Diversity and phylogenetic relationships of European species of Crepidostomum Braun, 1900 (Trematoda: Allocreadiidae) based on rDNA, with special reference to Crepidostomum oschmarini Zhokhov & Pugacheva, 1998

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Abstract

Background: Within the genus Crepidostomum Braun, 1900, identification of species and taxonomic decisions made only on the basis of adult morphology have resulted in great problems associated with evaluating actual diversity and validity of species. Life-cycle data, while equal in importance to adult characters, are scarce, controversial or incomplete for most Crepidostomum spp. In this study, rDNA sequences generated from adult and larval Crepidostomum spp. and some other allocreadiid species were analysed to reveal the diversity and phylogenetic relationships of the species and their host range. Detailed morphological description based on light microscopy, SEM tegumental surface topography and genetic data are provided for the poorly known trematode C. oschmarini Zhokhov & Pugacheva, 1998 found in the intestine of two teleost fish species, Barbatula barbatula (L.) and Cottus gobio L.

Results: We characterized 27 isolates of adult and larval parasites. Based on newly obtained 28S and ITS1-5.8S-ITS2 rDNA sequences, new intermediate and final hosts were ascertained, and life-cycles clarified for some allocreadids. New knowledge on the diversity and phylogenetic relationships of European Crepidostomum spp. was gained. The validity of C. oschmarini was verified based on comparative sequence analysis. Ophthalmoxiphidiocercariae of C. oschmarini were recorded in sphaeriid bivalves Pisidium (Euglesa) casertanum (Poli). Additionally, morphological differences between gravid specimens of C. oschmarini and other related species were observed.

Conclusions: Species of the Allocreadiidae parasitizing fishes in Europe are distributed among two monophyletic genera, Allocreadium and Bunodera, and two paraphyletic Crepidostomum clades. A complex of Crepidostomum metoecus (syn. C. nemachilus), C. oschmarini and Crepidostomum sp. 2 clustered in one clade, and a complex of C. farionis, Crepidostomum sp. 1 and, probably, C. wikgreni in the other. Molecular data indicated that C. oschmarini and Crepidostomum sp. 2 presumably have a wide geographical distribution in Europe. The new data provided evidence that Crepidostomum is a more diverse genus than can be judged from morphological data and host switching in this genus may occur independently of fish-host phylogeny.

Keywords: Crepidostomum oschmarini, ITS2 rDNA, 28S, Molecular phylogeny, Life-cycles, Tegumental topography, Morphology, Stone loach Barbatula barbatula, European bullhead Cottus gobio

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Background
Trematodes of the genus *Crepidostomum* Braun, 1900 are common parasites in the intestine of freshwater teleosts in the Holarctic [1, 2]. Including many nominal species, the taxonomy of this genus still lacks clarity and the actual diversity and validity of some species is still questioned. Although most taxonomic decisions have been made based on adult morphology, it should be noted that a number of species are morphologically very similar and there exist only a few morphological features useful for distinguishing species [3, 4]. *Crepidostomum farionis* (Müller, 1784) and *C. metoecus* Braun, 1900 are among the most common and widely distributed freshwater parasites of salmonid fishes in Europe. Occasionally they are also found in *Cottus* spp. (Cottidae), *Barbatula barbatula* (L.) (Nemacheilidae) and some other fishes [5–7]. Reliable features for differentiating these two species were obtained only in the second half of the 20th century (see [7]) and formerly *C. metoecus* was frequently mistaken for *C. farionis*. Additionally, both species have been known by numerous synonyms (see [1, 8]). Prior to the present study, *C. farionis* and *C. metoecus* were the only representatives of the genus to have been recorded in Lithuania and neighboring regions [6, 9].

*Crepidostomum oschmarini* Zhokhov & Pugacheva, 1998 was described from the stone loach *Barbatula barbatula* (as *Nemacheilus barbatulus*) (Nemacheilidae) from the small Sutki River in the upper Volga River basin, Russia [10]. Later, the validity of this species was questioned based on a comparative study of the morphological variability of *Crepidostomum* spp., and *C. oschmarini* was synonymized with *C. metoecus* [11]. However, the definitive hosts of *C. metoecus* (salmonid fishes), have never been found in the Sutki River and the exact taxonomic status of *C. oschmarini* remained unresolved. Here, we re-visit the taxonomic status of *C. oschmarini* based on material from a new host, the European bullhead *Cottus gobio* (Cottidae, Scorpaeniformes), collected in the Il’d River, Russia. The main purpose of this study was to gain new knowledge on the diversity and phylogenetic relationships of European *Crepidostomum* spp. and to determine whether *C. oschmarini* and *C. metoecus* are distinct or synonymous by comparing the 5.8S–ITS2 rDNA cluster and partial 28S rDNA gene sequences, as well as to investigate the phylogenetic relationships of *C. oschmarini* within the Allocreadiidae. In addition to the molecular evidence provided, a detailed morphological study of *C. oschmarini* based on light and scanning electron microscopy (SEM) was accomplished. Despite the small number of studies on SEM morphology of *Crepidostomum* spp. it has been demonstrated that some surface features, such as the distribution of sensory endings, may provide additional specific characters for their identification [1, 7, 12, 13].

Life-cycle data and larval characters are equal in importance to adult characters for resolving some difficulties in taxonomy [1, 14]. Unfortunately, data for cercariae are lacking or incomplete for most ‘recognized’ *Crepidostomum* species [1]. Bivalves rather than gastropods are utilized as first intermediate hosts [2]. Known allocreadiid cercariae belong to the ophthalmoxiphidiocercariae type (i.e. with eye-spots and stylet) and develop in rediae [2]. The development of *Crepidostomum farionis* was elucidated by Brown [15]. Larval stages of *C. metoecus* were studied and described by Stenko [16] in Crimea (River Burulcha). The sphaeriid clam *Pisidium* (*Euglæsa*) *casertanum* (Poli) was recorded as the first intermediate host, while larvae of the ephemeropteran *Ameletus* sp. served as the second intermediate host in experimental infection. Meanwhile, Awachie [17] found that the gastropod *Radix peregra* (O. F. Müller) (as *Lymnæa peregra*) serves as the first intermediate host for *C. metoecus* in North Wales and cercariae encysted in the amphipod *Gammarus pulex* (L.) as the second intermediate host (cercariae of allocreadiids encyst in aquatic arthropods). Due to this discrepancy between the two studies, it is likely that the authors were in fact dealing with different species.

The identification of intramolluscan stages of trematodes using morphological characters alone is difficult given their overall body plasticity and small size in relation to their complexity. The descriptions of cercariae of many related species render them morphologically indistinguishable. With molecular genetic methods having become standard practice for parasite identification, molecular data have become essential for matching different stages of digenean life-cycles. However, not a single life-cycle of a *Crepidostomum* species has been proven by molecular methods and only recently has a molecular study on trematodes in a sub-Arctic lake provided some molecular data on the diversity of developmental stages of some *Crepidostomum* spp. and their hosts in Europe [18].

During a parasite study of sphaerid bivalves collected from different populations in Lithuania, Crimea and Norway, we found clams naturally infected with rediae and ophthalmoxiphidiocercariae consistent with the diagnosis and descriptions for allocreadiid cercariae. In the present study, rDNA markers of larval and adult allocreadiid stages were obtained and compared to known rDNA markers available for allocreadiid trematodes with the aim to clarify life-cycles, host specificity and phylogenetic relationships.

Methods
Adult specimens of *C. oschmarini* were recovered from the intestine of *B. barbatula* and *C. gobio*. The fish hosts were caught in the Il’d River in the upper Volga River basin, Russia. Specimens of a few other adult allocreadiids,
25 adult and gravid specimens of *C. oschmarini* were fixed live in hot 10% buffered formalin. A total of 25 killed fish. For molecular studies, the worms were rinsed in quid CO₂ and mounted on stubs, sputter-coated with gold-palladium and examined using a JEOL JSM 6510LV scanning electron microscope (SEM) operating at 30 kV.

Genomic DNA was extracted from individual ethanol-fixed specimens following the protocol of Stunženas et al. [19] with a slight modification described in Petkevičiūtė et al. [20]. DNA fragments spanning the 3' end of the 5.8S rRNA gene, the complete internal transcribed spacer 2 region (ITS2) and a small section at the 5' end of the 28S gene were amplified using universal primers for flatworms, the forward primer 3S (5'-CGG TGG ATC ACT CGG CTC GTG-3') [21] and the reverse primerITS2.2 (5'-CCT GGT TAG TTT CTT TTC CTC CGC-3') [22]. Using a new primer pair designed for species of the Allocreadiidae, the end of the internal transcribed spacer 1 (ITS1), the complete 5.8S rRNA gene were amplified using universal primers for flatworms, the forward primer AlJe-F (5'-GTC CGG CTC GTG-3') [22] and the reverse primer AlJe-R (5'-CTG AG (AG) GAA ACT TCG-3') [23]. A fragment at the 5' end of the 28S rRNA gene was amplified using the forward primers Digl2 (5'-AAG CAT ATC ACT AAG C-3') or ZX-1 (5'-ACC CGC TGA ATT TAA GCA TAT-3') [23] and the reverse primers L0 (5'-GCT ATC CTT AG (AG) GAA ACT TCG-3') or 1500R (5'-GCT ATC CTT AGG GAA ACT TCG-3') [25, 26]. Amplification protocols were described in Petkevičiūtė et al. [20]. The amplification protocol for the newly designed primers AlJe-F and AlJe-R is identical as for the primer pair Digl2-L0. PCR products were purified and sequenced in both directions at BaseClear B.V. (Leiden, Netherlands) using the PCR primers. Contiguous sequences were assembled using Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, USA). Sequences generated in this study have been deposited in the GenBank database (see accession numbers in Table 1).

Additional rDNA sequences for species of the Allocreadiidae and outgroup taxa (Table 1) were downloaded from GenBank and included in pairwise sequence comparisons and phylogenetic analyses. For phylogenetic analyses, the sequences were aligned using multiple sequence alignment software MAFFT version 7 [27] with iterative refinement method of G-INS-i. The best-fit model of sequence evolution for phylogenetic analysis was estimated using jModeltest v.0.1.1 software [28]. Maximum likelihood (ML) phylogenetic trees were obtained and analyzed using MEGA v.6 [29]. Branch support was estimated by bootstrap analyses with 1000 pseudoreplicates. The ML trees were obtained using the general time reversible model with a gamma distribution rate and a proportion of invariant sites (GTR + G + I) for both the ITS2 and the 28S gene datasets. Gamma shape and the number of invariant sites were estimated from the data. Parsimony analysis based on subtree pruning and regrafting (SPR) was used with default parsimony settings. If two or more sequences belonged to one species, they were collapsed into one branch, except those newly obtained in this study. Estimates of mean evolutionary divergence over sequence pairs within and between groups were calculated using MEGA v.6.

**Results**

**Family Allocreadiidae Looss, 1902**

**Genus Crepidostomum Braun, 1900**

*Crepidostomum oschmarini* Zhokhov & Pugacheva, 1998

**Type-host:** *Barbatula barbatula* (L.) (Cypriniformes: Nemacheiliidae).

**Other host:** *Cottus gobio* L. (Scorpaeniformes: Cottidae).

**Type-locality:** River Sutki, Il’d River (the upper Volga River basin), Russia.

**Site in host:** Intestine.

**First intermediate host:** *Pisidium (Euglesa) casertanum* (Poli) (Veneroida: Sphaeriidae).

**Voucher material:** Four voucher specimens ex *C. gobio* on 2 slides [No. 1/9(6–7)] and 6 voucher specimens ex *B. barbatula* on 2 slides [No. 1/9(10–11)] were deposited in the Parasite Collection of the Institute for Biology of Inland Waters RAS, Russia.
| Species            | Host                      | Locality                        | GenBank ID | Reference |
|--------------------|---------------------------|---------------------------------|------------|-----------|
| Allocreadium sp.a  | Sphaerium corneum         | Ukraine: River Belka, Dnieper River basin | GU462121 [44] | FJ874919 [44] |
| Allocreadium sp.a  | Pisidium amnicum          | Russia: River Tvertsa, upper Volga River basin | FJ874923 [44] |
| Allocreadium gotoi | Misgurnus anguillicaudatus | Japan: Nagano, Iyama, Midori     | LC215274 [61] |
| Allocreadium isoporum | Albunus albumus        | Russia: Lake Oster, Karelia      | GU462125, GU462126 [44] | FJ874921 [44] |
| Allocreadium isoporum | Barbatula barbatula       | Russia: River Tvertsa, upper Volga River basin | MH143102, MH143096 |
| Allocreadium isoporum | Pisidium sp.             | Norway: Lake Sagelvvatn          | MH143105, MH143077 |
| Allocreadium neotenicum | Oreolydes sammarkii       | Norway: Lake Takvatn             | KY513133 [18] |
| Allocreadium neotenicum | Pisidium casertanum       | Ukraine: River Burulcha, Crimea  | MH143103, MH143075 |
| Allocreadium neotenicum | P. casertanum            | Norway: Lake Takvatn             | MH143104, MH143076 |
| Allocreadium neotenicum | Pisidium sp.             | Norway: Lake Nordersjoen         | MH143105 | MH143077 |
| Auriculostoma sp. | Astyanax mexicanus        | Mexico: Filipinas, Veracruz      | KF631425, KF631426 [63] |
| Auriculostoma asyanace | Astyanax aeneus           | Costa Rica: Temquisquito River, Guanacaste | HQ833707 [64] |
| Bunodera sp.       | Bycon guatemalensis       | Mexico: Mangal Lagoon, Tabasco   | KX954172 [51] |
| Bunodera acerinae | Gymnocephalus cernuus     | Russia: Lake Segozero, Karelia   | GU462114 [44] | FJ874914 [44] |
| Bunodera acerinae | P. amnicum                | Russia: River Tvertsa, upper Volga River basin | GU462112, GU462113, GU462122 [44] | FJ874911 [44] |
| Bunodera luciopercae | Perca flavescens          | USA: Steamboat Lake              | HQ833704 [64] |
| Bunodera luciopercae | P. fluviatilis           | Lithuania: Curonian Lagoon       | MH143101, MH143097 |
| Bunodera luciopercae | P. fluviatilis           | Russia: Lake Segozero, Karelia   | GU462115 [44] | FJ874917 [44] |
| Bunodera luciopercae | P. fluviatilis           | Russia: River Tvertsa, upper Volga River basin | GU462123 [44] | FJ874918 [44] |
| Bunodera luciopercae | Sphaerium nivicola        | Lithuania: dammed up River Nemunas near Kaunas | GU462116 [44] | FJ874916 [44] |
| Bunodera luciopercae | S. rivcola                | Ukraine: River Teterive          | GU462111 [44] | FJ874915 [44] |
| Cercariaeum crassum | P. amnicum                | Lithuania: River Ola             | GU462120 [65] | JF261148 [65] |
| Crepidostomum sp. 1a | Sphaerium sp.             | Norway: Lake Takvatn             | KYS13149 [18] |
| Crepidostomum sp. 1a | Siphlonurus lacustris      | Norway: Lake Takvatn             | KY513150 [18] |
| Crepidostomum sp. 1 | Salmo trutta              | Norway: Lake Sagelvvatn          | MH143113, MH143114 |
| Crepidostomum sp. 1a | P. casertanum             | Norway: Lake Sagelvvatn          | MH143111, MH143112 |
| Crepidostomum sp. 1a | P. amnicum                | Lithuania: River Ola             | GU462120 [65] | JF261148 [65] |
| Crepidostomum sp. 1a | Sphaerium nitidum         | Norway: Lake Kykkelvatn          | KYS13149 [18] |
| Crepidostomum sp. 2 | P. casertanum             | Ukraine: River Burulcha, Crimea  | MH143117, MH143118, MH143119 |
| Crepidostomum sp. 2a | P. casertanum             | Norway: Lake Sagelvvatn          | MH143111, MH143115, MH143116 |
| Crepidostomum sp. 2 | S. trutta                 | Norway: Lake Takvatn             | KYS13154 [18] |
| Crepidostomum sp. 2a | S. lacustris              | Norway: Lake Takvatn             | KYS13151 [18] |
Representative DNA sequences: ITS2 rDNA (MH143090-MH143095); 28S rDNA (MH159989-MH159994) (see also Table 1).

Description
[Based on 25 ovigerous worms; Fig. 1, Table 2.] Body elongate-oval, spindle-shaped, only slightly dorsoventrally flattened, with bluntly rounded extremities, 1332–1872 (1561) long, with maximum width at level of ventral sucker, 225–396 (284) (Fig. 1a, b). Body width to body length ratio 1:4.4–8.2 (5.8); forebody 270–369 (320) long; hindbody 909–1458 (1229) long, forebody to hindbody length ratio 1.3–4.5 (1:3.9). Tegument smooth. Eye-spot pigment present in all specimens, usually solid, rarely dispersed. Oral sucker ventro-terminal, 136–222 × 143–210 (180 × 168), provided with 6 muscular lobes arranged in ventro-lateral, dorso-median and dorso-lateral pairs; lobes approximately equal in size and well-separated at their bases. Pre-oral lobe very short, 22–44 (29). Ventral sucker round, scyphoid, semi-embedded, almost equal in size to oral sucker, 163–234 × 156–288 (191 × 197); sucker width ratio
1–1.6 (1.3). Prepharynx short; pharynx muscular, elongate-oval, 55–101 × 68–92 (77 × 76); oesophagus short, 15–44 (26); intestinal bifurcation immediately posterior to pharynx, at approximately mid-way between suckers; caeca long, terminating blindly near posterior extremity of body.

Testes 2, oval or globular, tandem, entire, contiguous or slightly separated; near middle of hindbody. Anterior testis 158–246 × 121–209 (210 × 164), smaller than posterior testis, latter 176–330 × 132–220 (233 × 171). Cirrus-sac elongate, well developed, 242–450 × 48–82 (327 × 66), with anterior end curved ventrally posterior to ventral sucker (Fig. 1c), extends posteriorly from level of intestinal bifurcation to ovarian region (up to posterior margin of ovary); contains coiled seminal vesicle, pars prostatica and ejaculatory duct. Seminal vesicle elongated, variable in size. Pars prostatica rectilinear, near middle of cirrus-sac; prostatic cells sparse, surround pars prostatica and extend throughout anterior half of cirrus-sac. Cirrus tubular, unarmed, 183–198 in length (n = 2). Genital atrium absent. Common genital pore median, between pharynx and ventral sucker, typically at level of intestinal bifurcation.

Ovary subspherical, entire, usually about equidistant between ventral sucker and anterior testis, dextrally or sinistrally submedian or in some specimens median, 92–176 × 99–163 (135 × 126). Proximal female genitalia not clearly observed. Seminal receptacle discerned in some specimens, 46–82 × 44–99 (57 × 66), rounded or somewhat transversely-elongate, immediately posterior to ovary. Uterus short, coiled between anterior testis and ventral sucker, overlapping ovary ventrally, only rarely extending slightly into testicular region; runs ventral to male duct; opens through common genital pore ventrally to male duct. Eggs not numerous, 4–26 (10 on average), operculate, thin-shelled, elongate-oval, 33–70 × 26–37 (59 × 33).

Vitellarium follicular, follicles in 2 lateral fields from level of posterior margin of pharynx to almost posterior extremity of body; fields sparsely confluent dorsally and ventrally in anterior caecal field, confluent dorsally and ventrally in post-testicular region, slightly overlapping testes, but not encroaching laterally between testes; no follicles present dorsal to ovary. Vitelline reservoir large, 55–92 × 57–110 (71 × 82), between ovary and anterior testis. Excretory vesicle tubular, elongate, I-shaped,
reaches anterior margin of anterior testis. Excretory pore subterminal.

Remarks
The specimens of *C. oschmarini* from *B. barbatula* are similar to those from *C. gobio* except in some morphological details that we do not consider to be of taxonomic importance. The worms from *C. gobio* have larger values for body length, body width to length ratio, oral sucker, muscular lobes on the oral sucker, pharynx, ovary, testes and eggs than the worms from *B. barbatula*. *Crepidostomum oschmarini* from *B. barbatula* also differs from the worms from *C. gobio* in the more rounded shape of testes.

Four species of the genus *Crepidostomum* (*C. metoecus, C. farionis, C. latum, C. wikgreni*) have been recorded from freshwater fishes in Europe. Here we do not consider *C. auriculatum* as this species is a specific parasite of sturgeons [1, 5] and appears to be much closer to *Bunodera*

| Table 2 Measurements (in μm) of *Crepidostomum oschmarini* from *Barbatula barbatula* (n = 15) and *Cottus gobio* (n = 10) |
| Host species | *Barbatula barbatula* | *Cottus gobio* |
|--------------|-----------------------|----------------|
| Variable     | Range | Mean | Range | Mean |
| Body length  | 1332–1800 | 1432 | 1476–1872 | 1652 |
| Maximum body width | 264–396 | 309 | 225–300 | 263 |
| Body width/length | 4.4–8.2 | 5.1 | 4.9–8 | 6.4 |
| Forebody length | 270–342 | 305 | 297–369 | 334 |
| Hindbody length | 909–1300 | 1150 | 1170–1458 | 1307 |
| Hindbody/forebody length | 3–4.3 | 3.8 | 3.6–4.5 | 4 |
| Pre-oral lobe length | 22–66 | 44 | 22–44 | 29 |
| Oral sucker length | 136–189 | 156 | 180–222 | 201 |
| Oral sucker width | 143–185 | 154 | 144–210 | 180 |
| Muscular lobes length | 40–46 | 43 | 46–68 | 57 |
| Muscular lobes width | 22–24 | 23 | 22–35 | 29 |
| Pharynx length | 55–90 | 71 | 68–101 | 83 |
| Pharynx width | 68–77 | 73 | 68–92 | 79 |
| Oesophagus length | 26–44 | 33 | 15–26 | 20 |
| Ventral sucker length | 163–222 | 184 | 165–234 | 197 |
| Ventral sucker width | 156–288 | 203 | 165–228 | 192 |
| Ovary length | 92–136 | 113 | 128–176 | 155 |
| Ovary width | 99–139 | 112 | 121–163 | 138 |
| Seminal receptacle length | 55–59 | 57 | 46–82 | 57 |
| Seminal receptacle width | 44–73 | 59 | 55–99 | 74 |
| Vitelline reservoir length | 59–92 | 72 | 55–82 | 70 |
| Vitelline reservoir width | 79–99 | 87 | 57–110 | 76 |
| Anterior testis length | 158–233 | 183 | 216–246 | 235 |
| Anterior testis width | 121–209 | 156 | 130–198 | 170 |
| Posterior testis length | 176–242 | 200 | 222–330 | 262 |
| Posterior testis width | 132–220 | 163 | 132–210 | 178 |
| Seminal vesicle length | 66–209 | 146 | 66–143 | 109 |
| Seminal vesicle width | 44–88 | 70 | 30–66 | 47 |
| Cirrus-sac length | 244–420 | 332 | 242–450 | 322 |
| Cirrus-sac width | 48–82 | 71 | 48–77 | 60 |
| Egg length | 48–68 | 58 | 33–70 | 60 |
| Egg width | 26–35 | 32 | 29–37 | 34 |
| Ventral sucker/oral sucker width | 1–1.6 | 1.3 | 0.9–1.4 | 1.1 |
| Oral sucker/pharynx length | 1.9–2.6 | 2.2 | 2.2–2.8 | 2.5 |
| No. of eggs | 4–26 | 11 | 4–14 | 9 |
Crepidostomum metoecus differs in the larger size of the body and cirrus-sac, smaller hindbody to forebody length ratio of 1:2.2, longer oesophagus, larger number of eggs in uterus, 8–79 (mean 30) [1], and in the position of the genital pore (posterior to intestinal bifurcation) [1, 31]. The ovigerous worms from central Europe lack eye-spot pigment [31–34], the worms from Britain, Japan and USA have small scattered eye-spot pigment [1, 8, 35].

Crepidostomum farionis differs in the much larger size of the body, suckers, pharynx and eggs, and in having a much longer oesophagus and cirrus-sac, smaller forebody to hindbody length ratio of 1:2.5, as well as in the possession of separate genital pores which open anterior to the intestinal bifurcation [1, 36]. The ovigerous worms lack eye-spot pigment in the forebody [33, 34, 37–39], or eye-spot pigment is small and scattered [1, 8, 35, 40]. The uterus in C. farionis often extends into the testicular region (up to middle of anterior testes), the excretory vesicle is Y-shaped and eggs are numerous, up to 230 [34, 37, 38].

Crepidostomum latum Figulewsky, 1931 has a wider body with a much shorter forebody and much larger size of eggs [41]. It differs from C. oschmarini by its shorter S-shaped cirrus-sac not reaching the posterior margin of the ventral sucker; shorter lateral fields of the vitellarium reaching anterior to the posterior margin of the ventral sucker; uterus extending into testicular region and lateral fields around the ventral sucker; testes which are almost equal in size.

Crepidostomum wikgreni is closest to C. oschmarini from which can be distinguished by its larger size of the body, pharynx, ventral sucker, ovary, testes, eggs, and in having a much longer oesophagus and shorter cirrus-sac and a specific microhabitat in the host (gall-bladder). Additionally, the ovigerous worms lack eye-spot pigment, the common genital pore is pre-bifurcal and the number of eggs in uterus is larger than in C. oschmarini (< 50 [42]).

The main morphological difference distinguishing C. oschmarini from the species listed above is the eye-spot pigment in the forebody; this character was clearly present in all specimens. Furthermore, the very short oesophagus differentiates C. oschmarini from its congeners parasitizing salmonid fish hosts.

Tegumental topography of Crepidostomum oschmarini

Under SEM, the ventral and dorsal tegumental surface of C. oschmarini is unarmored and possesses transverse ridges (Figs. 2a and 3c, f). The presence of cobblestone-like protrusions on the body surface was apparent at a higher magnification (Fig. 3g). The anterior extremity of the body bears a ventro-terminal oral sucker provided with six protruding, muscular lobes (Fig. 2a, c, d). These lobes are arranged in three symmetrical pairs, ventro-lateral, dorso-lateral and dorso-median (Fig. 2c, d). The dorso-median and dorso-lateral lobes are approximately equal in size, whereas the ventro-lateral lobes are slightly wider. As they are continuous with the margin of the oral sucker, the anterior region of the ventro-lateral lobes form the anterior part of the oral sucker rim (Fig. 2b–c). There are numerous sensory endings in the form of so-called “papillae and minute sensory receptors” [1, 7, 12] on the oral sucker rim, around the oral sucker rim and along the interlobular field dorsally to the oral sucker (Fig. 2b–d). Five papillate sensory endings (c. 6.5 μm in diameter) occur evenly spaced, about 40 μm apart from each other, and consistently associated with the posterior portion of the oral sucker rim (Fig. 2b, c). Underneath the ventro-lateral papilla of the oral sucker, groups of 8 papillae are visible on either side of the rim (Fig. 2c, f). In all of the specimens studied, there is a constant pattern of 3 pairs of symmetrical papillae, which vary in size, located at the middle of the anterior rim of the oral sucker (Fig. 2b–d, e). On each side of this symmetrical arrangement, the posteriormost papilla (c. 7 μm in diameter) is situated about 5 μm from the middle papilla (c. 4.5 μm in diameter), which, in turn, is situated about 4 μm from the anteriormost papilla (c. 3.5 μm in diameter) (Fig. 2e). The distance between posteriormost papillae on each side is 21 μm, between the middle papillae 32 μm and between anteriormost papillae 17 μm (Fig. 2e). An additional papilla (c. 4.5 μm in diameter) is situated on the surface of each vento-lateral lobe (Fig. 2d, g). Two kinds of sensory endings, papillate and non-papillate, are scattered irregularly on the interlobular field of the body (Fig. 2c, d). A group of non-papillate sensory endings occurs close to the 3 pairs of symmetrical papillae (Fig. 2d). Variability exists in the numbers and arrangement of additional papillae in this region (Fig. 2c, d). Not far from the margins of the dorso-lateral lobes, groups of 3–5 papillae are present (Fig. 2d, h); furthermore, between the ventro-lateral and dorso-lateral lobes, 6 papillae and 3–5 non-papillate sensory endings are apparent (Fig. 2d). Non-papillate sensory endings are also present close to the margins of the dorso-median lobes. All of the sensory endings (papillate and non-papillate) on the anterior body surface are ciliate receptors (Fig. 2i, j).

The ventral sucker is protruded ventrally. Its rim exhibits surface corrugations arranged radially (Fig. 3d) and bears six large dome-shaped papillae (c. 5.5 μm in diameter) regularly distributed around the rim (Fig. 3c–e).

The common genital pore is median between the two suckers (Fig. 5a, c); no tegumental papillae were observed around the genital pore (Fig. 3b–d). The tegument around the genital pore may form a weak elevation at the base of the everted cirrus (Fig. 3b, c). The surface of the cirrus is smooth (Fig. 3d). Everted cirrus was observed, measuring between 65–160 μm in length at different degrees of evagination (Fig. 3a, c, d).
Papillae are present on the forebody surface but are more abundant ventrally; they exhibit a tendency for bilateral symmetry (Fig. 3a, b). A gathering of ciliated and dome-shaped papillate sensory endings, arranged in two longitudinal, symmetrical rows, occurs on the ventral surface between the oral and ventral suckers (Fig. 3b). There are also a few papillae present in ventro-lateral and dorso-lateral areas of the forebody (Fig. 3a). The hindbody lacks papillae (Figs. 2a, 3f). Situated at the posterior extremity of the body is the excretory pore (Fig. 3f).

Fig. 2 SEM micrographs of the surface topography of the anterior region of the body of Crepidostomum oschmarini. a Ventral view of mature worm. b The constant pattern of 5 papillae on the posterior rim of the oral sucker, 3 symmetrical pairs of papillae in the middle of the anterior rim (white circle) and a group of 8 papillae underneath the sucker rim (black circle). c 3 paired symmetrical papillae (white circle) and the distribution of irregular papillae on the anterior rim. d Interlobular field with marked (white circle) of 6 symmetrical papillae, irregular papillate and non-papillate sensory endings and single papilla on each ventro-lateral lobe (black circles). e Regular pattern of different sizes of the posteriormost, middle and anteriormost pairs of 6 symmetrical papillae on anterior rim. f Regular arrangement of 8 papillae ventro-lateral to the posterior sucker rim (black circle). g Single papilla (black circle) on the surface of a ventro-lateral lobe. h Papillae close to the base of a dorso-lateral lobe. i Ciliated papillate and non-papillate sensory endings on interlobular field. j Ciliated papilla. Abbreviations: ap, anteriormost papilla; c, cilium; cr, cirrus; dll, dorso-lateral lobe; dml, dorso-median lobe; fp, forebody papillae; hb, hindbody; ep, excretory pore; l, lobe; mp, middle papilla; np, non-papillate sensory ending; oa, oral aperture; os, oral sucker; p, ciliated papilla; pp, posteriormost papilla; pr, posterior rim of oral sucker; tr, transverse tegumental ridges; vll, ventro-lateral lobe; vs, ventral sucker.
Molecular differentiation and phylogenetic analysis

Two genetically different *Crepidotostomum* cercariae were collected from *P. casertanum* in Lake Sagelvøt in Norway, corresponding to *Crepidotostomum* sp. 1 and *Crepidotostomum* sp. 2 sensu Soldánová et al. (2017) [18] recorded in Lake Takvatn, Norway. One of these isolates was genetically identical to the metacercariae of *Crepidotostomum* sp. 2 ex mayfly *Siphlonurus lacustris* and to adult ex *Salmo trutta* [18]. In Crimea, *Crepidotostomum* sp. cercariae were found in *P. casertanum* collected in the River Burulcha. This cercaria has been described previously as the larva of *C. metoecus* [16], although the divergence between these two species ranged between 0.6–0.7% (7–8 bp) in the alignment of the 28S gene. The divergence between the Crimean *Crepidotostomum* sp. and the sub-Arctic *Crepidotostomum* sp. 2 can be regarded as intraspecific, with 2–3 bp (0.2–0.3%) difference in the 28S and only 1 bp (0.16%) in the 5.8S-ITS2.

**Fig. 3** SEM micrographs of the surface topography of the forebody and hindbody of *Crepidotostomum oschmarini*. a Two symmetrical longitudinal fields of papillae (white circles), the protruded cirrus and the ventral sucker on the forebody. b Ciliated and dome-shaped sensory endings in the two longitudinal fields of papillae. c Ventral sucker with 6 dome-shaped papillae on its rim (white circles). d Radially arranged surface corrugations on the rim of the ventral sucker and the dome-shaped papillae. e Dome-shaped papilla (white circle) on the ventral sucker rim. f Posterior extremity of the body with the terminal excretory pore. g Cobblestone-like protrusions of the body surface. Abbreviations: cp, cobblestone-like protrusion; cr, cirrus; dp, dome-shaped papilla; ep, excretory pore; lp, lateral papilla; p, ciliated papilla; pe, elevation around genital pore; tr, transverse tegumental ridges; vs, ventral sucker.
Adult Crepidostomum specimens obtained from S. trutta in Lake Sagelvatn were genetically identical to samples of cercariae from P. casertanum and Pisidium sp. collected in this lake and from Sphaerium nitidum in Lake Kykkelvatn. The sequences of the 28S rRNA gene obtained from all of these samples were identical to Crepidostomum sp. 1 samples collected from Sphaerium sp. and S. lacustris in Lake Takvatn [18]. Intraspecific variation was detected in Lake Sagelvatn, but only a single nucleotide in the ITS2 was different in one specimen from S. trutta and in one isolate from Pisidium sp.; also this site was heterozygotic in one sample from P. casertanum, obviously as a result of hybridization of two genetically different lineages.

The newly generated rDNA sequences of C. oschmarini sampled from B. barbatula and C. gobio in Russia, and from P. casertanum in Lithuania were identical. The 28S sequences were aligned with those of closely related species in an alignment of 1150 bp. The divergence between C. oschmarini and C. metoecus was 8 and 9 bp (0.7–0.8%), the divergence between C. oschmarini and the sub-Arctic Crepidostomum sp. 2 was 11 and 12 bp (0.96–1%). However, C. oschmarini was less different from the Crimean Crepidostomum sp. 2 (9 bp, 0.8%).

Some specimens of P. casertanum, collected in the River Burulcha and in Lake Takvatn, Norway, as well as Pisidium sp. from Lake Nordersjoen, were infected with Allocreadium neotenicum. All samples were genetically identical. In a 28S alignment of 1150 bp, no nucleotide differences were detected between our samples and A. neotenicum collected from the dytiscid beetles Oerodytes sannmarkii and Hydropterus rufifrons in the sub-Arctic Lake Takvatn and the Lake District in Cumbria, UK, respectively [18, 43].

New rDNA sequences were obtained for A. isoporum, collected from B. barbatula in the upper Volga River basin, Russia. These sequences were identical to rDNA of A. isoporum collected from Alburnus alburnus in Lake Oster, Karelia, Russia [44], with one difference in a single nucleotide of the ITS2.

New rDNA sequences were obtained for B. luciopercae, collected from Perca fluviatilis in the Curonian Lagoon, Lithuania. The divergence between sequences from this specimen and sequences of B. luciopercae specimens from a previous study [44] was 2 and 3 bp in a 28S alignment of 1067 bp and only 1 bp in the ITS2 alignment of 394 bp.

The newly obtained sequences and relevant allocreadiid sequences of ITS2 rDNA and partial 28S rDNA from the GenBank database were used for phylogenetic analysis. Alignment of the ITS2 and partial 28S data yielded 394 and 1050 characters for analysis, respectively.

Phylogenetic analyses of the ITS2 and 28S datasets produced several strongly supported clades and some weakly or not supported clades in both phylogenetic trees (Figs. 4, 5). Adult C. oschmarini from two fish species, B. barbatula and C. gobio, and allocreadiid cercariae from the sphaeriid bivalve P. casertanum, formed a strongly supported monophyletic subclade (Figs. 4, 5). Sub-Arctic Crepidostomum sp. 2 together with Crimean Crepidostomum sp. formed the other subclade. These two subclades nested in to a well-supported monophyletic clade; in the 28S tree C. metoecus is included into this clade. Crepidostomum sp. 1 formed the other monophyletic clade (Fig. 5); in the 28S tree, together with C. farionis (Fig. 4). This clade was nested as sister to the clade formed by C. oschmarini + Crepidostomum sp. 2 + C. metoecus but the relationship was not supported (Fig. 4). Unfortunately, ITS2 data for C. metoecus and for C. farionis are not yet available. Nearctic Crepidostomum species nested into a separate monophyletic clade together with species from the allocreadiid genera Auriculostoma and Creptotrema in the two trees and, additionally, with Cryptotrema and Paracryptotremaoides in the 28S tree. Species of Allocreadium, as well as species of Bunodera, nested into two separate monophyletic clades (Figs. 4, 5). However, the relationships among all these clades showed some differences in the different trees. In the 28S rDNA tree, all these clades and the branch of Acrolichanus auriculatum were united into one strongly supported main clade, but did not form supported higher-level clades inside the main clade. The main difference between the ITS2 and 28S rDNA trees was in the relationships of Crepidostomum species. In the ITS2 tree, the strongly supported clade of Nearctic Crepidostomum spp. and the clade of C. oschmarini-Crepidostomum sp. 2 nested into a well-supported higher-level clade (Fig. 5), while in the 28S tree (Fig. 4), the clade of Nearctic Crepidostomum spp. was not strongly supported and its relationships with other clades of Crepidostomum was not supported.

**Discussion**

The results of this study shed new light on the diversity of trematodes of the genus Crepidostomum in Europe. The existing genetic data was based on analysis of partial sequences of the 28S rRNA gene of Crepidostomum species that use salmonid fishes as final hosts [18]. Crepidostomum oschmarini was found from two sympatric but phylogenetically distant fish host species, B. barbatula (Cypriniformes) and C. gobio (Scorpaeniformes). Although host switching is probably easier among related host species [45], host switches between unrelated hosts can also take place [46–48]. While B. barbatula and C. gobio are phylogenetically distant, their feeding habitats and food preferences are similar. Predominant preys are small benthic arthropods (insect larvae, i.e. ephemeropterans, plecopterans, trichopterans and crustaceans [49,
It is likely that a feeding overlap produced by food items that are involved in the life-cycle of the parasite, resulted in the infection of both fish with the same Crepidostomum species.

Comparative sequence analysis in this study confirmed the link between the redial and cercarial isolates ex P. casertanum from the River Nedzingė, Lithuania, and the adult stages of C. oschmarini parasitizing B. barbatula and C. gobio from the River Il’d, Russia. No intraspecific variation was detected between these isolates, despite the considerable geographical distance (~ 1000 km) between the Lithuanian and Russian populations.

The level of differences found between the partial 28S rDNA sequences of C. oschmarini and C. metoecus (0.6–0.7%) clearly demonstrates that these two forms are different species. The observed level of differences is similar to the levels of interspecific variability reported for allocreadiid digeneans [4, 18, 30, 44]. It is notable that occasionally relatively low interspecific genetic divergence can be discovered in related allocreadiid species; for instance, only 0.29% divergence was found between Auriculostoma lobata Hernández-Mena, Lynggaard, Mendoza-Garfias & Pérez-Ponce de León, 2016 and its sister species A. astyanace Scholz, Aguirre-Macedo & Choudhury, 2004 (Allocreadiidae) [51]. Despite the conservative nature of the 28S rDNA gene region, it segregates well-supported subclades of C. metoecus, Crepidostomum sp. 2 and C. oschmarini, within a single clade (Fig. 4).

Recently, the existence of cryptic species was uncovered among Crepidostomum spp. infecting salmonid fishes and different first and second intermediate hosts in the sub-Arctic Lake Takvatn [18]. Hence, molecular results evidenced that species diversity in
Crepidostomum is underestimated. Molecular data obtained from analysis of 28S rDNA partial sequences disclosed two pairs of genetically closely related species, i.e. C. farionis - Crepidostomum sp. 1, and C. metoecus-Crepidostomum sp. 2. Considering that there are only five nominal European species included into the Fauna Europaea database [52], i.e. C. auriculatum Wedd, 1858, C. farionis, C. latum, C. metoecus and C. wikgreni Gibson & Valtonen, 1988, the finding was surprising and shows that diversity in this genus and in this otherwise depauperate freshwater ecosystem is higher than was presumed. Crepidostomum farionis and C. metoecus were known as the only two species of this genus parasitizing salmonids in Europe [5, 34]. Crepidostomum wikgreni described from the gall-bladder and intestine of the whitefish Coregonus acronius (Salmonidae) in Lake Yli-Kitka, northeast Finland, has never been recorded elsewhere and is regarded as an endemic form [42]. No sequence data are available for this species and it could not be included in molecular phylogenies. The gross morphology of C. wikgreni appeared very similar to C. farionis and it was suggested that C. wikgreni has probably evolved from C. farionis after deglaciation and since c.8400 BP when the waters of the Kitka Lake system were isolated [42]. These two species presumably represent closely related sister taxa.

Crepidostomum nemachilus was originally described from Nemachilus barbatulus toni (Nemacheilidae) on Sakhalin Island in the Russian Far East [53]. The genetic identity of C. nemachilus and C. metoecus was revealed in comparative analysis of 28S sequences, but distinctions observed in morphology of the two species were regarded as an argument to refrain from deciding upon synonymy of the two taxa [30]. However, recent morphological reexamination of C. nemachilus from the type-host Barbatula toni (Dybowskii) (syn. Nemachilus toni) showed that it is consistent with the specimens of C. metoecus including those found in B. toni in every essential feature [8]. Thus, both molecular and morphological findings demonstrated that C. nemachilus is a synonym of C. metoecus.

Acrolichanus auriculatum (syn. Crepidostomum auriculatum), a parasite of sturgeons, comprise a separate branch in the molecular 28S rDNA phylogenetic tree, distantly related to other Crepidostomum spp. [18, 30].

**Fig. 5** Phylogenetic tree based on maximum likelihood analysis of the ITS2 nuclear rDNA region. Bootstrap support values lower than 70% are not shown. GenBank accession numbers of sequences in collapsed clades are provided in Table 1. The species sequenced in this study are indicated in bold.
Wedl [54] described this species as *Distoma auriculatum*. Since then, it has undergone many taxonomic revisions (see [1, 55]). Skwartzoff [56] conducted a comprehensive morphological analysis of the species based on a large amount of material collected from *Acipenser ruthenus* from the Volga River and Oka River, concluding that they should be assigned to the genus *Acrolichaenus* Ward, 1917. Thus, there have been opposing opinions on the validity of *Acrolichaenus*. Some authors, along with Hopkins [57], are of the opinion that this taxon is insufficiently distinct from *Crepidostomum* and must be placed within the latter genus. However, molecular data support the opinion of Skryabin & Koval [55] and Bykhovskaya-Pavlovskaya & Kulakova [5] that *A. auriculatum* is distinct enough from samples of the other Palaearctic and Nearctic *Crepidostomum* spp. to be assigned to another genus. In 28S rDNA based phylogenies *A. auriculatum* appears to be much closer to *Bunodera* spp. than to *Crepidostomum* spp.

*Crepidostomum latum* is a little-known species described by Pigulewsky [41] based on only two specimens from the intestine of the rudd, *Scardinius erythrophthalmus* (L.) (Cyprinidae), in the River Sozh (in the upper course of the River Dnepr, Ukraine). The species has not been encountered since and its validity is questionable [5].

The 28S rDNA based phylogenetic tree generated here agrees in general topology with recently published estimates of phylogeny for the Allocreadiidae [18, 58]. In these studies, the species of the genera *Allocreadium* and *Bunodera* formed two monophyletic clades. The different situation concerns the genus *Crepidostomum*. In the present analyses *C. oschmarini, C. metoecus* (syn. *C. nemachilus* Krotov, 1959) and *Crepidostomum* sp. 2 represented closely related sister taxa in the 28S rDNA-based phylogeny. We refer to this clade as the *C. metoecus* complex. The second clade, including European *Crepidostomum* isolates was comprised of *C. farionis* and *Crepidostomum* sp. 1. Our sequences for isolates sampled from *Sphaerium nitidum, Pisidium* sp., *P. casertanum* and *S. trutta* from Norway matched the sequences of *Crepidostomum* sp. 1 of Soldánová et al. [18]. However, a monophyletic origin of the two *Crepidostomum* clades is not supported in the 28S rDNA-based phylogeny. DNA sequences, unconstrained by function, as the internal transcribed spacer 2 (ITS2), usually experience higher rates of genetic change than encoding regions, as the 28S. In the ITS2-based phylogeny, the *Crepidostomum* sp. 1 clade was distant from all other allocreadiid clades, but the *C. metoecus* complex and Nearctic *Crepidostomum* clade formed well-supported higher-level clade (Fig. 5). It is interesting that the Nearctic *Crepidostomum* clade combines some species of the other allocreadiid genera, i.e. *Auriculostoma* and *Creptotrematina* in the ITS2 tree, and even more genera comprise this clade in the 28S tree. The results of the phylogenetic analyses led us to a presumption that at least two groups of *Crepidostomum* species are paraphyletic. There are more cases known when molecular analysis using the 28S rDNA gene revealed paralogy in a group of allocreacid trematodes, conventionally regarded as a monophyletic assemblage. Hence, recently the genus *Paracreptotrema* Choudhury, Pérez-Ponce de León, Brooks & Daverdin, 2006 was shown to be a paraphyletic and two new genera were erected to accommodate the taxonomy with the results of molecular phylogeny [58].

The sequences of isolates of the intramolluscan stages from *P. casertanum* collected in the River Burulcha, Crimea, formed a robustly supported subclade from metacercarial and adult isolates of *Crepidostomum* sp. 2 sensu Soldánová et al. (2017) [18] from a sub-Arctic lake in Norway. This finding did not match our expectations, as the upper stream of the River Burulcha, Crimea, is the type-locality for the cercarial material used by Stenko [16] for experimental life-cycle studies on *C. metoecus*. Notably, the second intermediate hosts recorded in the study of Stenko [16] were nymphs of the mayfly (Ephemeroptera) and stonefly (Plecoptera). Nymphs of these insects were found infected with metacercariae of *Crepidostomum* sp. 2 in the molecular study of Soldánová et al. [18]. On the other hand, amphipods *Gammarus lacustris* were found to be the second intermediate host of *C. metoecus* in Lake Takvatn in Norway [18]. Based on these facts, we can assume that Stenko [16] was dealing with *Crepidostomum* sp. 2. Adult specimens of *Crepidostomum* sp. 2 were recorded in brown trout, *S. trutta*, in the molecular study of Soldánová et al. [18], and Stenko [16] noted that *S. trutta fario* was naturally infected with “*C. metoecus*” in the River Burulcha. Speciation patterns of parasites may be directly associated with their hosts, though in the case of parasites with complex life-cycles it is often less clear which host may have the most influence on parasite speciation. The life-cycle peculiarities of two closely related species, *C. metoecus* and *Crepidostomum* sp. 2, would suggest that the speciation was driven by factors associated with the second intermediate hosts, phylogenetically distant arthropods, while the first intermediate and definitive hosts are shared between these two trematode species. However, a more accurate knowledge of life-cycles is necessary to explain the pattern of cryptic diversity observed in *Crepidostomum* spp.

The molecular segregation of *C. oschmarini* and *C. metoecus* prompted us to compare these worms using SEM and to try to identify diagnostic morphological features for the species. The present SEM study of the surface morphology of *C. oschmarini* revealed both common
and specific patterns in the number and arrangement of tegumental papillae as compared with other similarly studied species of *Crepidostomum* [1, 7, 12, 13].

The five large papillae detected on the posterior rim of the oral sucker of *C. oschmarini* are common to all of the *Crepidostomum* species examined to date and can be seen in SEM photos of *C. metoe cus*, *C. farionis*, *C. illinoense* Faust, 1918, *C. ictaluri* (Surber, 1928) and *C. cooperi* Hopkins, 1931, published by Caira [1], as well as in *C. farionis* and *C. metoe cus* examined by Moravec [7] and Žďárská & Nebesárová [13] and also in *C. opeongoensis* Caira, 1985 studied by Choudhury & Nelson [12]. Five characteristic larger papillae are also visible on the posterior rim of the oral sucker in other allocreadiid species, e.g. *Bunodera sacculata* Van Cleave & Mueller, 1932 and *B. mediowitella* Tsimbaliuk & Roitman, 1966 studied by Caira [1].

The presence of six dome-shaped papillae on the rim of the ventral sucker revealed in *C. oschmarini* has also been observed in *C. metoe cus* by Moravec [7] and in *C. opeongoensis* by Choudhury & Nelson [12]. However, no papillae were observed on the ventral sucker in *C. farionis* [7].

A consistent pattern in the sensory papillae arrangement was found to occur in the anterior body region and ventral forebody surface of all specimens of *C. oschmarini*. First, there are three paired, symmetrically distributed, differently-sized papillae situated in the centre of the anterior rim of the oral sucker. Such a pattern has not been reported for any of the other *Crepidostomum* species studied to date by SEM [1, 7, 12, 13]. In *C. metoe cus*, a species most closely related to *C. oschmarini*, only two pairs of symmetrically arranged papillae are visible in the illustrations of Moravec [7] and Žďárská & Nebesárová [13].

Secondly, a local concentration of eight papillae located ventro-laterally underneath the rim of the oral sucker is characteristic for *C. oschmarini*. It is worth noting, however, that the presence of a similar but smaller group of papillae can be seen in published SEM photos of some previously studied species, i.e. three papillae in *C. metoe cus* and five papillae in *C. cooperi* [1].

Thirdly, a single large papilla is associated with each ventro-lateral lobe in *C. oschmarini*. In contrast, the presence of two papillae on the surface of each ventro-lateral lobe is apparent in the SEM photos of *C. metoe cus* presented by Moravec [7].

Fourthly, the arrangement of ciliated and non-ciliated sensory endings in two longitudinal symmetrical rows on the ventro-median surface of the forebody was revealed in *C. oschmarini*. Judging from the available SEM data on the papillae distribution in *Crepidostomum* spp., two fields of “tegumental bosses” (non-ciliated sensory endings) are situated laterally along the forebody in *C. metoe cus* [7] and, there are four pairs of papillae on the ventral forebody in *C. opeongoensis* [12].

The present SEM study clearly demonstrates the distinction between two sister taxa, *C. oschmarini* and *C. metoe cus*, which have been shown, using our molecular data, to be closely related. This and previous SEM studies on the surface topography of species of *Crepidostomum* suggest that the arrangement of the sensory endings of adult specimens exhibit interspecific differences which represent useful additional taxonomic criteria for understanding this genus.

**Conclusions**

According to available data, we suggest that two complexes of *Crepidostomum* species parasitize freshwater fishes in Europe. The *Crepidostomum metoe cus* complex consists of *C. metoe cus* (syn. *C. nemachilus*), *C. oschmarini* and *Crepidostomum* sp. 2, while the *C. farionis* complex includes *C. farionis*, *Crepidostomum* sp. 1 and, probably, *C. wikgreni*. Morphological and molecular evidence together indicated the validity of *C. oschmarini* and provided clear criteria for its separation from *C. metoe cus* and other congeneric species. The phylogenetic study supported that some *Crepidostomum* species are euryxenous, so host switching in this genus may occur independently of fish-host phylogeny. Our phylogenetic analyses confirm the prediction that there are large numbers of cryptic parasite species to be discovered [59] and reinforce the idea that trematodes are a much more diverse group than is judged from morphological data and once again confirm the observation that studies based on comparison of nuclear DNA markers are more likely to uncover cryptic species among trematodes than other groups of helminths [60]. This study demonstrates the value of steadily adding relevant parasitological and sequence data to a growing database for allocreadiids as well as for any other group of trematodes. No matter which life-cycle stage has been obtained or from what hosts and geographical localities, from such specimens we will gain the framework needed to connect and clarify life-cycles and gain a more complete understanding of the existing diversity, host specificity and ecology of trematodes under consideration.

**Abbreviations**

GTR + G + I: Gamma distribution of rates and a proportion of invariant sites; ITS2: Internal transcribed spacer 2; ML: Maximum likelihood; SEM: Scanning electron microscopy; SPR: Subtree pruning and regrafting

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**Availability of data and materials**
Nucleotide sequences obtained in the present study have been deposited into the GenBank database under the accession numbers MH143101-MH143119, MH159989-MH159994 (2BS) and MH143075-MH143100 (ITS2).

**Authors’ contributions**
RP designed the study, RP, VS and GS performed the field and laboratory research and analyzed data. AEZ conducted field collections and carried out morphological research. LGP performed micromorphological research. VS and GS extracted DNA for PGR and sequencing. Molecular analyses were carried out by VS. All authors actively contributed to the interpretation of the findings and development of the final manuscript. All authors read and approved the final manuscript.

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**References**

1. Caia JN. A revision of the North American papillose Allocreadiidae (Digenea) with independent cladistics analyses of larval and adult forms. Bull Univ Nebr State Mus. 1989;91:1–58.
2. Caia JN, Bogue T. Family Allocreadiidae Loos, 1902. In: Jones A, Gibson DI, Bray RA, editors. Keys to the Trematoda, Volume 2. Wallingford: CAB International Publishing and the Natural History Museum, 2005. p. 417–36.
3. Gibson DI. Trematoda. In: Margolis L, Kabata Z, editors. Guide to the Parasites of Fishes of Canada, part IV. Canadian Special Publication of Fisheries and Aquatic Sciences, Vol. 124. Ottawa: NRC Research Press, 1996.
4. Tkach W, Curran SS, Bell JA. Overstreet RM. A new species of Crepidostomum (Digenea: Allocreadiidae) from Hiodon tergisus in Mississippi and molecular comparison with three congeners. J Parasitol. 2013;99:1114–20.
5. Bykhoivskaya-Pavlovskaya IE, Kulakova AP. Trematoda. In: Bauer ON, editor. Maps and institutional affiliations. Canadian Special Publication of Fisheries and Aquatic Sciences, Vol. 124. Ottawa: NRC Research Press, 1996.
6. Caia JN, Bogue T. Family Allocreadiidae Loos, 1902. In: Jones A, Gibson DI, Bray RA, editors. Keys to the Trematoda, Volume 2. Wallingford: CAB International Publishing and the Natural History Museum, 2005. p. 417–36.
7. Gibson DI. Trematoda. In: Margolis L, Kabata Z, editors. Guide to the Parasites of Fishes of Canada, part IV. Canadian Special Publication of Fisheries and Aquatic Sciences, Vol. 124. Ottawa: NRC Research Press, 1996.
8. Tkach W, Curran SS, Bell JA, Overstreet RM. A new species of Crepidostomum (Digenea: Allocreadiidae) from Hiodon tergisus in Mississippi and molecular comparison with three congeners. J Parasitol. 2013;99:1114–20.
9. Bykhoivskaya-Pavlovskaya IE, Kulakova AP. Trematoda. In: Bauer ON, editor. Maps and institutional affiliations. Canadian Special Publication of Fisheries and Aquatic Sciences, Vol. 124. Ottawa: NRC Research Press, 1996.
10. Zhihkov AE, Pugacheva MN. Crepidostomum oscharini sp. n. (Trematoda, Bunoderae) of Nemacheilus barbatulus (Cobitidae) from the upper Volga River basin. Zool Zh. 1998;77:1239–31. (In Russian).
11. Sokolov SG. Fish parasites of the Upper Volga basin (taxonomic and ecological diversity and zoogeography). Extended abstract of Cand Sci (Biol) dissertation. Moscow: INPA RAN; 2000.
12. Choudhury A, Nelson PA. Redescription of Crepidostomum opeongensis Caia, 1985 (Trematoda, Allocreadiidae) from fish hosts Hiodon alisoniides and Hiodon tergisus (Osteichthyes: Hiodontidae). J Parasitol. 2000;86:1305–12.
13. Zďaršák Z, Nebesťárová J. Transmission electron microscopy of presumed sensory receptors of the forebody papillae of Crepidostomum metoecus (Digenea: Allocreadiidae). Folia Parasitol. 2004;51:127–32.
14. Cannon UB. Life cycles of Bunodera sacculata and B. luciopecora (Trematoda: Allocreadiidae) in Algonquin Park, Ontario. Can J Zool. 1971;49:1417–29.
15. Brown FJ. On Crepidostomum farionis O.F. Mull. (= Stephanophiala laeuenta Zeder), a diestome parasite of the trout and grayling. I. The life history. Parasitology. 1927;1986–99.
16. Stenko RP. On the life cycle of Crepidostomum metoecus (Trematoda: Bunoderae). In: Polyansky J, editor. Parasites and Parasitosis of Man and Animals. Kiev: Naukova Dumka, 1982. p. 198–204. (In Russian).
17. Awachie JBE. On the bionomics of Crepidostomum metoecus (Braun, 1900) and Crepidostomum farionis (Müller, 1764) (Trematoda: Allocreadiidae). Parasitology. 1968;55:307–24.
18. Boldanová M, Georgievna S, Roháčová J, Knudsen R, Kuhn JA, Hennekken E, et al. Molecular analyses reveal high species diversity of trematodes in a sub-Arctic lake. Int J Parasitol. 2017;47:327–45.
19. Stunčiūtė V, Petkevičiūtė R, Stanevičiūtė G. Phylogeny of Spharum solidum (Bivalvia) based on karyotype and sequences of 16S and ITS1 rDNA. Cent Eur J Biol. 2011;6:105–17.
20. Petkevičiūtė R, Stunčiūtė V, Stanevičiūtė G. Differentiation of European freshwater bucephalids (Digenaea: Bucephalidae) based on karyotypes and DNA sequences. Syst Parasitol. 2011;80:199–212.
21. Bowles J, Blair D, McManus DP. A molecular phylogeny of the human schistosomes. Mol Phylogenet Evol. 1995;4:103–9.
22. Cribb TH, Anderson GR, Adlard RD, Bray RA. A DNA-based demonstration of a three-host life-cycle for the Bivasculariae (Platyhelminthes: Digenea). Int J Parasitol. 1998;28:1791–5.
23. Scholz T, De Chambrer J, Kuchta R, Littlewood DTJ, Waeschenbach A. Macrobothriotenia fca (Cestoda: Proteocephalidea), a parasite of sunbeam snake (Xenopeltis unicolor); example of convergent evolution. ZooTaxa. 2013; 3640:485–99.
24. Tkach V, Graba-Kuzubskaya B, Pawlowski J, Swiderski Z. Molecular and morphological evidence for close phylogeographic affinities of the genera Macroderina, Leptophallus, Mesocephalophallus, and Parapodopema (Digenea, Plagiorchiidae). Acta Parasitol. 1999;44:170–9.
25. Olson PD, Cribb TH, Tkach W, Bray RA, Littlewood DTJ. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). Int J Parasitol. 2003;33:733–55.
26. Tkach V, Littlewood DTJ, Olson PD, Kinsella MJ, Swiderski Z. Molecular phylogenetic analysis of the Microphalloidea Morozov, 1955 (Trematoda: Digenea): a bigger data set resolves fewer genera. Syst Parasitol. 2003;56:1–15.
27. Katch K, Standley DM. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in performance and usability. Mol Biol Evol. 2013; 30:722–80.
28. Posada D. ModelTest: phylogenetic modelling averaging. Mol Biol Evol. 2008;25:1253–6.
29. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–9.
30. Atopkin DM, Shedko MB. Genetic characterization of far eastern species of the genus Crepidostomum (Trematoda: Allocreadiidae) by means of 28S ribosomal DNA sequences. Adv Bocsi Biotechnol. 2014;5:209–15.
31. Nybelin O. Crepidostomum suecicum n. sp. – ein Trematode mit ungewöhnlich morphologischer variationsbreite. Ark Zool. 1932;25:1–6.
32. Braun M. Einige bemerkungen über die Fascioliden der Chiroptera. Zool Anz. 1900;23:387–91.
33. Dinulescu G. Bemerkungen über das helminthen-schmarotzer- und anderen aus den bergflüssen Rumäniens. Neue arten aus Trematoden und Nematoden. An ins Cerict Pesc României. 1942;1:7–25.
34. Šušarski W. The adult Digenea from Salmonidae of the basin of the Vistula and of the South Baltic. Acta Parasitol Pol. 1958;6:247–58 (In Polish).
35. Thomas JD. Studies on Crepidostomum metoecus (Braun) and C. fischeri (Müller), parasitic in Salmo trutta L. and S. salar L. in Britain. Parasitology. 1958;48:336–52.
36. Linton E. Trematodes from fishes mainly from the Woods Hole region, Massachusetts. Proc US Nat Mus. 1940;88:1–72.
37. Nikol W. Studies on the structure and classification of the digenetic trematodes. Quart J Microsc Sci. 1905;53:391–487.
38. Faust EC. Studies on American Stephanocephaline. Trans Amer Microsoc Soc. 1891;37:185–98.
39. Layman EM. Parasitic worms of fish of Baikal Lake. Proc Baikal Limnol Station. 1933;45:98 (In Russian).
40. Markiewich OP. Parastosfauna of Freshwater Fishes of Ukraine. Kiev: Izdatelstvo Akademii Nauk SSR; 1951 (In Russian).
41. Pigulewsky SW. Neue arten von Trematoden aus fischen des Dnjeprbassins. Zool Anz. 1931;96:9–18.
42. Gibson DI, Valtonen ET. A new species of Crepidostomum (Allocreadiidae): Digenea) from north-eastern Finland, with comments on its possible origin. Syst Parasitol. 1998;12:31–40.
43. Bray RA, Foster GN, Waeschenbach A, Littlewood DTJ. The discovery of progenetic Allocreadium neustinum Peters, 1957 (Digenea: Allocreadiidae) in water beetles (Coleoptera: Dytiscidae) in Great Britain. Zootaxa. 2012;3578:56–70.
44. Petkevičius R, Stunčienė V, Stanevičius G, Sokolov SG. Comparison of the developmental stages of some European allocreadiid trematode species and a clarification of their life-cycles based on ITS2 and 28S sequences. Syst Parasitol. 2010;76:169–78.
45. Poulin R. Evolutionary Ecology of Parasites. 2nd ed. Princeton: Princeton University Press; 2007.
46. Stunčienė V, Petkevičius R, Podrubnya LG, Stanevičius G, Zhokhov AE. Host specificity, molecular phylogeny and morphological differences of Phyllodistomum pseudololum Nybelin, 1926 and Phyllodistomum angulatum Linstow, 1897 (Trematoda: Gorgoderidae) with notes on Eurasian ruffe as final host for Phyllodistomum sp. Parasit Vectors. 2017;10:286.
47. Brabec J, Waenschenbach A, Scholz T, Littlewood DTJ, Kuchta R. Molecular phylogeny of the Bothriocephalidea (Cestoda): molecular data challenge switching, with notes on cestodes of Palaearctic hedgehogs (Erinaceidae). J Parasitol. 2018;104:361–72.
48. Shimazu T. Digeneans parasitic in freshwater fishes (Oslechthyes) of Japan. XI. A list of the papers of the series, a key to the families in Japan, a parasite-host list, a host-parasite list, Addenda, and Errata. Bull Natl Mus Nat Sci Ser A. 1927;43:129–43.
49. Curran SS, Tkach W, Overstreet RM. A review of Polylekithum Arnold, 1934 and its familial affinities using morphological and molecular data, with description of Polylekithum catahoulensis sp. nov. Acta Parasitol. 2006;51:23–48.
50. Razo-Mendivil U, Mendoza-Garfilas B, Pérez-Ponce de León G, Rubio-Godoy M. A new species of Auriculostoma (Digenea: Allocreadiidae) in the Mexican Tetra A. mexicanus (Actinopterygii: Characidae) from Central Veracruz, Mexico, described with the use of morphological and molecular data. J Parasitol. 2014;100:67–70.
51. Curran SS, Tkach W, Overstreet RM. Phylogenetic affinities of Auriculostoma (Digenea: Allocreadiidae), with descriptions of two new species from Peru. J Parasitol. 2011;97:661–70.
52. Petkevičius R, Stunčienė V, Stanevičius G. Clarification of the systematic position of Ceracanthum crusius Weisenberg-Lund, 1934 (Digenea), based on karyological analysis and DNA sequences. J Helminthol. 2012;86:293–301.
53. Curran SS, Pulis EE, Hugg DO, Brown JP, Manuel LC, Overstreet RM. Phylogenetic position of Cryptotrema funduli in the Allocreadiidae based on partial 28S rDNA sequences. J. Parasitol. 2012;98:873–5.
54. Stunčienė V, Cryan JR, Molloy DP. Comparison of DNA sequences from colchicine treated and untreated tissues. Parasitol Int. 2004;53:223–8.