderson *et al.*, 2020; Mack *et al.*, 2019; Iwama *et al.*, 2019). However, the countries sampled are all in relatively close proximity. It would be most useful to add samples from central and eastern Russia. Unfortunately, a 12S sequence for *A. peledina* in the Baikal region of Russia from a recent publication was not made publicly available (Bolbat *et al.*, 2019).

#### MORPHOLOGY

Our morphological examination and comparison of *Paracanthobdella* and populations of *Acanthobdella* help to characterize these species further. The scanning electron micrographs (Figs 6–9) and morphometry of facial hooks ( $N = 1280$ ) help to accentuate the main external differences between the two species: (1) the presence or absence of a cup-shaped depression between rows of chaetae (anterior sucker); and (2) the chaetal dimensions and shape (Fig. 3; Table 3). The differences in both these characteristics become more notable as the species mature. The deep cup-shaped anterior sucker, which is viewed as the most important distinguishing feature of *P. livanowi*, develops gradually through ontogeny from a flat state characteristic of juvenile individuals of the species. In *A. peledina*, the anterior end does not form a clearly separated sucker even in fully grown specimens, but a deep cavity appears between chaetae concomitantly with the growth of the animal. The shape of the chaetae differs between species: in *A. peledina*, chaetae are

bent at a right angle and the breadth of the chaetae is similar in all rows, whereas in *P. livanowi* the angle is obtuse and chaetae in rows 4 and 5 have substantially greater breadth (Fig. 3; Table 3). The well-developed prostomium, which has been considered as another distinguishing feature of the species and the genus, was found to be less prominent and conspicuous than presented in previous studies (Epstein, 1987).

Other studies have examined the internal morphology of these species (Bielecki *et al.*, 2014); known differences from this work and others are summarized in Table 1.

*Acanthobdella peledina* has the same morphology across the Nordic and Alaskan populations examined here and seems to be indistinguishable from Siberian populations (I. A. Kaygorodova & P. Świątek, unpublished scanning electron microscopy data) in other studies (Kaygorodova *et al.*, 2012). Nevertheless, although we did not find differences between *A. peledina* from Alaska and Eurasia, it is entirely possible that detailed internal examinations might unearth differences, given that these populations appear to be divergent genetically.

#### CONCLUSION AND FUTURE DIRECTIONS

Our results help to shed light on the hook-faced fish worms (Acanthobdellida): an ancient lineage that is most closely related to leeches, and demonstrate that Acanthobdellida species and populations have diverged fairly recently. It is even possible that there are multiple species within *A. peledina*. Specifically, the American and Nordic populations appear to be distinct genetically and are likely to be isolated reproductively. However, there are important gaps to fill in the knowledge of the populations of this species before definitive action is taken on determining whether they represent the same species. Those gaps are as follows: (1) adding specimens from localities for central and eastern Russia; (2) obtaining additional genetic data (i.e. COI and additional nuclear data) for Alaskan samples; and (3) looking for internal morphological differences between populations.

#### ACKNOWLEDGEMENTS

D. B. de Carle and Ł. Gajda contributed equally to this work. We thank those who have helped to provide specimens for this study or aided in our fieldwork. P. Świątek thanks M.Sc. Marzena Zmarzły, Dr Mariusz Kanturski and Dr Izabela Potocka from the Institute of Biology, Biotechnology and Environmental Protection, University of Silesia in Katowice, Katowice, Poland for help with stereo microscopy and scanning electron microscopy analysis. D. B. de Carle thanks Kristen

Choffe for her assistance with sequencing at the Royal Ontario Museum. A. Bielecki thanks M.Sc. Monika Szostek for photographing *Acanthobdellida* chaetae. S. Utevsky thanks Andriy Utevsky for photographing the large specimen of *P. livanowi*. We thank our four anonymous reviewers for their enthusiastic and constructive comments. We also thank those who have helped to fund our work: National Science Foundation (#1601562, awarded to M. Tessler), Natural Sciences and Engineering Research Council of Canada Postgraduate Doctoral Scholarship (PGSD2-518435-2018), awarded to D. B. de Carle), the Science Research Mentoring Program (SRMP) at the American Museum of Natural History and the Ministry of Science and Higher Education of Poland within the statutory activities of Faculty of Natural Sciences, University of Silesia in Katowice.

#### CONFLICT OF INTEREST

The authors do not report any conflict of interest.

#### DATA AVAILABILITY

The primers used for DNA amplification and sequencing are detailed in Table S1. Molecular sequence data used in this study are available on GenBank (see Tables 2 & S2 for accession numbers). The alignment used to conduct phylogenetic analyses is available in the supporting information (File S1).

#### REFERENCES

- Anderson K, Braoudakis G, Kvist S. 2020. Genetic variation, pseudocryptic diversity and phylogeny of *Erpobdella* (Annelida: Hirudinida: Erpobdelliformes), with emphasis on Canadian species. *Molecular Phylogenetics and Evolution* **143**: 106688.
- Andersson E. 1988. The biology of the fish leech *Acanthobdella peledina* Grube. *Zoologische Beiträge* **32**: 31–50.
- Apakupakul K, Siddall ME, Burreson EM. 1999. Higher level relationships of leeches (Annelida: Clitellata: Euhirudinea) based on morphology and gene sequences. *Molecular Phylogenetics and Evolution* **12**: 350–359.
- Bely AE, Wray GA. 2004. Molecular phylogeny of naidid worms (Annelida: Clitellata) based on cytochrome oxidase I. *Molecular Phylogenetics and Evolution* **30**: 50–63.
- Bielecki A, Cichocka JM, Jeleń I, Świątek P, Płachno BJ, Piłka D. 2014. New data about the functional morphology of the chaetiferous leech-like annelids *Acanthobdella peledina* (Grube, 1851) and *Paracanthobdella livanowi* (Epshtein, 1966) (Clitellata, Acanthobdellida). *Journal of Morphology* **275**: 528–539.

- Bolbat AV, Sorokovikova NV, Kaygorodova IA. 2019.** Assessing efficiency of the mitochondrial 12S marker fragment for the use in reconstruction of the phylogeny of acanthobdellids. *Russian Journal of Genetics* **55**: 1554–1558.
- Bolbat A, Vasiliev G, Kaygorodova I. 2020.** The first mitochondrial genome of the relic *Acanthobdella peledina* (Annelida, Acanthobdellida). *Mitochondrial DNA Part B: Resources* **5**: 3300–3301.
- Brinkhurst RO. 1982.** Evolution in the Annelida. *Canadian Journal of Zoology* **60**: 1043–1059.
- Brinkhurst RO. 1999.** Lumbriculids, branchiobdellidans and leeches: an overview of recent progress in phylogenetic research on clitellates. In: Healy BM, Reynoldson TB, Coates KA, eds. *Aquatic oligochaetes*. Berlin: Springer, 281–290.
- Brinkhurst RO, Gelder S. 1989.** Did the lumbriculids provide the ancestors of the branchiobdellidans, acanthobdellidans and leeches? *Hydrobiologia* **180**: 7–15.
- de Carle D, Ocegüera-Figueroa A, Tessler M, Siddall ME, Kvist S. 2017.** Phylogenetic analysis of *Placobdella* (Hirudinea: Rhynchobdellida: Glossiphoniidae) with consideration of *COI* variation. *Molecular Phylogenetics and Evolution* **114**: 234–248.
- Chevaldonné P, Jollivet D, Desbruyères D, Lutz RA, Vrijenhoek RC. 2002.** Sister-species of eastern Pacific hydrothermal vent worms (Ampharetidae, Alvinellidae, Vestimentifera) provide new mitochondrial *COI* clock calibration. *Cahiers de Biologie Marine* **43**: 367–370.
- Cichočka JM, Bielecki A, Świątek P, Jabłońska-Barna I, Kobak J, Hildebrand J, Dmitryjuk M, Strużyński W, Rost-Roszkowska M. 2021.** The activity of hydrolytic enzymes in the digestive system of *Acanthobdella*, *Branchiobdella* and *Hirudinida* (Annelida, Clitellata) – considerations on similarity and phylogeny. *European Zoological Journal* **88**: 26–43.
- Dahm AG. 1962.** Distribution and biological patterns of *Acanthobdella peledina* Grube from Sweden: (Hirudinea, Acanthobdellae). *Acta Universitatis Lundensis* **58**: 1–35.
- Epstein VM. 1966.** *Acanthobdella livanowi* sp. n.—a new species of the ancient leeches (Archihirudinea) from Kamchatka. *Doklady Akademii Nauk SSSR* **168**: 955–958.
- Epstein VM. 1987.** Tip KOLCHATYÉ CHEVRI – Annelida [Phylum RINGED WORMS – Annelida]. In: Bauer ON, ed. *Opređelitel parazitov presnovodnykh ryb fauny SSSR* [Identification key to parasites of the freshwater fishes of the fauna of the USSR]. Leningrad: Akademiya Nauk SSSR, Zoologicheskii Institut, Izdatelstvo Nauka, 340–372.
- Erséus C, Källersjö M. 2004.** 18S rDNA phylogeny of Clitellata (Annelida). *Zoologica Scripta* **33**: 187–196.
- Esin EV, Markevich GN. 2017.** *Charrs of the genus Salvelinus of Asian North Pacific: origin, evolution and modern diversity*. Petropavlovsk-Kamchatsky: Kamchatpress.
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994.** DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**: 294–299.
- Gelder SR, Siddall ME. 2001.** Phylogenetic assessment of the Branchiobdellidae (Annelida, Clitellata) using 18S rDNA, mitochondrial cytochrome *c* oxidase subunit I and morphological characters. *Zoologica Scripta* **30**: 215–222.
- Hauck AK, Fallon MJ, Burger CV. 1979.** New host and geographical records for the leech *Acanthobdella peledina* Grube 1851 (Hirudinea, Acanthobdellidae). *Journal of Parasitology* **65**: 989.
- Holmquist C. 1974.** A fish leech of the genus *Acanthobdella* found in North America. *Hydrobiologia* **44**: 241–245.
- Iwama RE, Ocegüera-Figueroa A, de Carle D, Manglicmot C, Erséus C, Miles NMS, Siddall ME, Kvist S. 2019.** Broad geographic sampling and DNA barcoding do not support the presence of *Helobdella stagnalis* (Linnaeus, 1758) (Clitellata: Glossiphoniidae) in North America. *Zootaxa* **4671**: 1–25.
- Iwama RE, Tessler M, Kvist S. 2022.** Leech anticoagulants are ancestral and likely to be multifunctional. *Zoological Journal of the Linnean Society* zlab126.
- Iwama RE, Tessler M, Siddall ME, Kvist S. 2021.** The origin and evolution of antistasin-like proteins in leeches (Hirudinida, Clitellata). *Genome Biology and Evolution* **13**: evaa242.
- James SW, Davidson SK. 2012.** Molecular phylogeny of earthworms (Annelida: Crassicitellata) based on 28S, 18S and 16S gene sequences. *Invertebrate Systematics* **26**: 213.
- Katoh K, Standley DM. 2013.** MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.
- Kaygorodova IA, Dzyuba EV. 2018.** New information on the distribution pattern of *Acanthobdella peledina* (Annelida, Acanthobdellida) in eastern Siberia. *Zootaxa* **4399**: 123–126.
- Kaygorodova IA, Dzyuba EV, Pronin NM. 2012.** Leech-like parasites (Clitellata, Acanthobdellida) infecting native and endemic eastern Siberian salmon fishes. *The Scientific World Journal* **2012**: 652827.
- Kaygorodova IA, Sherbakov DY. 2006.** Molecular phylogenetic study of the systematic position of Baikalian oligochaetes in Clitellata. *Russian Journal of Genetics* **42**: 1390–1397.
- Kowalevsky A. 1896.** Etude sur l'anatomie de l'*Acanthobdella peledina*. *Bulletin de l'Académie Impériale des Sciences de St. Petersburg* **5**: 263–274.
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018.** MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**: 1547–1549.
- Kutschera U, Epshtein VM. 2006.** Nikolaj A. Livanov (1876 – 1974) and the living relict *Acanthobdella peledina* (Annelida, Clitellata). *Annals of the History and Philosophy of Biology* **11**: 85–98.
- Kvist S. 2015.** Does a global DNA barcoding gap exist in Annelida? *Mitochondrial DNA. Part A, DNA Mapping, Sequencing, and Analysis* **27**: 2241–2252.
- Livanov N. 1906.** *Acanthobdella peledina* Grube, 1851. *Zoologische Jahrbücher. Abteilung für Anatomie* **22**: 637–866.
- Livanov N. 1931.** Die Organisation der Hirudineen und die Beziehungen dieser Gruppe zu den Oligochaeten. *Ergebnisse und Fortschritte der Zoologie* **7**: 387–484.

- Mack J, de Carle D, Kvist S. 2019.** Prey, populations, and the Pleistocene: evidence for low *COI* variation in a widespread North American leech. *Mitochondrial DNA. Part A, DNA Mapping, Sequencing, and Analysis* **30**: 749–763.
- Makhrov AA, Bolotov IN, Spitsyn VM, Gofarov MY, Artamonova VS. 2019.** Resident and anadromous forms of Arctic charr (*Salvelinus alpinus*) from north-east Europe: an example of high ecological variability without speciation. *Doklady. Biochemistry and Biophysics* **485**: 119–122.
- Marotta R, Ferraguti M, Erséus C, Gustavsson LM. 2008.** Combined-data phylogenetics and character evolution of Clitellata (Annelida) using 18S rDNA and morphology. *Zoological Journal of the Linnean Society* **154**: 1–26.
- Martin P. 2001.** On the origin of the Hirudinea and the demise of the Oligochaeta. *Proceedings of the Royal Society B: Biological Sciences* **268**: 1089–1098.
- Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R. 2020.** IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution* **37**: 1530–1534.
- Mitenev VK, Šul’man BS. 1999.** *Parazity ryb vodoemov Murmanskoy Oblasti. [Fish parasites in waters of the Murmansk District]*. Murmansk: Izdat. PINRO.
- Oceguera-Figueroa A, León-Règagnon, Siddall ME. 2010.** DNA barcoding reveals Mexican diversity within the freshwater leech genus *Helobdella* (Annelida: Glossiphoniidae). *Mitochondrial DNA* **21 Suppl 1**: 24–29.
- Phillips AJ, Dornburg A, Zapfe KL, Anderson FE, James SW, Erséus C, Lemmon EM, Lemmon AR, Williams BW. 2019a.** Editor’s note: phylogenomic analysis of a putative missing link sparks reinterpretation of leech evolution. *Genome Biology and Evolution* **11**: 3275.
- Phillips AJ, Dornburg A, Zapfe KL, Anderson FE, James SW, Erséus C, Moriarty Lemmon E, Lemmon AR, Williams BW. 2019b.** Phylogenomic analysis of a putative missing link sparks reinterpretation of leech evolution. *Genome Biology and Evolution* **11**: 3082–3093.
- Purschke G, Westheide W, Rohde D, Brinkhurst RO. 1993.** Morphological reinvestigation and phylogenetic relationship of *Acanthobdella peledina* (Annelida, Clitellata). *Zoomorphology* **113**: 91–101.
- Rota E, Martin P, Erséus C. 2001.** Soil-dwelling polychaetes: enigmatic as ever? Some hints on their phylogenetic relationships as suggested by a maximum parsimony analysis of 18S rRNA gene sequences. *Contributions to Zoology* **70**: 127–138.
- Rousset V, Plaisance L, Erséus C, Siddall ME, Rouse GW. 2008.** Evolution of habitat preference in Clitellata (Annelida). *Biological Journal of the Linnean Society* **95**: 447–464.
- Sawyer RT. 1986.** *Leech biology and behaviour*. Wotton-under-Edge: Clarendon Press.
- Siddall ME, Apakupakul K, Burreson EM, Coates KA, Erséus C, Gelder SR, Källersjö M, Trapido-Rosenthal H. 2001.** Validating Livanow: molecular data agree that leeches, Branchiobdellidans, and *Acanthobdella peledina* form a monophyletic group of oligochaetes. *Molecular Phylogenetics and Evolution* **21**: 346–351.
- Siddall ME, Burreson EM. 1998.** Phylogeny of leeches (Hirudinea) based on mitochondrial cytochrome *c* oxidase subunit I. *Molecular Phylogenetics and Evolution* **9**: 156–162.
- Świątek P, Urbisz AZ, Strużyński W, Płachno BJ, Bielecki A, Cios S, Salonen E, Klag J. 2012.** Ovary architecture of two branchiobdellid species and *Acanthobdella peledina* (Annelida, Clitellata). *Zoologischer Anzeiger* **251**: 71–82.
- Tessler M, de Carle D, Voiklis ML, Gresham OA, Neumann JS, Cios S, Siddall ME. 2018a.** Worms that suck: phylogenetic analysis of Hirudinea solidifies the position of Acanthobdellida and necessitates the dissolution of Rhynchobdellida. *Molecular Phylogenetics and Evolution* **127**: 129–134.
- Tessler M, Siddall ME, Oceguera-Figueroa A. 2018b.** Leeches from Chiapas, Mexico, with a new species of *Erpobdella* (Hirudinida: Erpobdellidae). *American Museum Novitates* **3895**: 1–15.
- Tessler M, Weiskopf SR, Berniker L, Hersch R, Mccarthy KP, Yu DW, Siddall ME. 2018c.** Bloodlines: mammals, leeches, and conservation in southern Asia. *Systematics and Biodiversity* **16**: 488–496.
- Trontelj P, Sket B, Steinbrück G. 1999.** Molecular phylogeny of leeches: congruence of nuclear and mitochondrial rDNA data sets and the origin of bloodsucking. *Journal of Zoological Systematics and Evolutionary Research* **37**: 141–147.
- Utevsky SY, Sokolov SG, Shedko MB. 2013.** New records of the chaetiferous leech-like annelid *Paracanthobdella livanowi* (Epshtein, 1966) (Annelida: Clitellata: Acanthobdellida) from Kamchatka, Russia. *Systematic Parasitology* **84**: 71–79.
- Westheide W. 1997.** The direction of evolution within the Polychaeta. *Journal of Natural History* **31**: 1–15.
- Williams BW, Gelder SR, Proctor HC, Coltman DW. 2013.** Molecular phylogeny of North American Branchiobdellida (Annelida: Clitellata). *Molecular Phylogenetics and Evolution* **66**: 30–42.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** Polymerase chain reaction primers and thermal profiles.

**Table S2.** Sequences used in phylogenetic analysis.

**File S1.** Concatenated alignment used for phylogenetic analysis (in NEXUS format).

**File S2.** Maximum likelihood tree inferred using mitochondrial loci (*COI*, 12S and 16S) only.

**File S3.** Maximum likelihood tree inferred using nuclear loci (18S and 28S) only.

**File S4.** Maximum likelihood tree inferred using all loci concatenated (*COI*, 12S, 16S, 18S and 28S).