Proteocephalidean tapeworms (Cestoda: Onchoproteocephalidea) of loaches (Cobitoidea):
evidence for monophyly and high endemism of parasites in the Far East

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ABSTRACT The parasite fauna of loaches (Cypriniformes: Cobitoidea), a group of small
bottom-dwelling freshwater fishes with a mostly Eurasian distribution, remains a largely
unknown quantity. Here we revise the taxonomy of tapeworms of the genus *Proteocephalus*
Weinland, 1858 (Cestoda: Proteocephalidae) that had been found in loaches from the
Palearctic Region (Central Europe, Japan and Russia [Primorsky Region]). Molecular
phylogenetic analysis based on two nuclear (*ssr*- and *lsrDNA*) and two mitochondrial genes
(*cox1* and *rrnL*) revealed a monophyletic group consisting of four valid species nesting within
the *Proteocephalus*-aggregate: (i) *Proteocephalus sagittus* (Grimm, 1872) from *Barbatula*
barbatula (Europe, Russia and Tadjikistan), (ii) *Proteocephalus demshini* n. sp. from

Barbatula toni (Russian Far East – Primorsky Region), (iii) *Proteocephalus midoriensis* Shimazu, 1990 from *Lefua echigonia* (Japan) and *L. costata* (Russia) (new host and geographical record), and (iv) *Proteocephalus misgurni* n. sp. from *Misgurnus anguillicaudatus* (Russia; Primorsky Region). *Proteocephalus sagittus* and *P. demshini*, and *P. midoriensis* and *P. misgurni* were recovered as sister taxa, respectively. *Proteocephalus sagittus* and *P. demshini* are characterized by having proglottids that are wider than long, an elongate to pyriform cirrus-sac and the vitelline follicles that form wide lateral bands. *Proteocephalus midoriensis* and *P. misgurni* are characterized by having proglottids that are more elongate and an ovoid to almost spherical cirrus-sac and the vitelline follicles forming narrow lateral bands. *Proteocephalus demshini* differs from *P. sagittus* in the posterolateral extent of the vitelline follicles, whereas *P. misgurni* can be distinguished from *P. midoriensis* mainly by the relative size of the ovary, posterior extent of the vitelline follicles and width of the scolex. Unlike most species of the *Proteocephalus*-aggregate that possess an apical sucker, all species from loaches are devoid of any apical organ. The existence of two new species in loaches from the Primorsky Region of Russia indicates high endemism of fish parasites in this region. A key to the identification of recognised species from loaches is provided.

Keywords: Taxonomy, morphology, 16S rDNA, 18S rDNA, 28S rDNA, *cox1* *Proteocephalidae*, *Nemacheilidae*, *Cobitidae*, Palearctic Region

1. Introduction

Loaches are small, bottom-dwelling, scavenging freshwater fishes belonging to the families Balitoridae, Cobitidae and Nemacheilidae of the superfamily Cobitoidea (order

Cypriniformes). Their main distribution is throughout Eurasia, although two species have been reported from Africa (Morocco and Ethiopia). Most of them inhabit rivers and streams in hilly areas (up to 5,200 m above sea level) but they are also known from streams in rainforest, from the black waters of peat swamp forests, and from desert oases [1, 2].

The Cobitoidea are diverse in terms of species number (1,043 valid species; [1]) and ecological valence, yet, data on their parasites are scarce and fragmentary, probably because of their generally low economic value. The best-studied species are the stone loach, *Barbatula barbatula* (Linnaeus, 1758) (Nemacheilidae), distributed throughout Europe, and the weather loach, *Misgurnus anguillicaudatus* (Cantor, 1842) (Cobitidae), distributed throughout East Asia [3]. Available information indicates that the helminth fauna of loaches is relatively depauperate but includes parasites that are specific to these fishes, such as caryophyllidean cestodes of the genus *Paracaryophyllaeus* Kulakovskaya, 1961, which harbors five valid species and other putative undescribed taxa [4].

Cestodes of the genus *Proteocephalus* Weinland, 1858 (Proteocephalidae) are common parasites of a wide spectrum of freshwater teleosts globally [5, 6], but the genus is an artificial assemblage of at least seven unrelated lineages [7]. In contrast, species of the *Proteocephalus*-aggregate de Chambrier, Zehnder, Vaucher and Mariaux, 2004, which occur only in the Holarctic Region, form a monophyletic lineage [7–9]. Two species of the *Proteocephalus*-aggregate have been found in loaches, namely *Proteocephalus sagittus* (Grimm, 1872) in the stone loach, *B. barbatula* (type host), and other loaches, and *Proteocephalus midoriensis* Shimazu, 1990, in the Japanese eight-barbel loach, *Lefua echigonia* Jordan and Richardson, 1907 (Nemacheilidae). Phylogenetic analyses based on 5.8S rDNA + ITS2, the V4 region of the small subunit nuclear ribosomal RNA (*ssrDNA*) and the D1–D3 region of the large subunit nuclear ribosomal RNA (*lsrDNA*) revealed invariably close relatedness of these two species [10, 11]. Furthermore, they formed a lineage separate

from the other species of *Proteocephalus*, including *Proteocephalus torulosus* (Batsch, 1786), a parasite of cyprinids (Cypriniformes) in the Holarctic Region. *Proteocephalus sagittus* was erroneously synonymized with *P. torulosus* by Scholz and Hanzelová (1998) [12] (see also [13]).

In 2010, new material of *Proteocephalus* cestodes was collected by three of the present authors (T. S., A. C. and A. E.) in two localities of the River Amur basin near Khanka Lake, Primorsky Region, Russia. Evaluation of this new material, which includes two separate taxa, as well as specimens from museum collections, made it possible to critically evaluate the species composition of proteocephalids parasitizing loaches. In the present paper, a survey of all valid species is provided together with description of two new species from the Russian Far East. A key, enabling their identification based on morphological characteristics, is also given. In addition, phylogenetic relationships of these species were reconstructed based on data from almost complete *ssrDNA* (= 18S rDNA), partial (D1–D3 variable domains) *lsrDNA* (= 28S rDNA), partial cytochrome *c* oxidase subunit I (*cox1*) and partial large mitochondrial ribosomal RNA subunit (*rrnL*) (= 16S rDNA).

2. Materials and methods

2.1. Morphological evaluation

The present survey is based on the evaluation of museum material and newly collected specimens from the Russian Far East (River Amur basin in the Primorsky Region) and Japan: (1) 6 whole mounts of *P. sagittus* from *B. barbatula*, River Dřevnice near Vyškov, Czech Republic, collected by B. Koubková from April to July 2001 (deposited at the Helminthological Collection of the Institute of Parasitology, Biology Centre of the Czech

Academy of Sciences, České Budějovice, Czech Republic – Coll. No. IPCAS C-33/1; see also [13]);

(2) 12 whole mounts of *P. midoriensis* from *L. echigonia*, a small river and small irrigation canals in the paddy field at Midori, Iiyama City, Nagano Prefecture, Japan, collected by T. Shimazu on 6 August 1998 (Coll. No. IPCAS C-362/1);

(3) 2 whole mounts designated as “*Proteocephalus* sp. n.” from *L. costata* (Kessler, 1876) (host field numbers 562-1 and 590-10), River Razdolnaya basin (43°37'N, 131°52'E°), collected by A. Ermolenko on 22 July 1983 and 12 October 1983 (Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia; no collection number);

(4) 4 whole mounts of specimens from *Barbatula toni* (Dybowski, 1869), River Komissarovka, Khanka Lake basin, Primorsky Region, Russia, collected by T. Scholz, A. de Chambrier and A. Ermolenko on 28 June 2011 (host field numbers RUS 190, 191, 192 and 195; Coll. Nos. IPCAS C-727 and Natural History Museum, Geneva, Switzerland – Coll. No. MHNG-PLAT 79145, 79149, 79150); 2 scoleces of specimens from *B. toni* used for scanning electron microscopical (SEM) observations (RUS 192 and 193).

(5) 15 whole mounts of specimens from *M. anguillicaudatus*, River Ilistaya near Chernigovka, Primorsky Region, Russia, collected by T. Scholz, A. de Chambrier and A. Ermolenko on 21–23 June 2011 (host field numbers RUS 74–78, 91–93, 95, 97–100 and 121; Coll. Nos. IPCAS C-728 and MHNG-PLAT 79135, 79136, 79139–79141, 79156 and 79158); three slides with cross sections of specimens (RUS 93; Coll. No. MHNG-PLAT 79140); two scoleces used for SEM observation (RUS 121).

Tapeworms found in the intestine of fish that were euthanized just before dissection were gently detached from the intestinal wall and rinsed in 0.9% NaCl solution. A small piece, usually comprising a few posterior-most gravid proglottids, was excised and preserved

in 96% molecular-grade ethanol for DNA sequencing (see below). Morphological vouchers, i.e. hologenophores (the specimen from which the molecular sample was taken; see [14] for terminology), were fixed with hot 4% formaldehyde solution.

Tapeworms for morphological evaluation were stored in 70% ethanol after fixation and then stained with Mayer's hydrochloric carmine solution, dehydrated in an ethanol series, cleared with eugenol (clove oil) and mounted in Canada balsam. Pieces of the strobila were embedded in paraffin wax, cross sectioned (thickness of 12–15 μm), stained with Weigert's haematoxylin, and counterstained with 1% eosin B following protocol outlined by [15].

For SEM observations, scoleces were prepared using the method described by [16] and observed with a Zeiss 940a electron microscope at the Natural History Museum, Geneva, and a JEOL JSM 6700F scanning electron microscope at the Institute of Parasitology, BC AS CR, České Budějovice, Czech Republic. Microthrix terminology follows [17]. All measurements are given in micrometres unless otherwise indicated. Abbreviations used in the descriptions are $\bar{x}$ = mean and n = number of measurements.

2.2. Molecular phylogeny

Genomic DNA isolation, PCR amplification and sequencing of almost complete *ssrDNA* and partial (D1–D3) *lsrDNA*, *rrnL* and *cox1* followed the protocols published by [19]. Newly generated data for were combined with already published data (see Table 1 for details). Outgroup choice was informed by the tree topology published in [7] and consisted of *Glanitaenia osculata* (Goeze, 1781) and *Paraproteocephalus parasiluri* (Zmееv, 1936). The ingroup consisted of the following members of the *Proteocephalus*-aggregate: *P. torulosus*, *P. gobiorum* Dogiel and Bychowsky, 1939, *P. fluviatilis* Bangham, 1925 (this Nearctic species has been accidentally introduced with its host fish from North America to Japan – [18]), *P.*

plecoglossi Yamaguti, 1934, , *P. longicollis* (Zeder, 1800), *P. tetrastomus* (Rudolphi, 1810),
P. pinguis La Rue, 1911, *P. filicollis* (Rudolphi, 1802), *P. macrocephalus* (Creplin, 1825) and
P. percae (Müller, 1780), and the following *Proteocephalus* species from loaches: *P. sagittus*,
P. midoriensis, *Proteocephalus misgurni*, and *Proteocephalus demshini* (see below for new
species descriptions of the latter two). For localities, host species and GenBank accession
numbers – see Table 1.

Sequences were aligned using MAFFT version 7.149b [18], with 1,000 cycles of
iterative refinement and the genafpair algorithm. An alignment mask, excluding sites of
uncertain positional homology, was generated using ZORRO [21]. ZORRO uses a pair
Hidden Markov Model and a weighted sum of pairs scheme (guided by a guide tree) that
sums up the probability that a given alignment column appears over the total alignment
landscape, thus providing an objective estimate whether positions consist of correctly aligned,
homologous residues.

Default settings were used, except for the invocation of the sample option, which
calculates the alignment columns posterior probabilities based on a random sampling of pairs
of sequences, rather than exhaustively sampling each pair, thus speeding up the running time.
Positions with confidence scores < 0.4 were excluded from subsequent analyses. MrModeltest
v. 2.3 [22] was used to select models of sequence evolution using the Akaike Information
Criterion. Bayesian inference (BI) analysis was performed using MrBayes version 3.2.1 [23].
Maximum likelihood (ML) bootstrap values for 100 replicates were obtained using the
Genetic Algorithm for Rapid Likelihood Inference (GARLI) Version 2.01 [24] using default
settings, except for setting ‘Genthreshfortopoterm’ to 10,000 generations. Clades were
considered to have high nodal support if BI posterior probability (pp) was $\geq 95\%$ and
bootstrap values $\geq 75\%$; all other nodes were collapsed. Uncorrected p-distances were
calculated using PAUP* v4.0b10 [25].

The data were partitioned into five character sets: (1) *ssrDNA*, (2) *lsrDNA*, (3) *rrnL* (4) *coxI*, and (5) the concatenated 4-gene data. BI and ML analyses were carried out using the GTR + I + G and GTR + I models of sequence evolution for partitions, *ssrDNA*, *rrnL* and *coxI*, and *lsrDNA*, respectively. Two parallel BI runs were performed for 10,000,000 generations and sampled every 1,000 generations. In the concatenated dataset, parameters were estimated separately for each gene. The burn-in was defined as the point at which the average standard deviation of split frequencies were < 0.01.

3. Results

3.1. Molecular phylogeny

The *ssrDNA* alignment was composed of 1961 positions (sequence length ranged from 454 to 1956 bp), of which 14 were excluded. The *lsrDNA* alignment was composed of 1552 positions (sequence length ranged from 1001 to 1518 bp), of which 50 were excluded. The *rrnL* alignment was composed of 851 positions (sequence length ranged from 424 to 842 bp), of which six were excluded. The *coxI* alignment was composed of 555 positions (sequence length ranged from 441 to 555 bp), of which none was excluded. Thus, the concatenated alignment was composed of 4919 positions, of which 70 were excluded.

The topology for the concatenated 4-gene (*ssrDNA* + *lsrDNA* + *rrnL* + *coxI*) dataset (Fig. 1) was not in conflict with any of the topologies based on the individual gene partitions (Supplementary Figs. 1–4). Any differences in topology and nodal support were attributable to differential taxon sampling between data partitions. Specifically, for *P. longicollis*, *P. tetrastomus*, *P. pinguis*, *P. filicollis* and *P. macrocephalus* only *lsrDNA* data were available

(Table 1). Thus, the results described below are based on the concatenated 4-gene analysis (Fig. 1).

All *Proteocephalus* species formed a well-supported monophyletic group. The positions of *P. torulosus*, *P. pinguis* and *P. fluviatilis* were unresolved. However, in the *lsrDNA*-only analysis *P. pinguis* and *P. fluviatilis* formed a well-supported clade with *P. filicollis* and *P. macrocephalus* in which the latter two were sister-taxa (Supplementary Fig. 2). This sister-group relationship was also present in the concatenated 4-gene analysis (Fig. 1). The remainder of the tree was well resolved: *P. gobiorum* formed the sister group to a clade composed of *P. sagittus*, *P. demshini*, *P. misgurni* and *P. midoriensis*. *Proteocephalus sagittus* and *P. demshini*, and *P. misgurni* and *P. midoriensis* were sister taxa, respectively. *P. percae* and *P. longicollis* were sister taxa in a clade which also included *P. plecoglossi* and *P. tetrastomus* (Fig. 1).

3.2. Morphological study

Taxonomic evaluation of museum and newly collected tapeworms from several species of loaches in the Palearctic Region has revealed the presence of four species, parasitizing two host families (Cobitidae and Nemacheilidae), which form two pairs of species: (1) *P. sagittus*, a parasite of *B. barbatula* (type host) in Europe and other loaches in Asia (Russia, Tadjikistan), and *P. demshini* from *B. toni* in Russia (Primorsky Region), and (2) *P. midoriensis* from *Lefua* spp. in Japan and Primorsky Region, and *P. misgurni* from *M. anguillicaudatus* in Russia (Primorsky Region). These pairs differ morphologically from each other in the shape of proglottids, shape of the cirrus-sac and the width and posterior extent of the lateral bands of the vitelline follicles. Herein, a survey of species of *Proteocephalus* from

loaches is provided including their differential diagnoses; species are listed chronologically, with the two new taxa listed in alphabetical order.

3.2.1. *Proteocephalus sagittus* (Grimm, 1872) La Rue, 1911 Fig. 2

Syns.: *Ichthyotaenia sagitta* Grimm, 1872; *Proteocephalus pamirensis* Dzhililov and Ashurova, 1971

Taxonomic summary

Type host: *Barbatula barbatula* (Linnaeus, 1758) (Cypriniformes: Nemacheilidae).

Other hosts: *Cobitis* ‘*taenia*’ Linnaeus, 1758 (Cypriniformes: Cobitidae) (several species of loaches have been reported under this name – [2]); *Triplophysa stoliczkai* (Steindachner, 1866) (Cypriniformes: Nemacheilidae).

Site of infection: intestine.

Type locality: Sanint Petersburg, Russia.

Type material: not known to exist.

Distribution: Europe (British Isles, Czech Republic, Hungary, Latvia, Russia, Slovak Republic, Ukraine), Palaearctic Asia (Russia – basins of the rivers Volga, Ob and Irtysh, Tadjikistan).

Selected references: Grimm (1872) [26], Freze (1965) [5], Dzhililov and Ashurova (1971) [27], Scholz and Hanzelová (1998) [10], Scholz et al. (2003, 2007) [11, 13], Jarkovský et al. (2004) [28], Hypša et al. (2005) [10].

Remarks

This species was described as *Ichthyotaenia sagitta* by Grimm (1872) [26] and transferred to *Proteocephalus* by La Rue (1911) [29]. Scholz and Hanzelová (1998) [12] synonymized this species with *P. torulosus*, a specific parasite of cyprinid fishes in the

Holarctic Region [12]. However, a subsequent study of new material as well as a comparative analysis of partial *ssrDNA* and 5.8S-ITS2 rRNA gene sequences delineated these as separate species [11, 13]. The present study provides strong support for the validity of *P. sagittus* and made it possible to supplement species diagnosis by additional measurements not reported in the previous morphological descriptions (see [12, 13, 30]; Table 2).

Proteocephalus sagittus has been found in Europe and parts of Palaearctic Asia [5, 12]. *Barbatula barbatula*, which is the type host of this cestode, is found in most parts of Europe [1], but records of *P. sagittus* from this fish host are relatively scarce [3]. *Proteocephalus sagittus* has also been found in the cobitid *Cobitis 'taenia'* in Europe and nemacheilid *Triplophysa stoliczkai* in Tadjikistan, but no material suitable for molecular evaluation was available to test whether tapeworms from these hosts are actually conspecific with *P. sagittus*.

Records of *P. sagittus* from fishes other than loaches, such as *Cottus gobio* (Perciformes: Cottidae), *Gobio gobio*, *Leuciscus leuciscus* (Cypriniformes: Cyprinidae), and *Salvelinus alpinus* (Salmoniformes: Salmonidae) (e.g. [31, 32]; see [3] for exhaustive list of records), are considered erroneous.

3.2.2. *Proteocephalus midoriensis* Shimazu, 1990 Fig. 3

Taxonomic summary

Type host: *Lefua echigonia* Jordan and Richardson, 1907 (Cypriniformes: Nemacheilidae).

Other host: *Lefua costata* (Kessler, 1876) (new host record – see Remarks).

Site of infection: Intestine.

Type locality: Midori, Iiyama City, Nagano Prefecture, Japan (36°53' N, 138°21' E).

273 *Type material*: National Museum of Nature and Science, Tsukuba, Ibaraki Prefecture, Japan
274 (formerly National Science Museum, Tokyo; holotype – NSMT-P1 3658; paratypes – NSMT-
275 P1 3642 and 3652–3661).

276 *Distribution*: Palaearctic Asia (Japan – Nagano and Shiga Prefectures, Primorsky Region of
277 Russia – new geographical record).

278 *Selected references*: Shimazu (1990) [18], Hypša et al. (2005) [10], Scholz et al. (2007) [11].

279 **Remarks**

280 The species was described by Shimazu (1990) [18] from specimens found in *L.*
281 *echigonia* from a small river and small irrigation canals in the paddy field at Midori, Iiyama
282 City, Nagano Prefecture, Japan. In the present study, specimens from the type host and type
283 locality were studied, which made it possible to supplement the original description with
284 previously unreported measurements (see Table 2). In addition, the number of proglottids was
285 counted (30–35 in total, with 19–30 immature, only 1 mature and 6–11 pregravid; gravid
286 proglottids, i.e. those with eggs containing fully formed hexacanth larvae, were not observed)
287 and uterine development of type 2 of de Chambrier et al. (2004; see below and [9]) was
288 observed.

289 As in *P. misgurni*, which is newly described here from *M. anguillicaudatus* (see
290 below), the proglottids of *P. midoriensis* are longer than wide except for the first immature
291 ones – Fig. 3C (versus wider than long or rectangular in *P. sagittus* and *P. demshini*, which is
292 newly described here from *B. toni* [see below]), the cirrus-sac is ovoid to almost spherical –
293 Fig. 3E (versus pyriform to elongate), and the vitelline follicles form a narrow lateral band
294 composed of few (usually 1 or 2) follicles – Fig. 3C–E (versus wide bands composed of
295 several follicles at the same level in both species from *Barbatula* spp.), which may overlap
296 the ovary on its ventral side – Fig. 3D (only exceptionally some follicles overlap the ovarian
297 lobes in the other species). Unlike *P. misgurni*, the ovary of *P. midoriensis* is large, with very

wide (high), but short (horizontally) lateral wings, and a considerable part of the ovary (up to its half-length) may be overlapped by the vitelline follicles ventrally (Fig. 3C, D). The scolex of *P. midoriensis* is similar to that of *P. misgurni* (Fig. 5C), but it is less conspicuously widened at its apical part, and suckers are smaller in relation to the scolex width (Fig. 3A; Table 2).

Tapeworms from *L. costata*, collected by the late N.I. Demshin and designated by him as '*Proteocephalus* sp. n.', were found to be indistinguishable from those of *P. midoriensis* from congeneric fish hosts and are considered conspecific, even though the quality of all specimens is poor due to their *post mortem* decomposition and improper fixation by strong flattening (Fig. 3B).

3.2.3. *Proteocephalus demshini* n. sp. Figs. 4, 6

Description (based on 5 whole-mounted specimens and 2 scoleces studied using SEM; for morphometrical data – see Table 2): Proteocephalidae, Proteocephalinae. Testes, vitelline follicles, ovary and uterus medullary. Medium-sized tapeworm, body length 18–33 mm (n = 5), maximum width up to 1.1 mm (n = 5). Strobila acraspedote, anapolytic, consisting of about 80–100 proglottids: 55–60 immature, 1 mature, 16–24 pregravid and 6–13 gravid proglottids. Immature, mature and pregravid proglottids wider than long, gravid longer than wide.

Scolex wider than neck. Suckers spherical, with deep cavity (Fig. 4A). Apical organ absent; apex of scolex and region posteromedian to suckers with gland cells (Fig. 4A). Neck (proliferation zone) 360 wide. Scolex covered with acicular filitriches and gladiate spinitriches (on suckers – Fig. 6F) and acicular filitriches (between suckers and on scolex apex); neck covered with gladiate spinitriches. Inner longitudinal internal musculature developed, anastomosed. Ventral osmoregulatory canals thin-walled, 15–25 wide, ventral to

lateral bands of vitelline follicles (Fig. 4C). Dorsal osmoregulatory canals thick-walled, narrow, about 5–8 wide.

Testes 62–89 in number, spherical, in two irregular layers, form one compact field between lateral bands of vitelline follicles and ovary (Fig. 4B), present also in gravid proglottids. Vas deferens strongly coiled, with loops forming elongated field crossing median line of proglottid (Fig. 4B). Cirrus-sac pyriform to elongate (Fig. 4C), thick-walled. Sperm duct (internal vas deferens) coiled. Cirrus muscular. Common genital atrium narrow, deep (Fig. 4C). Genital pores alternating irregularly, pre-equatorial (Fig. 4B).

Ovary medullary, bilobed, with long and narrow lateral wings. Relative size of ovary, i.e. its surface in relation to surface of pregravid and gravid proglottids (see [33]), about 17%. Mehlis' gland 85–160 in diameter.

Vaginal canal almost straight in distal part, with terminal part (*pars copulatrix vaginae*) surrounded by chromophilic cells; vaginal sphincter absent (Fig. 4C). Vitelline follicles medullary, forming two wide lateral bands interrupted ventrally on poral side at level of cirrus-sac and vagina; bands widened posteriorly and reaching to anterior margin of ovary only, rarely with few follicles slightly (less than by 10%) overlapping ovarian wings on ventral side (Fig. 4B).

Uterus medullary, with development of type 2 [9]. Lumen of uterine stem occupies up to 49% of proglottid width in last immature proglottids. In mature proglottids, lumen occupies up to 55% of proglottid width; apex lined with numerous chromophilic cells (Fig. 4C). In gravid proglottids, uterine diverticula occupy up to 90% of proglottid width.

Eggs spherical, with hyaline outer envelope and bilayered embryophore; oncosphere containing embryonic hooks (Fig. 4D).

Taxonomic summary

348 *Type and only host:* *Barbatula toni* (Dybowski, 1869) (Cypriniformes: Nemacheilidae).

349 *Site of infection:* intestine.

350 *Type locality:* River Komissarovka near Kamen-Rybolov, Primorsky Region, Russia

351 (44°57'22"N, 131°42'00"E).

352 *Prevalence and intensity of infection (mean with range and number of fish examined in*

353 *parentheses):* 19% (n = 32); 1.7 (1–4).

354 *Deposition of specimens:* Holotype (one whole-mounted specimen from host field No. RUS

355 190 collected on 28 June 2011; Coll. No. MHNG-PLAT 79145); three paratypes (whole-

356 mounted specimens from hosts field Nos. RUS 191, 194a and 195 collected on 28 June 2011;

357 IPCAS C-727; MHNG-PLAT 79150); one voucher (whole-mounted specimen from host field

358 Nos. RUS 190 collected on 28 June 2011; Coll. No. IPCAS C-727); 2 scoleces from host field

359 No. RUS 192 (MHNG-PLAT 79149) used for SEM observations.

360 *Etymology:* The species is named in honour of the late N.I. Demshin, Vladivostok, Russia, for

361 his extraordinary contribution to helminthology in the Russian Far East, especially life-cycles

362 of cestodes.

363 *Differential diagnosis.* The new species differs from *P. midoriensis* and *P. misgurni* from *M.*

364 *anguillicaudatus* (see below) by the shape of the proglottids and the cirrus-sac, width of the

365 lateral bands of the vitelline follicles and their posterior extent (see Remarks on *P.*

366 *midoriensis* and compare Figs. 3–5). *Proteocephalus demshini* is strikingly similar to *P.*

367 *sagittus* from the congeneric host and a few other loaches in its morphology including the

368 shape of proglottids, which are wider than long except for the last gravid proglottids, wide

369 bands of the vitelline follicles widened posteriorly, and an elongate to pyriform cirrus-sac.

370 These two species can be distinguished from each other by (i) the number of uterine

371 diverticles on each side, 3–8 in *P. demshini* versus 9–14 in *P. sagittus*; (ii) the ratio of the

372 width of the band of the vitelline follicles at the level of the ovary in relation to the width of

the proglottid, which is 15–19% ($x = 17\%$) in the new species versus 8–14% in *P. sagittus* ($x = 10\%$); (iii) shape of the anterior end of the body, with the neck region widened anteriorly in *P. demshini* versus of the same width in *P. sagittus*; and (iv) the posterior extent of the vitelline follicles, which overlap the ovary only slightly in *P. demshini* (up to 1/10 of the length of the ovary) versus up to 1/5 in *P. sagittus* (Table 2).

Discrete species status of these apparent sibling species is supported by molecular data (see Fig. 1 and Suppl. Figs. 2 and 4); uncorrected p-distances are 0.5% and 9.9% for *lsrDNA* and *cox1*, respectively (Suppl. Table 1).

The type host of this new species, *B. toni*, occurs in Asia and lives in all rivers flowing to the Arctic and Pacific Oceans between the rivers Ob and Huang He [1]. Its helminth fauna is relatively depauperate [3, 34–36], but includes also three species of cestodes, yet none of them being specific to, or typical of, this fish. Scholz and Ergens (1990) [37] reported juveniles of a species of *Proteocephalus* that possessed a vestigial muscular apical sucker, thus apparently not belonging to *P. demshini*, which is devoid of an apical sucker. T. Shimazu (unpubl. data) examined 100 specimens of *B. toni* from Hokkaido in 1984 and 2011 but no proteocephalidean cestodes were found, which indicates that *P. demshini* is endemic to the Primorsky Region of Russia.

3.2.4. *Proteocephalus misgurni* sp. n. Figs. 5, 6

Description (based on 11 whole-mounted specimens and 2 scoleces studied using SEM; for morphometrical data – see Table 2): Proteocephalidae, Proteocephalinae. Testes, vitelline follicles, ovary and uterus medullary. Medium-sized tapeworm, body 9–21 mm long ($n = 5$), maximum width up to 530 ($n = 10$). Strobila acraspedote, anapolytic, consisting of 52–58 proglottids: 33–39 immature, 2 mature, 3–12 pregravid; 3–6 gravid proglottids (Fig.

5A). Immature proglottids and mature proglottids wider than long to longer than wide.

Pregravid proglottids rectangular to longer than wide, gravid proglottids longer than wide.

Scolex 250–350 long ($n = 4$), wider than neck (Figs. 5C, 6B, E). Suckers spherical, with deep cavity (Fig. 6b, e). Neck (proliferation zone) widened, 340–470 wide. Scolex covered with acicular filitriches and gladiate spinitriches (on suckers) and acicular filitriches (between suckers – Fig. 6G, and on scolex apex); neck covered with gladiate spinitriches (Fig. 6H).. Inner longitudinal internal musculature well-developed, anastomosed. Ventral osmoregulatory canals thin-walled, 10–12 wide, overlapping lateral bands of vitelline follicles, sometimes anastomosed, slightly sinuous (Fig. 5E). Dorsal osmoregulatory canals thick-walled, narrow, about 5 wide, difficult to observe in most mature or pregravid proglottids.

Testes 32–44 in number, spherical, small, in two irregular layers (Fig. 5E), forming one field separated aporally by well-developed sperm duct (vas deferens) (Fig. 5E). Testes present also in gravid proglottids. Vas deferens strongly coiled, with loops forming elongated field crossing median line of proglottids (Fig. 5D, E). Cirrus-sac ovoid to almost spherical, thick-walled; sperm duct (internal vas deferens) coiled. Cirrus muscular. Common genital atrium narrow, deep (Fig. 5D). Genital pores alternating irregularly, pre-equatorial to equatorial.

Ovary medullary, bilobed. Relative size of ovary (see [9]) about 15% of size of pregravid proglottids. Mehlis' gland 60–75 in diameter. Vaginal canal slightly sinuous in distal part; terminal part of vaginal canal (*pars copulatrix vaginae*) surrounded by chromophilic cells, without sphincter (Fig. 5D).

Vitelline follicles medullary, forming two long, narrow lateral bands slightly widened posteriorly at ovarian level (Fig. 5E). Follicles absent at level of cirrus-sac and vagina on ventral side (Fig. 5E). Posteriorly, follicles may overlap ovary ventrally (Fig. 5E). Uterus

423 medullary, with development of type 2 according to [9]. Uterus occupies up to 38% of
424 proglottid width in last immature proglottids, up to 87% in pregravid proglottids and up to
425 92% of gravid proglottids.

426 Eggs spherical, with hyaline outer envelope and bilayered embryophore; oncosphere
427 containing six embryonic hooks 15–17 long (Fig. 5B).

428

429 *Taxonomic summary*

430 *Type and only host: Misgurnus anguillicaudatus* (Cantor, 1842) (Cypriniformes: Cobitidae).

431 *Site of infection:* intestine.

432 *Type locality:* River Ilistaya near Chernigovka, Primorsky Region, Russia (44°26'47"N,
433 132°25'20"E).

434 *Prevalence and intensity of infection (mean with range and number of fish examined in*
435 *parentheses):* 29% (n = 73); 1.3 (1–3).

436 *Type material:* Holotype (one whole-mounted specimen from host field No. RUS 77 collected
437 on 21 June 2011; Coll. No. MHNG-PLAT 79136); two paratypes (whole-mounted specimens
438 from hosts field Nos. RUS 75 and RUS 100 collected on 21 and 22 June 2011, respectively;
439 MHNG-PLAT 79135 and 79142); two paratypes (whole-mounted specimens from host field
440 Nos. RUS 91 and 121 collected on 22 and 23 June 2011, respectively; IPCAS C-728).

441 *Etymology:* the specific name is given according to the host's genus.

442

443 *Differential diagnosis.* *Proteocephalus misgurni* is most similar to *P. midoriensis* (see below),
444 and differs from *P. sagittus* and *P. demshini*, in a similar shape of the body, with the club-
445 shaped scolex conspicuously wider than immature and mature proglottids, the strobila
446 composed of well-separated proglottids that are rectangular (most immature ones) or longer
447 than wide (all remaining proglottids – Fig. 5A), relatively few vitelline follicles that form

narrow lateral bands, with posterior follicles overlapping the ovarian wings on the ventral side, a subspherical to almost spherical cirrus-sac, and low number of testes (less than 50) (Figs. 2–5; Table 2).

This new species can be easily distinguished from *P. midorensis* by (i) its size (it is about twice longer and wider – see Table 2) including a much wider scolex (width 425–535 µm versus 285–310 µm); (ii) less elongate proglottids (see Figs. 3C and 5E); (iii) longer (horizontally) and narrower (in vertical direction, i.e. height) lateral wings of the ovary versus wings longer than wide in *P. midoriensis* (Figs. 3C and 5E); (iv) posterior extent of vitelline follicles, which overlap the ovary ventrally only up to its fifth in this new species (versus up to its half in *P. midoriensis*), and width of their lateral bands (bands represent only 10–12% of the width of proglottids in *P. misgurni* versus 19–22% in *P. midoriensis*); and (v) other metrical data such as the diameter of oncospheres, relative size of the suckers and relative position of the genital pore (see Table 2).

Proposal of *P. misgurni* as a new species is supported by molecular data (see Fig. 1, Suppl. Figs. 1, 2, 4, Suppl. Table 1); uncorrected p-distances between this species and its sister species, *P. midoriensis*, are 1.5% for *ssrDNA*, 0.6% for *lsrDNA* and 2.5% for *rrnL*, respectively (Suppl. Table 1).

This new species seems to be a specific parasite of *M. anguillicaudatus*, which is native to Siberia (Tugur and Amur drainages), Sakhalin, Korea, Japan, China south to northern Vietnam, but has been introduced to several localities in the River Rhine (Germany) and Ticino (Italy, north of Milano) drainages, the Aral Sea basin, North America, Australia and Hawaii. This species proved successful in the aquarium fish trade and at least one country reports adverse ecological impact after introduction [1, 2]. The parasite fauna of weather loaches is rather depauperate and cestodes were represented only by species of the caryophyllidean genus *Paracaryophyllaeus* Kulakovskaya, 1961 (see [4]). The present

473 authors and their co-workers (T. Shimazu in Japan and T. Scholz, M. Oros and his Slovak co-
 474 workers in China) examined as many as about 500 and 700 weather loaches in Japan and
 475 China, respectively, but no proteocephalidean cestodes were found. This indicates that *P.*
 476 *misgurni* is also endemic to the Primorsky Region of Russia as *P. demshini*.

477

478 3.3. Identification key

479

480 An identification key to the four species of the *Proteocephalus*-aggregate that
 481 parasitize loaches (Cobitoidei) is provided below.

482

483 1a. Most proglottids elongate, longer than wide; cirrus-sac ovoid to almost spherical; lateral
 484 bands of vitelline follicles narrow, formed by a few (usually only 1 or 2) follicles. 2

485 1b. Most proglottids wider than long; cirrus-sac pyriform to elongate; lateral bands of vitelline
 486 follicles wide, formed by several follicles. 3

487 2a. Body smaller (less than 18 mm long); ovary with long, almost rectangular lateral wings,
 488 overlapped by vitelline follicles up to its half-width on the ventral side. In *Lefua echigonia*
 489 and *L. costata* (Nemacheilidae). *P. midoriensis* Shimazu, 1990

490 2a. Body larger (up to 33 mm long); ovary with shorter, transversely elongate lateral wings,
 491 overlapped by vitelline follicles only up to fifth of its width. In *Misgurnus anguillicaudatus*
 492 (Cobitidae) *P. misgurni* n.
 493 sp.

494 3a. Anterior part with the neck region wider in its anterior part and narrowing posteriorly;
 495 band of vitelline follicles posteriorly wider (15–19% of proglottid width); follicles overlap the
 496 ovary ventrally only slightly (less than 10% of the width of the ovarian wings). In *Barbatula*
 497 *toni* (Nemacheilidae). *P. demshini* n. sp.

3b. Anterior part with the neck region of the same width; band of vitelline follicles posteriorly narrower (8–14% of proglottid width); follicles overlap the ovary ventrally up to the fifth of its length. In *Barbatula barbatula*, *Triplophysa stoliczkai* (Nemacheilidae) and *Cobitis* ‘*taenia*’ (Cobitidae). *P. sagittus* (Grimm, 1872)

4. Discussion

In the present study, tapeworms of the *Proteocephalus*-aggregate specific to loaches (Cobitoidea) were surveyed and two new species, both probably endemic to the Russian Far East, have been described based on molecular and morphological results. All four species currently recognized as parasites of loaches form a monophyletic assemblage of two pairs of Palaearctic species (Fig. 7) that differ from each other in their morphology and host use: one group is formed by two sibling species which are parasites of nemacheilids of the genus *Barbatula* and other loaches, whereas the two remaining morphologically similar species parasitize fish of different families: *P. midoriensis* is a parasite of nemacheilids of the genus *Lefua* and *P. misgurni* seems to be specific to the cobitid *M. anguillicaudatus*. Genetic and morphological similarity of tapeworms of the former group indicates that they should be considered sibling species that have diversified relatively recently, based on the recent divergence of their fish hosts.

Molecular data also indicate that *P. gobiorum* from gobies, which is the only species of the *Proteocephalus*-aggregate known to occur exclusively in brackish waters, is a sister taxon to the lineage formed by the four species from loaches. Despite phylogenetic unrelatedness of hosts of these taxa (gobies of the order Perciformes versus loaches of the order Cypriniformes), their cestodes are devoid of any apical structure in the scolex, which is uncommon among species of the aggregate (most species have a muscular, yet vestigial apical

sucker – [12]). Instead, the apical part of the scolex of *P. gobiorum* and all species from loaches contains gland cells that are grouped to form a relatively regular structure (see fig. 12 in [12]) and the region posterior to the scolex contains a large concentration of large gland cells in that species. In contrast, gland cells in the scolex apex of species from loaches are distributed sparsely and irregularly posterior to the suckers (e.g., Figs. 3A, 5C).

Species of *Proteocephalus* from loaches can be distinguished from each other using several morphological characteristics, but some differences, especially those between the two sibling species, *P. sagittus* and *P. demshini*, are in fact quite subtle. Arrangement of the vitelline follicles, especially the width of their lateral bands and posterior extent, represents one of a few morphological characters that distinguish these species from each other. Baer (1950) [39] pointed out taxonomic importance of the disposition of the vitelline follicles in tapeworms, which is also valid for proteocephalideans.

Regarding host associations, the available data indicate slightly different patterns in host use among species of loaches. Both new species, *P. demshini* and *P. misgurni*, may be strictly specific (oioxenous) to their type and only hosts, *B. toni* and *M. anguillicaudatus*, whereas the two remaining taxa seem to have a broader host specificity. *Proteocephalus midoriensis* has been found in two species of *Lefua* Herzenstein, 1888, provided that tapeworms found by N.I. Demshin in *L. costata* in the Russian Far East are actually conspecific as assumed based on morphological evidence. The widest spectrum of definitive hosts is allegedly seen in *P. sagittus*, which has been reported from species of three genera, namely *Barbatula* Linck, 1790, *Triplophysa* Rendahl, 1933 (both Nemacheilidae) and *Cobitis* Linnaeus, 1758 (Cobitidae). However, conspecificity of tapeworms in the two latter genera has to be confirmed by molecular data.

The fact that both new species of *Proteocephalus* were found in the Russian Far East may appear surprising, considering the fact that researchers of the former Soviet-Union

studied fish parasites intensively, including those in fishes of the River Amur basin (e.g. [43–45]. However, these authors often failed to fix tapeworms adequately (they usually fixed them under pressure, which caused worms’ deformation and artificial changes to their measurements) and thus subtle morphological differences between species could not be recognized reliably. Moreover, these pioneering studies on the Russian Far Eastern cestode fauna predate the use of molecular markers.

The host phylogeny has recently been studied intensively [e.g., 42–44]. These studies have also revealed that the genus *Misgurnus* Lacepède, is polyphyletic and *M. anguillicaudatus* seems to be complex of species [45]. This may explain that *P. misgurni* was found only in the Russian Far East.

The degree of endemism in the River Amur basin is quite high and this is also valid for freshwater fishes and their parasites as was confirmed in trematodes [36]. A recent taxonomic study on caryophyllidean cestodes of *Paracaryophyllaeus* Kulakovskaya, 1961, specific parasites of loaches, has indicated the existence of cryptic species in *M. anguillicaudatus* from eastern Asia (China). According to some ichthyologists and Russian fish parasitologists, the River Amur basin should be considered a separate zoogeographical region or at least as an intermediate region between the Palaearctic and Oriental zoogeographical region [46, 47].

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Figure legends

Fig. 1. Bayesian analysis of the concatenated 4-gene alignment (ssrDNA, lsrDNA, rrnL, cox1) constructed using MrBayes 3.2.1 using the GTR + I + G (ssrDNA, rrnL, cox1) and GTR + I (lsrDNA) models of nucleotide evolution. The analysis was run for 10 million generations; 7 million generations were discarded as burn-in. Posterior probabilities and maximum likelihood bootstrap values (obtained using GARLI) are shown above and below branches, respectively. All nodes with < 0.95 posterior probabilities have been collapsed. The branch-length scale indicates number of substitutions per site.

Fig. 2. *Proteocephalus sagittus* (Grimm, 1872) from *Barbatula barbatula*, Czech Republic (IPCAS C-33/1). **A.** Scolex. **B.** Mature proglottid.

Abbreviations (for all figures): cc = chromophilic cells; ci = cirrus; cp = pars copulatrix vaginae; cs = cirrus-sac; doc = dorsal osmoregulatory canal; du = uterine diverticles; eh = embryonic hooks; em = bilayered embryophore; ga = genital atrium; gc = glandular cells, probably of exocrine type; lm = internal longitudinal musculature; ln = longitudinal lateral nerves; mg = Mehlis' gland; oe = outer envelope; on = oncosphere; oo = oocapt; ov = ovary;

742 sc = subtegumental cells; sd = sperm duct (vas deferens); st = subtegumental muscle fibres; te
743 = testes; tg = tegument; ud = uterine ducts; us = uterine stem; ut = uterus; vc = vaginal canal;
744 vd = vitelloduct; vf = vitelline follicles; voc = ventral osmoregulatory canal; vs = vaginal
745 sphincter.

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748 **Fig. 3.** *Proteocephalus midoriensis* Shimazu, 1990 from *Lefua echigonia*, Japan (IPCAS C-
749 362/1) (A, C–E) and from *Lefua costata*, Russia (“*Proteocephalus* sp. n.” of N.I. Demshin;
750 not accessioned) (B). **A, B.** Scoleces. **C.** Mature proglottid, dorsal view, note vitelline follicles
751 overpassing the ovary ventrally; uterus not illustrated. **D.** Detail of the posterior part of the
752 proglottid, ventral view. **E.** Detail of terminal genitalia, ventral view.

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754 **Fig. 4.** *Proteocephalus demshini* n. sp. from *Barbatula toni*, Russia. **A.** Scolex (host field No.
755 RUS 195; IPCAS C-727). **B.** Mature proglottid, ventral view (host field No. RUS 191; IPCAS
756 C-727); uterus not illustrated. **C.** Detail of terminal genitalia, ventral view (host field No.
757 RUS 192; MHNG-PLAT 79149). **D.** Eggs drawn in distilled water (host field No. RUS 190;
758 IPCAS C-727).

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760 **Fig. 65.** *Proteocephalus misgurni* n. sp. from *Misgurnus anguillicaudatus*. **A.** Holotype, total
761 view, ventrally (host field No. RUS 77; MHNG-PLAT 79136). **B.** Eggs drawn in distilled
762 water (host field No. RUS 93; MHNG-PLAT 79140). **C.** Paratype 1, scolex (host field No.
763 RUS 100; MHNG-PLAT 79142); note the proliferation zone widened anteriorly. **D.** Paratype
764 2, detail of terminal genitalia, dorsal view (host field No. RUS 75; MHNG-PLAT 79135;
765 uterus not illustrated. **E.** Holotype, mature proglottid, ventral view (host field No. RUS 77;
766 MHNG-PLAT 79136); uterus not illustrated.

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Fig. 6. Scanning electron micrograph of *Proteocephalus demshini* n. sp. from *Barbatula toni*, Russia (A, C, D, F) and *Proteocephalus misgurni* n. sp. from *Misgurnus anguillicaudatus*, Russia (B, E, G, H). A, B – dorsoventral view, C – apical view, D – lateral view, E – subapical view, F – acicular filitriches and gladiate spinitriches on internal rim of suckers, G – acicular filitriches between suckers, H – gladiate spinitriches on neck region. Small letters in A and B indicate position of microtriches on F–H).

Fig. 7. Geographical distribution of species of *Proteocephalus* from loaches.

A **B**

Table 1

List of specimens used in the molecular phylogeny. Emboldened GenBank accession numbers indicate sequences generated in the present study.

Species	Collection code	Host species	Country	<i>ssrDNA</i>	<i>lsrDNA</i>	<i>rrnL</i>	<i>cox1</i>
Outgroup							
<i>Glanitaenia osculata</i>	PBI-670 ¹	<i>Silurus glanis</i>	Switzerland	KX768929	KX768937	KX768923	KX768943
<i>Paraproteocephalus parasiluri</i>	PBI-519 ¹	<i>Silurus asotus</i>	Russia	KX768930	KX768938	-	-
<i>Proteocephalus</i>-aggregate							
<i>Proteocephalus torulosus</i>	IPCAS C-32 ²	<i>Squalius cephalus</i>	Czech Republic	AF335511	-	AJ389482	-
<i>Proteocephalus gobiorum</i>	PBI-445 ¹	<i>Apollonia fluviatilis</i>	Ukraine	KX768931	KP729393	KX768924	KX768944
<i>Proteocephalus fluviatilis</i>	PBI-436 ¹	<i>Micropterus dolomieu</i>	Japan	KX768932	KP729390	KX768925	KX768945
<i>Proteocephalus plecoglossi</i>	PBI-395 ¹	<i>Plecoglossus altivelis</i>	Japan	KX768933	AJ388606	KX768926	KX768946
<i>Proteocephalus percae</i>	PBI-650 ¹	<i>Perca fluviatilis</i>	Switzerland	KX768934	AJ388594	KX768927	KX768947
<i>Proteocephalus longicollis</i>	MHNG-PLAT-21681 ³	<i>Coregonus</i> sp.	Switzerland	-	AJ388626	-	-
<i>Proteocephalus tetrastomus</i>	MHNG-PLAT-22429 ³	<i>Hypomeus nipponensis</i>	Japan	-	AJ388635	-	-
<i>Proteocephalus pinguis</i>	IPCAS C-679 ²	<i>Esox lucius</i>	USA	-	KP729395	-	-
<i>Proteocephalus filicollis</i>	MHNG-PLAT-24081 ³	<i>Gasterosteus aculeatus</i>	UK	-	AJ388636	-	-
<i>Proteocephalus macrocephalus</i>	N/A	<i>Anguilla anguilla</i>	Czech Republic	-	AJ388609	-	-
<i>Proteocephalus</i> from loaches							
<i>Proteocephalus sagittus</i>	PBI-437 ¹	<i>Barbatula barbatula</i>	Czech Republic	KX768935	KP729391	-	KX768948
<i>Proteocephalus midoriensis</i>	IPCAS C-362 ²	<i>Lefua echigonia</i>	Japan	AY551130	AJ388610	AJ389481	-
<i>Proteocephalus misgurni</i> n. sp.	PBI-394 ¹	<i>Misgurnus anguillicaudatus</i>	Russia	KX768936	KX768941	KX768928	KX768949
<i>Proteocephalus demshini</i> n. sp.	RUS-191	<i>Barbatula toni</i>	Russia	-	KX768942	-	KX768950

¹ PBI numbers indicate specimens that were sequenced as part of the National Science Foundation Planetary Biodiversity Inventory program 'A survey of tapeworms from vertebrate bowels of the earth' (PBI award Nos. 0818696 and 0818823; see <http://www.tapewormdb.uconn.edu/> for data on molecular specimens); ² Accession numbers of the Helminthological Collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences (<http://www.paru.cas.cz/en/collections/>); ³ Accession numbers of the Parasitic Platyhelminth Collections of the Muséum d'Histoire Naturelle de la Ville de Genève.

Table 2

Comparative measurements of species of *Proteocephalus* (Cestoda: Proteocephalidea) from loaches (Cobitoidea) in the Palaearctic region. Measurements in micrometres unless otherwise stated.

Species	<i>P. sagittus</i> *	<i>P. demshini</i> n. sp.	<i>P. midoriensis</i> **	<i>P. misgurni</i> n. sp.
Authors	(Grimm, 1872)	This paper	Shimazu, 1990	This paper
Hosts	<i>Barbatula barbatula</i> (Linnaeus, 1758)	<i>Barbatula toni</i> (Dybowski, 1869)	<i>Lefua echigonia</i> Jordan & Richardson, 1907	<i>Misgurnus anguilli</i> – <i>caudatus</i> (Cantor, 1842)
Total length	up to 43 mm	18–33 mm (n=5)	9–17 mm	> 33 mm
Width of proglottids (WP)	1.37 mm	1.09 mm (n=5)	370	530
Width of scolex	324–675	420–445 (n=2)	285–310	425–535 (n=4)
Diameter of suckers	122–244	170–190 (n=4)	115–140	195–285 (n=16)
Ratio of width of suckers to width of scolex	32–50%	40–44% (n=8)	45–47%	42–49% (n=16)
Width of neck	240–370	360	215–300	300–470
Number of testes	41–94	62–89 (x=78, n=9)	32–52	32–44 (x=38, n=8)
Length of testes	45–60 × 35–40	45–60 × 45–50 (n=15)	30–60	35–55
Size of cirrus-sac (CS)	100–172 × 53–107	110–145 × 50–60	140–220 × 60–70	80–105 × 60–85
Ratio of length of CS/WP	16–35%	15–25% (x=19, n=14)	20–27%	19–32% (x=25%, n=12)
Ratio of CS length/width	36–41%	37–50% (x = 44%, n=9)	57–85% (x = 70%)	75–93% (x = 83%, n=10)
Length of cirrus/CS length	65–83%	up to 65%	< 50%	< 35%
Relative position of genital pore ¹	33–54% (x=43%)	27–45% (x=33%, n=10)	41–50%	28–41% (x=35%, n=13)
Relative width of ovary ²	72–83%	76–83% (x=79%, n=13)	75–82%	74–88% (x=79%, n=13)
Relative length of ovary ³	23–30% (x=26%, n=12)	26–41% (x=34%, n=26)	27–33% (x=30%, n=20)	24–41% (x=32%, n=20)

Ovary superficie ⁴	13.2%	16.6%	16.5%	14.6%
Part of ovary overlapped by vitelline follicles (VF)	17–21%	0–9%	39–52%	16–20%
Relative size of Mehlis gland ⁵	13–13% (x=14%, n=8)	14–23% (x=17%, n=12)	19–29% (x=23%)	17–25% (x=21%)
Position of vagina to CS	anterodorsal or anterior	anterior (n=84)	anterior	anterior (n=57)
Ratio of width of band of VF to width of proglottid	8–14% (x=10%, n=17)	15–19% (x=17%, n=8)	19–22%	10–12% (n=2)
Ratio of length of poral band of VF to length of proglottid	72–81% (x=74%, n=9)	71–83% (x=77%, n=13)	69–77% (x=74%, n=6)	65–71% (x=68%, n=11)
Ratio of length of aporal band of VF to length of proglottid	69–81% (x=74%, n=8)	68–86% (x=81%, n=13)	68–83% (x=74%, n=6)	67–74% (x=72%, n=11)
Uterine branches	9–14 on each side	3–8 on each side	9–12 on each side	6–11 on each side
Diameter of embryophore	16–27	23–25	26–29	25–29
Diameter of oncosphere	12–20	15–16	21–22	15–17
Length of embryonic hooks	N/A	5–7	8	8–10

*Data from Scholz et al. (2003) supplemented by new measurements; **Data from Shimazu (1990) supplemented by new measurements; ¹Ratio of distance of the genital pore from the anterior margin of the proglottid to its length; ²Ratio of width of the ovary to the width of the proglottid; ³Ratio of the length of the ovary to the length of the proglottid; ⁴Ratio of the surface of the ovary to the surface of the proglottid (see de Chambrier et al., 2012 for method of calculation); ⁵Ratio of the length of Mehlis' gland to the length of the proglottid; N/A – not available. Measurements that differentiate sister species from each other, i.e. *P. demshini* n. sp. from *P. sagittus*, and *P. misgurni* from *P. midoriensis*, respectively, are highlighted by bold.