Molecular analyses reveal high species diversity of trematodes in a sub-Arctic lake☆

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ABSTRACT

To identify trematode diversity and life-cycles in the sub-Arctic lake Takvatn, we characterised 120 trematode isolates from mollusc first intermediate hosts and metacercariae from second intermediate host fishes and invertebrates using molecular techniques. Phylogenies based on nuclear and/or mitochondrial DNA revealed high species richness (24 species or species-level genetic lineages), and uncovered trematode diversity (16 putative new species) from five families typical in lake ecosystems (Allocreadiidae, Diplostomidae, Plagiorchiidae, Schistosomatidae and Strigeidae). Sampling potential invertebrate hosts allowed matching sequence data for different stages, thus achieving molecular elucidation of trematode life-cycles. Phylogenetic analyses also helped identify three major mollusc intermediate hosts (*Radix balthica*, *Pisidium casertanum* and *Sphaerium* sp.) in the lake. Our findings increase the known trematode diversity at the sub-Arctic lake Takvatn, showing that digenean diversity is high in this otherwise depauperate sub-Arctic freshwater ecosystem, and indicate that sub-Arctic and Arctic ecosystems may be characterised by unique trematode assemblages.

Keywords: Trematode diversity, intermediate hosts, phylogeny, mitochondrial DNA, nuclear DNA, Takvatn, Norway, sub-Arctic

1. Introduction

Arctic and sub-Arctic ecosystems are often regarded as relatively simple and species poor due to past glaciations and extreme seasonality (Hoberg et al., 2012). Such low host diversity should translate to low parasite diversity (Hechinger and Lafferty, 2005; Kamiya et al., 2014; Poulin, 2014). However, taxonomically complex and diverse parasite assemblages can occur at high latitudes (e.g. Storer 2000; 2002; Hemmingsen and MacKenzie 2001; Muzzafar and Jones 2004; Perdiguero-Alonso et al., 2008; Kutz et al., 2012; for a detailed review see Hoberg, 2013). Notwithstanding, our knowledge of parasite diversity at high latitudes stems from research on terrestrial and marine host-parasite systems, and data from the freshwater environment are scarce.

Digenetic trematodes are an important and species-rich group in lakes and other aquatic systems (Choudhury et al., 2016; Faltýnková et al., 2016; Scholz et al., 2016). Due to the sequential use of different host species throughout complex life-cycles, digenean diversity and abundance in the first intermediate mollusc hosts is inherently linked to host diversity and abundance and reflects the dynamics of the trophic web at the ecosystem level (Hechinger and Lafferty, 2005; Lafferty et al., 2006, 2008). Digeneans are easily sampled in their intermediate hosts and are usually transmitted to their definitive hosts *via* predation; they can thus serve as indicators capturing host diversity, trophic interactions and food web function in an ecosystem. However, it can be difficult to identify larval stages and link life-cycle stages in intermediate hosts and sexually mature adults (Nolan and Cribb, 2005; Faltýnková et al., 2016).

Molecular methods using accumulated sequence data make possible rapid molecular identification in large-scale digenean surveys in North America (Brant and Locker, 2009; Detwiler et al., 2010, 2012; Locke et al., 2010a, b, 2011) and Europe (Kostadinova et al., 2003; Aldhoun et al., 2009a, b; Jouet et al., 2010; Georgieva et al., 2013a, b, 2014; Blasco-Costa et al., 2014; Faltýnková et al., 2014; Pérez-del-Olmo et al., 2014; Selbach et al., 2014, 2015; Zikmundová et al., 2014). For instance, morphological and molecular genetic approaches detected several novel species within the Diplostomidae (five species, see Blasco-Costa et al., 2014; Faltýnková et al., 2014), Schistosomatidae (four species, see Aldhoun et al., 2009a, b; Jouet et al., 2010) and Echinostomatidae (two species, see Georgieva et al., 2012; 2013a) in Iceland. These data indicate unexpected digenean diversity in high latitude ecosystems (Blasco-Costa et al., 2014). However, these diversity data result from systematic sampling of specific taxonomic groups and, to date, no attempt has been made to assess digenean biodiversity baselines in a single freshwater ecosystem in the Arctic.

Here, using recent European morphological and sequence datasets, we present the first known estimates of digenean diversity, transmission pathways and host associations in a sub-

Arctic lake. While assessing benthic macroinvertebrates and their parasites in the littoral food web in Takvatn (Norway), we examined samples of several free-living animal taxa potentially acting as intermediate hosts for digeneans. Using coarse-grained identification, based on morphology and molecular approaches, we characterised digenean diversity across both first and second intermediate hosts, linked the parasite life-cycle stages in the first (mollusc), the second (invertebrate/vertebrate) intermediate and definitive hosts, and established digenean diversity baselines and genetic datasets for identifying and exploring host-parasite interactions and food web studies in Arctic lakes.

2. Materials and methods

2.1. Study lake

Takvatn is an oligotrophic, dimictic, sub-Arctic lake located in Målselv drainage, Troms County, northern Norway (69°07'N, 19°05'E; elevation 214 m; surface area of 14.2 km^2; maximum depth of *c.*80 m; for detailed environmental characteristics of the lake (see Amundsen et al., 2009). Faunal diversity and food web relationships in Takvatn have been studied for more than 30 years (e.g. Klemetsen et al., 2002; Amundsen et al., 2009; Klemetsen and Elliott, 2010; Klemetsen and Knudsen, 2013). Parasites in fish hosts have also been studied (e.g. Knudsen et al., 1996, 1997, 2002, 2003, 2008, 2010, 2014; Amundsen et al., 2013) but only with morphological identification (but see Kuhn et al., 2015).

The fish, zooplankton and parasites of the pelagic food web in Takvatn are well studied (see Amundsen et al., 2009 and references therein). A detailed three-year study on macroinvertebrate diversity in the rocky-intertidal zone demonstrated the presence of 25 taxa (18 insects and 7 non-insects (see Klemetsen and Elliott, 2010 for details). Of these, the gastropod *Radix peregra* (identified here as *R. balthica*), the amhipod *Gammarus lacustris* and oligochaetes were common non-insect taxa and mayfly, stonefly and chironomid larvae dominated among the insect taxa.

A few aquatic bird censuses during the breeding season over a period of 30 years listed 21 species (divers, ducks, gulls, terns and waders) in Takvatn (Klemetsen and Knudsen, 2013). Of these, six species were present in all censuses and breeding pairs were observed for 12 species: *Anas penelope*; *Anas platyrhynchos*; *Aythya fuligula*; *Bucephala clangula*; *Gavia arctica*; *Larus canus*; *Melanitta fusca*; *M. nigra*; *Mergus serrator*; *Sterna paradisaea*; *Tringa hypoleucos* and *T. totanus*. Two salmonids, the Arctic charr *Salvelinus alpinus* and the brown trout *Salmo trutta*, and the three-spined stickleback *Gasterosteus aculeatus* live in the lake (see Klemetsen et al., 2002).

2.2. Sampling

Whereas most studies on trematode diversity focus on snail hosts, we considered a range of first and second intermediate hosts (allowing us to find more species and discern life-cycles). In total, 3,496 macrozoobenthic invertebrate specimens of 51 species belonging to three phyla, five classes, 11 orders and 26 families were collected during the ice-free period in 2012 (August and October) and 2013 (June and September) from several sampling sites of the lake littoral (see Supplementary Table S1 for details).

Substantial sampling in the profundal zone (at depths of 20–40 m) in August 2012 found only 209 invertebrates. Therefore, subsequent sampling was focused on the littoral zones (depths of 3–8 m), characterised by the co-occurrence of dense mats of brittleworts (*Nitella* sp.) and mosses. At most sampling sites, invertebrates were collected using a sieve sampler pulled behind a boat through abundant submerged vegetation. We sampled by hand and/or with a strainer from the sediment surface and vegetation (*Equisetum* spp.), at two shallow sites at the southeastern part of the lake (0.5 m deep) where the snail *Radix balthica* was found in high densities.

In the laboratory, invertebrates were sorted to major taxonomic groups and identified to the lowest possible taxon (see Supplementary Table S1). Each specimen was given a unique code and provisional identification and examined for the presence of parasites. Annelids and arthropods were initially compressed between glass slides and infected specimens dissected. Molluscs were placed individually into containers with filtered lake water under a light source to stimulate cercarial emergence; if emergence was not observed within two days, the molluscs were dissected. Annelids and arthropods were identified according to Nilsson (1996, 1997) and molluscs according to Glöer (2002). Digenean life-cycle stages were initially examined live and photomicrographs were taken whenever possible. Preliminarily identification of the cercariae and metacercariae to familial/generic level was carried out using the keys of Faltýnková et al. (2007, 2008) and other relevant sources, e.g. Sudarikov et al. (2002). All isolates from the first samples were given provisional identification labels; these were consistently applied to the subsequent samples. Voucher material is deposited in the Helminthological Collection of the Institute of Parasitology (HCIP), Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice under accession numbers HCIP D-735–D-750. Representative photomicrographs for the metacercariae from which the molecular samples were directly derived (i.e. hologenophores *sensu* Pleijel et al., 2008) are provided in Supplementary Fig. S1.

Intramolluscan stages (parthenitae) were identified from molecular data. To facilitate connection of some life-cycle stages in molluscs and fishes, metacercariae from the eyes of three specimens of each of the three fish species present in the lake were sampled. Subsamples of digenean life-cycle stages from all provisionally identified parasite taxa were fixed in molecular grade ethanol for DNA isolation and sequencing. A few previously collected adult specimens of *Crepidostomum* sp. and metacercariae from *Diplostomum phoxini* collected from Lake Øvre Heimdalsvatnet (61°42'24.8"N, 8°86'75.12"E) were also used to generate molecular data. Foot tissue taken from infected *Radix* spp. and two morphotypes of small clams were examined for the presence of metacercariae, washed with distilled water and fixed in molecular grade ethanol for DNA isolation and sequencing.

2.3. Sequence generation

Total genomic DNA was isolated from single ethanol-fixed rediae, sporocysts, metacercariae and adults or from 50–100 pooled cercariae emerged from a single infected mollusc using the protocols described in Georgieva et al. (2013a). Tissue from snails and small clams was also used for DNA isolation and amplification. Polymerase chain reaction (PCR) amplifications were carried out in a total volume of 25 µl using illustra puReTaq Ready-To-Go PCR beads (GE Healthcare, UK) following the manufacturer's instructions. Partial fragments of the mitochondrial genes cytochrome *c* oxidase subunit 1 (*cox*1) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1), and the nuclear 28S rRNA gene (domains D1–D3) and the complete ribosomal internal transcribed spacer region ITS1-5.8S-ITS2 (or ITS2), were amplified depending on the parasite (or mollusc host) family-level group (see Supplementary Tables S2 and S3 for details on the primers and PCR conditions used).

PCR amplicons were purified using Qiagen QIAquick^{TM} PCR purification kit (Qiagen Ltd., UK) following the manufacturer's protocol and sequenced directly for both strands using the same primers (*cox*1, *nad*1 and ITS1-5.8S-ITS2) or with additional internal primers (28S) with ABI Big Dye chemistry (ABI Perkin-Elmer, UK) alcohol-precipitated and run on an ABI Prism 3130x1 automated sequencer. Contiguous sequences were assembled, quality checked and edited manually using MEGA v6 (Tamura et al., 2013) and compared with those available in the GenBank database using BLASTn. Unique haplotypes were identified with DnaSP (Rozas et al., 2003) against all published sequences for a given species/lineage. Pairwise genetic distances were calculated using the p-distance model (i.e. the percentage of pairwise character differences with pairwise deletion of gaps) implemented in MEGA v6. All sequences are submitted to the GenBank database under accession numbers XXXXXX-XXXXXXX (see Table 2 for details).

2.4. Alignments and phylogenetic analyses

Newly-generated and published sequences for each gene/taxonomic group were aligned with MUSCLE (Edgar, 2004) implemented in MEGA v6. The alignments for protein-coding genes included no insertions or deletions and were aligned with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code (translation table 9; http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi#SG9) (Telford et al., 2000). However, these alignments were analysed solely as nucleotides as insufficient variability was provided by the amino acids alone; first, second and third positions within the included codons were included in these analyses.

Eleven alignments were analysed for parasites (see Table 1 for details). These represented a total of 307 sequences retrieved from the GenBank database for 149 species or species-level genetic lineages from the taxonomic groups targeted based on our provisional sorting/identification of the isolates sequenced from Takvatn. We selected up to three representative published sequences (the longest possible) per species/lineage as determined in previous studies (see Supplementary Table S4 for details). The ITS alignment (*Trichobilharzia* spp., Alignment 11; see Table 1) represents a concatenated data set of the ITS1 (2,062 nt long) and ITS2 (380 nt long) fragments in order to include all sequences for species of *Trichobilharzia* available in the GenBank database. Concatenation was made in SEAVIEW (Galtier et al., 1996) and resulted in a 2,442 nt long alignment which included ambiguously aligned regions; these were detected with the aid of Gblocks v0.91b (Castresana, 2000) implemented in SEAVIEW with less stringent parameters, and omitted prior to phylogenetic analysis. The final alignment was 1,297 nt long.

Two alignments were analysed for the snail and clam hosts of the parasites sampled in Takvatn: Alignment 12 (ITS2 sequences for *Radix* spp.) and Alignment 13 (28S rDNA sequences for small clams) (see Table 1).

Molecular identification of the parasite and host isolates sequenced from Takvatn was achieved in Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic analyses. Prior to analyses, jModelTest 2.1.4 (Guindon and Gascuel, 2003; Darriba et al., 2012) was used to estimate the best-fitting models of nucleotide substitution based on Akaike Information Criteria (AIC); these are listed in Table 1. BI analyses were carried out with MrBayes version 3.2.6 (Ronquist et al., 2012) using Markov chain Monte Carlo (MCMC) searches on two simultaneous runs of four chains for 10^7 generations, sampling trees every 10^3 generations. The first 25% of the trees sampled were discarded as ‘burn-in’, determined by stationarity of lnL assessed using Tracer v. 1.5 (Rambaut and Drummond, 2009) and a consensus topology and nodal support estimated as posterior probability values (Huelsenbeck et al., 2001) were calculated from the remaining 75% of the trees. BI analyses were run on the Cipres Science Gateway v. 3.1 (http://www.phylo.org/sub_sections/portal/) (Miller et al., 2010), using MrBayes (3.2.6) on XSEDE. ML analyses were performed with PhyML 3.0 (Guindon et al., 2010) run on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/) with a non-parametric bootstrap validation based on 1,000 pseudoreplicates. The outgroup taxa used in the analyses are listed in Table 1.

3. Results

Of the 3,496 individual invertebrates (51 species, 26 families and 11 orders), 919 (19 species of 14 families and nine orders) were infected with digeneans (see Supplementary Table S1 for details). The most abundant invertebrates, *Gammarus lacustris* and *Radix balthica*, were also the most frequently infected hosts in the lake. The infected arthropods included 373 amphipods (*G. lacustris*) and 229 aquatic insects (15 spp.; predominantly larval stages, 13 spp.). Of the three snail species examined, *R. balthica* hosted most larval digeneans, whereas only two *Gyraulus acronicus* were infected and no parasites were found in the 14 *Valvata piscinalis* dissected (see Supplementary Table S1).

Our phylogenetic analyses based on 148 sequences for 120 digenean isolates from invertebrates and fish sampled from Takvatn revealed unexpected high species richness (24 species or species-level lineages) and uncovered substantial diversity of digeneans, including 16 putative new species, within five of the families typical in lake ecosystems, i.e. the Allocreadiidae, Diplostomidae, Plagiorchiidae, Schistosomatidae and Strigeidae (Faltýnková et al., 2016; Scholz et al., 2016). Molecular identification relied on (and now adds to) sequence and morphological databases for the European species of the last four families (Georgieva et al., 2013a, b, 2014; Blasco-Costa et al., 2014, Zikmundová et al., 2014; Selbach et al., 2015; Roháčová et al., unpublished data). Phylogenies developed here based on mitochondrial and nuclear DNA wherever applied, depicted the same distinct genetic lineages. Furthermore, the extensive sampling across a range of possible hosts allowed matching sequence data for different life-cycle stages thus achieving molecular elucidation of life-cycles for 14 species, more than 50% of the species discovered in the lake.

3.1. Family Allocreadiidae

Both, ML and BI analyses of the Allocreadiidae (Alignment 1 including sequence data for 25 species available in the GenBank database; see Tables 1, 2 and Supplementary Table S4 for details) resulted in consensus trees with similar topologies (Fig. 1). The newly-generated sequences from Takvatn fell into five distinct strongly supported monophyletic lineages, four within *Crepidostomum* and one within *Allocreadium*. Notably, *Crepidostomum* was resolved as polyphyletic with the five North American species (*C. affine*, *C. auritum*, *C. cooperi*, *C. cornutum* and *C. illinoisense*) included in a strongly supported clade comprising a range of allocreadiid taxa with a North American distribution whereas two Eurasian species did not join the main (albeit unsupported) cluster formed by *Crepidostomum* spp. from Europe and Asia. One unidentified isolate of *Crepidostomum* from Europe clustered with species of *Allocreadium* with strong support and an Asian isolate of *Crepidostomum auriculatum* appeared as earliest divergent to all allocreadiids (Fig. 1). Phylogenetic analysis of *Crepidostomum* spp. alone (Alignment 2 including sequence data for 11 species available in the GenBank database; see Tables 1, 2 and Supplementary Table S4 for details) revealed similar patterns and support but with *C. auriculatum* clustering as earlier divergent with *C. farionis* and *Crepidostomum* sp. 1 with strong support from BI analysis (see Supplementary Fig. S2).

The sequences for 21 isolates sampled from clams, insects, gammarids and fish (see Table 2 for details) in Takvatn formed four strongly supported reciprocally monophyletic lineages within the cluster of the Eurasian species of *Crepidostomum.* The sequences for two progenetic metacercariae from the dytiscid beetle *Oreodytes sanmarkii* clustered within the clade of *Allocreadium* spp. with a maximum support. These results indicate that two pairs of closely related *Crepidostomum* spp. complete their life-cycles in the lake: (i) *C. farionis* (using the clams *Pisidium casertanum* and *Sphaerium* sp. as first intermediate hosts) and the closely-related sister species *Crepidostomum* sp. 1 (using *Sphaerium* sp. as first intermediate host and nymphs of the mayfly *Siphlonurus lacustris* as second intermediate hosts); and (ii) *C. metoecus* (using *Pisidium casertanum* as first intermediate host, *G. lacustris* as second intermediate host and *Salmo trutta* as definitive host) and the closely related sister species *Crepidostomum* sp. 2 (using nymphs of the mayfly *Siphlonurus lacustris* and the stonefly *Diura bicaudata* as second intermediate hosts, and *S. trutta* as definitive host) (Fig. 1). Notably, intraspecific variation was detected only for *Crepidostomum* sp. 2 with a difference of a single nucleotide position. The interspecific divergence between the pairs of *Crepidostomum* spp. from Takvatn was 0.8% (6 nt) (*C. farionis* - *Crepidostomum* sp. 1) and between 0.8–1.0% (6–7 nt) (*C. metoecus* - *Crepidostomum* sp. 2). The interspecific divergence between the two main clades of the Eurasian species of *Crepidostomum* ranged between 3.8–4.5% (27–32 nt).

The sequences for the progenetic metacercarie ex *O. sanmarkii* were identical to a sequence for *Allocreadium neotenicum* from the UK (Bray et al., 2012). These isolates were, therefore, identified as *A. neotenicum*. Notably, the closest relative, the North American *A. lobatum,* differed by only two nucleotide positions.

3.2. Family Strigeidae

Phylogenetic reconstructions for representatives of the family Strigeidae were based on partial sequences for *cox*1 (Alignment 3 including data for 22 species/lineages available in the

GenBank database; see Tables 1, 2 and Supplementary Table S4 for details) and 28S rDNA (Alignment 4 including data for 8 species/lineages from GenBank; see Tables 1, 2 and Supplementary Table S4 for details). Individual gene analyses yielded tree topologies with congruent sister-group relationships among the available representatives of the family despite the different taxa composition (Fig. 2, Supplementary Fig. S3). Overall, the *cox*1 phylogeny comprising data for seven strigeid genera revealed the clade comprising *Cotylurus*, *Ichthyocotylurus* and *Cardiocephaloides* as earlier divergent (ML support only).

Species/lineages of *Apatemon* formed two clusters, one strongly supported and comprising five lineages sequenced in North America plus a lineage from Takvatn and the second supported from ML analysis only (84%) containing a lineage from Takvatn and an unidentified species from New Zealand, *Apatemon* sp. "jamiesoni". Additionally, there was no support for the genera *Australapatemon* and *Ichthyocotylurus*, and *Apharyngostrigea* was recovered as paraphyletic (Fig. 2).

The newly-generated *cox*1 sequences for isolates from Takvatn clustered in three strongly supported reciprocally monophyletic lineages (Fig. 2). Two of these clustered within *Apatemon* spp. clades: (i) *Apatemon gracilis* (using *R. balthica* as first intermediate host and *Gasterosteus aculeatus* as second intermediate host); and (ii) a novel species of *Apatemon* in the second intermediate host (two metacercariae ex *G. aculeatus*). Both lineages contained sequences generated recently for metacercariae ex *G. aculeatus* from Takvatn by Kuhn et al. (2015): three labelled as "Strigeidae gen. sp." (GenBank KM212057, KM212064, KM212065) fell within the clade representing *A. gracilis* and two labelled as *Apatemon* sp. (GenBank KM212028; KM212029) clustered with the sequences for the novel species of *Apatemon* from Takvatn. Both species exhibited low levels of intraspecific divergence (0–1.0% and 0.2–0.7%, respectively).

Sequences from sporocysts ex *R. balthica* and metacercariae ex *R. balthica* and *Gyraulus acronicus* represented two haplotypes (intraspecific divergence 0–0.7%) and formed a strongly supported lineage clustering with the only sequence for *Cotylurus* spp. available on GenBank (Fig. 2); this lineage was identified based on morphology and our unpublished sequences (Roháčová et al., unpublished data) as *Cotylurus cornutus*.

Phylogenetic analyses of the 28S rDNA dataset (Alignment 4; see Tables 1, 2 and Supplementary Table S4 for details) corroborated the distinct species status of the three strigeids from Takvatn (Supplementary Fig. S3). Notably, there was a strongly supported sister-group relationship between *A. gracilis* and *Apatemon* sp. "jamiesoni" sequenced in New Zealand in both *cox*1 (ML only, 84%) and 28S rDNA analyses. No 28S rDNA sequence is available on GenBank for *Cotylurus* spp. but both, ML and BI analyses depicted a strongly supported relationship between *C. cornutus* and an otherwise unpublished sequence for *Nematostrigea serpens* indicating that the latter has been misidentified (Supplementary Fig. S3).

3.3. Family Diplostomidae

The newly-generated sequences depicted six species of diplostomid completing their life-cycles in Takvatn with *R. balthica* and fishes acting as first and second intermediate hosts, respectively (Table 2). The *cox*1 phylogeny for *Diplostomum* spp. including data for 35 species/lineages available in the GenBank database (Alignment 5; see Tables 1, 2 and Supplementary Table S4 for details) demonstrated that the newly-sequenced isolates from Takvatn cluster into five strongly supported reciprocally monophyletic lineages (Fig. 3). These included *Diplostomum phoxini* (a cercarial isolate ex *R. balthica* and a metacercaria ex *Phoxinus phoxinus* from Lake Øvre Heimdalsvatnet, Norway; sequence divergence 0.2%) and four of the six lineages of *Diplostomum* recently discovered and described by Blasco-Costa et al. (2014) and Faltýnková et al. (2014) in Iceland.

Two of these lineages represented metacercariae in fish only: (i) *Diplostomum* sp. ‘Lineage 3’ of Blasco-Costa et al. (2014) comprising metacercariae from the eye vitreous humour of the two salmonids studied [four haplotypes including three novel (out of 18 currently known haplotypes); intra-lineage divergence 0.5–2.0%]; and (ii) *Diplostomum* sp. ‘Lineage 5’ of Blasco-Costa et al. (2014) comprising metacercariae from the eye vitreous humour of the two salmonids plus one metacercaria ex *G. aculeatus* [six haplotypes including five novel (out of 17); intra-lineage divergence 0–1.7%].

The two remaining lineages both contained sequences generated from cercariae ex *R. balthica* and metacercariae from the eye vitreous humour and retina of *G. aculeatus*. *Diplostomum* sp. ‘Lineage 4’ of Blasco-Costa et al. (2014) was represented by five haplotypes including four novel (out of 23; intra-lineage divergence 0–1.5%) and *Diplostomum* sp. ‘Lineage 6’ of Blasco-Costa et al. (2014) was represented by seven haplotypes including three novel (out of 20; intra-lineage divergence 0–1.7%). There was a strongly supported sister-group relationship between *Diplostomum* sp. ‘Lineage 3’ and *Diplostomum* sp. ‘Lineage 4’ and between *Diplostomum* sp. ‘Lineage 5’ and *Diplostomum* sp. 6 of Locke et al. (2010a) based on material from the St Lawrence River in Canada as shown in previous studies (see Georgieva et al., 2013b; Blasco-Costa et al., 2014) and *Diplostomum* sp. ‘Lineage 6’ clustered with four lineages of *Diplostomum* spp. (species 8, 9, 13 and 17 of Locke et al., 2010a) from the St Lawrence River, Canada.

Single haplotypes recovered within ‘Lineages 3–5’ of *Diplostomum* from Takvatn have recently been reported from fishes and snails in central Europe or sub-Arctic: (i) within

Diplostomum sp. ‘Lineage 3’, haplotype S847 was shared with an isolate ex *S. trutta* from the River Ruhr, Germany (JX986868; Georgieva et al., 2013b) and an isolate ex *S. alpinus* from Hafravatn, Iceland (KJ726463; Blasco-Costa et al., 2014); (ii) within *Diplostomum* sp. ‘Lineage 4’, haplotype S852 was shared with two isolates ex *Perca fluviatilis* from Lake Constance, Germany (JQ639182 and JQ639194; Behrmann-Godel, 2013) and three isolates ex *G. aculeatus* from Takvatn (KM212030, KM212032 and KM212033; Kuhn et al., 2015); (iii) within ‘Lineage 5’, haplotype S836 was shared with three isolates ex *S. trutta* from Hafravatn, Iceland (KJ726492–KJ726494; Blasco-Costa et al., 2014).

Finally, within *Diplostomum* sp. ‘Lineage 6’, four haplotypes were shared among isolates sampled in our study and previously published sequences from metacercariae ex *G. aculeatus* in Takvatn by Kuhn et al. (2015) as follows: (i) haplotype 1: isolate S858 ex *R. balthica* and four isolates (KM212035, KM212036, KM212043 and KM212052); (ii) haplotype 2: isolates S835 and S828 ex *G. aculeatus* and four isolates (KM212037, KM212040, KM212041 and KM212047); (iii) haplotype 3: isolates S854 and S859 ex *R. balthica* and five isolates (KM212039, KM212042, KM212045, KM212046 and KM212051); and (iv) haplotype 4: isolate S832 ex *G. aculeatus* and isolate KM212054 of Kuhn et al. (2015). Notably, two of these haplotypes have been first discovered in sub-Arctic lakes in Iceland by Blasco-Costa et al. (2014): (i) haplotype 2 ex *G. aculeatus* was shared with two isolates ex *R. balthica* (KJ726505 and KJ726506) from Lake Nordic House, Reykjavik; and (ii) haplotype 3 ex *R. balthica* was shared with one isolate ex *R. balthica* (KJ726497) and two isolates ex *G. aculeatus* (KJ726496 and KJ726498), all from Lake Nordic House, Reykjavik.

Phylogenetic analyses of the available *cox*1 sequence data for species/lineages of *Tylodelphys* (Alignment 6; 14 spp.; see Tables 1, 2 and Supplementary Table S4 for details) revealed three well-supported clades (Fig. 4), one containing four African species/lineages plus two widely distributed European species, *Tylodelphys clavata* and *T. excavata*; one representing three species from North and South America; and one containing the newly-sequenced metacercarial isolates from the vitreous humour of the two salmonids in Takvatn and the North American *Tylodelphys immer*. The two haplotypes of the novel lineage differed by 0.5%; both differed from the sister-species, *T. immer*, by 5.0–5.8%.

3.4. Family Plagiorchiidae

Large numbers of *R. balthica* were infected with *Plagiorchis* spp. The newly-generated *cox*1 sequences from selected cercarial isolates and three metacercariae ex *G. lacustris* and a larval cranefly *Tipula salicetorum* were aligned together with sequences for five European and one Korean species of *Plagiorchis* (Alignment 7; including sequence data for six species available in the GenBank database; see Tables 1, 2 and Supplementary Table S4 for details). Both BI and ML analyses depicted seven novel species-level lineages (Fig. 5A); of these, two (*Plagiorchis* sp. 2 and *Plagiorchis* sp. 3) included matching sequences from cercariae and metacercariae (ex *G. lacustris* and *T. salicetorum*, respectively). The novel *cox*1 sequences represented 22 haplotypes (18 unique) as follows: *Plagiorchis* sp. 1 (eight; six unique); *Plagiorchis* sp. 2 (four; two unique); *Plagiorchis* sp. 3 (four unique); *Plagiorchis* sp. 4 (two unique); *Plagiorchis* sp. 5 (two unique); *Plagiorchis* sp. 6 (one); and *Plagiorchis* sp. 7 (one). Within the dataset studied, the intraspecific divergence range was 0–2.1% and the range for interspecific divergence was 3.5–17.7%.

Analyses of 28S rDNA sequences for *Plagiorchis* spp. (Alignment 8; including data for seven species available in the GenBank database; see Tables 1, 2 and Supplementary Table S4 for details) confirmed that the lineages of *Plagiorchis* spp. are novel (Fig. 5B). Three lineages included matching sequences from cercariae ex *R. balthica* and metacercariae from benthic invertebrates as follows: *Plagiorchis* sp. 1 (larval *T. salicetorum*); *Plagiorchis* sp. 2 (*G. lacustris*), *Plagiorchis* sp. 3 (larval *T. salicetorum* and the dytiscid beetle *Oreodytes alpinus*), and *Plagiorchis* sp. 5 (larval alderfly *Sialis lutaria* and *O. alpinus*). However, the sequences for *Plagiorchis* sp. 4 and *Plagiorchis* sp. 6 were identical and there was no support for lineages *Plagiorchis* sp. 1, 2 and 3. The intraspecific sequence divergence between the lineages sampled at Takvatn was low (0–2 nt) but still below the minimum interspecific genetic divergence (4–22 nt; mean 15 nt).

3.5. Miscellaneous groups with single species

Sequences for *nad*1 were generated from metacercarial isolates ex *Pisidium casertanum* and *Sphaerium* sp. and a redia ex *R. balthica* provisionally assigned to the family Echinostomatidae. A preliminary analysis with a large number of echinostomatid sequences (data not shown) assigned the isolates from Takvatn to the genus *Echinoparyphium*. Analyses based on sequences for both *nad*1 (Alignment 9; see Tables 1, 2 and Supplementary Table S4 for details) and 28S rDNA (Alignment 10; see Tables 1, 2 and Supplementary Table S4 for details) for seven species of *Echinoparyphium* resulted in identification of the isolates from Takvatn as *Echinoparyphium recurvatum* (Fig. 6A, B). All new *nad*1 sequences represented novel haplotypes with intraspecific sequence divergence between 0.1 and 2.3%.

Identification of schistosome infections in *R. balthica* from Takvatn was attempted using concatenated sequences for the two internal transcribed spacers (ITS1 and ITS2) of the rRNA gene cluster (Alignment 11; see Tables 1, 2 and Supplementary Table S4 for details). Phylogenies inferred from BI and ML were congruent with similar tree topologies (Fig. 7). The newly-sequenced cercarial isolates clustered together with three isolates of the lineage *T. franki* haplotype “peregra” sampled in Iceland and considered by Jouet et al. (2010) to represent a distinct species based on analyses of sequences for the mitochondrial *cox*1 and nuclear (rRNA) genes. Genetic distances between Takvatn isolates ranged between 0 and 0.4% (0–5 nt) and between Takvatn and Icelandic isolates ranged between 0.1 and 0.4% (1–5 nt). The overall relationships among *Trichobilharzia* spp. were similar to those depicted by Brant and Loker (2009). There was a strong support for Clade Q *sensu* Brant and Loker (2009), a group of morphologically and genetically similar species from North America and Europe, and for the sister-group relationship between this clade and *Trichobilharzia regenti* (BI only). Notably, the isolates from Takvatn clustered with strong support (BI) together with an isolate (ex *Lymnaea stagnalis*) of the polyphyletic *T. franki* within Clade Q (Fig. 7).

Partial 28S rDNA sequence was obtained from a single isolate of *Notocotylus* sp. (Table 2). A BLASTn search of the GenBank nucleotide database indicated a 99% similarity (one gap; coverage 100%) with *Notocotylus* sp. BH-2008 (EU712725) ex *Physa gyrina* from Nebraska, USA (Hanelt, 2009) and an unidentified pronocephaloidean (EU371602) ex *Potamopyrgus antipodarum* from Wyoming, USA (Adema et al., 2009).

3.6. Mollusc hosts

Four ITS2 sequences from *R. balthica* sampled in Takvatn were aligned together (Alignment 12, see Tables 1, 2 for details) with 26 sequences for isolates of *Radix* spp. from Europe, including sub-Arctic lakes in Iceland. The isolates from Takvatn clustered together with two Icelandic isolates [isolate IS2F (GenBank HQ003228) from Botnsvatn, referred to as *R. balthica* in GenBank and *R. peregra* and *R. balthica* by Jouet et al. (2010), and the isolate radix3.1 (GenBank GU574213) from Osland, referred to as *R. peregra* by Huňová et al. (2012)] plus the isolate SnUK20 from Scotland, UK (GenBank KT337604, referred to as *R. balthica* by Lawton et al., 2015) in a clade sister to *Radix lagotis* sequenced by Huňová et al. (2012), joined by a sequence for *R. peregra* from France (GenBank AJ319635) sequenced by Bargues et al. (2001) (see Supplementary Fig. S4). Sequences from Takvatn were identical with those for the Icelandic isolate of Jouet et al. (2010) and the Scottish isolate and differed by one nucleotide from the Icelandic isolate of Huňová et al. (2012) and by two nucleotides from the French isolate of *R. balthica*. However, relationships among *Radix* spp. were unresolved (see Supplementary Fig. S4).

Representative partial 28S rDNA sequences for the two morphs of pea clams were analysed together with selected sequences for species of *Sphaerium*, *Pisidium* and *Musculium* (Alignment 13, see Tables 1, 2 for details). One of the morphotypes was resolved as a sister species to *Sphaerium* spp. (*S. corneum* and *S. nucleus*) with strong support from both BI and ML analyses and the second morphotype clustered with *Pisidium casertanum* (isolate from Greece; KF483338) (see Supplementary Fig. S5). The newly-generated sequence for *Sphaerium* sp. differed by 3 nt from the sequences for *S. corneum* and *S. nucleus* which were identical, and the new sequence for *Pisidium* sp. differed by 1 nt from *Pisidium casertanum*. Based on these results, the two species of pea clams are referred to as *Sphaerium* sp. and *Pisidium casertanum*.

4. Discussion

We found more digenean diversity in Takvatn than one might suspect for a sub-Arctic freshwater ecosystem: 24 species/species-level genetic lineages of ten genera and seven families, the latter being the most diverse and widely distributed suprageneric taxa in the freshwater environment (Faltýnková et al., 2016; Scholz et al., 2016). This high degree of digenean biodiversity is surprising given the restricted host fauna compared with other aquatic ecosystems and suggests that digenean diversity in the sub-Arctic freshwater environments is still vastly underestimated, even among parasites that use relatively well-studied fish hosts (Blasco-Costa et al., 2014).

Although fish parasites have been studied in Takvatn, only *Crepidostomum* spp. (assumed to be *C. farionis* and *C. metoecus*) had been recorded (e.g. Kristoffersen, 1995; Kuhn et al., 2016) and no attempts to identify metacercariae in fish had been made until recently (Kuhn et al., 2015; see below). We were surprised to find two pairs of genetically closely related species of *Crepidostomum* among the 21 isolates sequenced from Takvatn, considering that there are only four known European species of the genus, i.e. *C. auriculatum* (Wedl, 1858), *C. farionis*, *C. metoecus* and *C. wikgreni* Gibson & Valtonen, 1988. Further molecular studies focused on the adult stages might reveal more *Crepidostomum* spp. in sub-Arctic freshwater ecosystems.

It is worth noting that we sequenced few metacercariae from fishes. However, the novel *Apatemon* and *Tylodelphys*, species, *A. gracilis* and five *Diplostomum* species and the presence of similar or shared haplotypes with isolates from a previous extensive sampling of *Gasterosteus aculeatus* in Takvatn (*Apatemon gracilis*, *Apatemon* sp., *Diplostomum* sp. 'Lineage 4' and *Diplostomum* sp. 'Lineage 6'; see Fig. 3 and intensity data in Kuhn et al., 2015) indicate that metacercariae in fish represent a diverse assemblage with high transmission rates in the lake. The fish parasite diversity in Takvatn, revealed by the molecular and phylogenetic approaches applied here, is higher from sub-Arctic diversity baselines compiled from studies relying on morphological identification (e.g. Poulin et al., 2011; Wrona et al., 2013). Our study adds 9 and 7 species, respectively, to species richness estimates for parasites in *G. aculeatus* (1–11 species;

Poulin et al., 2011) and salmonid and coregonid hosts (4–18 spp.; Wrona et al., 2013) in the sub-Arctic and Arctic ecosystems.

Although we found 15 digenean species in *R. balthica*, this snail is the only compatible host for another four species (*Apatemon* sp., *Diplostomum* sp. 'Lineage 3', *Diplostomum* sp. 'Lineage 5' and *Tylodelphys* sp.) thus increasing the number of species to 19 (Table 3). Comparisons with the most comprehensive diversity baselines for digeneans in *Radix* spp. from Europe, reveal that digenean richness in *R. balthica* from Takvatn represents more than half of the species (58–68%) recorded in *R. peregra* (33 spp.), *R. ovata* (syn. of *R. balthica*; 31 spp.) and *R. auricularia* (28 spp.) between 1878 and 2012 (see Faltýnková et al., 2016). Notably, 39 of the 55 mollusc species in the dataset (based on 246 surveys in 22 European countries) analysed by Faltýnková et al. (2016) host one to five species, thus highlighting the extraordinary digenean diversity in a single snail in Takvatn. Diversity estimates vary locally (Faltýnková et al., 2016) but the 19 digenean species in *R. balthica* in Takvatn is high compared with 12 species (1–7 species per lake) in *R. auricularia* in four interconnected lakes of the River Ruhr in Germany (Soldánová et al., 2010) and with 3–19 digenean species in 2–5 snail species per lake in six high latitude lakes in central Alberta (Gordy et. al., 2016).

Notably, two-thirds of the genetically distinct digenean lineages in our dataset from Takvatn did not match any reference sequence, suggesting that the 16 novel lineages are new species, including four of the five novel *Diplostomum* lineages 'just' discovered from sub-Arctic lakes in Iceland (Blasco-Costa et al., 2014; Faltýnková et al., 2014). The remaining 12 species-level lineages could not be matched with confidence to existing described species and, therefore, await detailed morphological examination and descriptions.

Our results suggest that most species assemblages within the major freshwater families are unique to sub-Arctic and Arctic ecosystems. This is supported by the novel *Apatemon*, *Crepidostomum* and *Tylodelphys* lineages and by the fact that two of the novel *Diplostomum* spp. lineages (lineages 5 and 6) and the lineage *Trichobilharzia franki* haplotype “peregra" have so far been detected in Iceland only, despite extensive sampling in Europe (e.g. Jouet et al., 2010; Georgieva et al., 2013b; Pérez-del-Olmo et al., 2014; Selbach et al., 2015; see also Soldánová et al. 2013 for a review on records of *Trichobilharzia* spp.). Further, four *Trichobilharzia* spp. have been recorded and molecularly characterised in snails and birds in Iceland [*T. anseri* (FJ469790, FJ469791, FJ469784); *T. franki* haplotype “peregra" (HM131185/ HM131168; HM131186/ HM131169; HM131187/ HM131171; present study); *T. mergi* (FJ469807, FJ469799); and *Trichobilharzia* sp. 3 (FJ469803, FJ469804) of Aldhoun et al. (2009a) (see Aldhoun et al., 2009a, b; Jouet et al., 2010)] compared with but three species (i.e. *T. franki*, *T. regenti* and *T. szidati*) reported in central Europe despite a much higher sampling effort there. Finally, *Plagiorchis* diversity in sub-Arctic lakes in Iceland (Roháčová et al., unpublished data) includes five of the novel species-level lineages reported here, thus reinforcing our suggestion that our observations extend beyond Takvatn across a broader sub-Arctic geographic range. Unfortunately, the sequence data of Gordy et al. (2016) cannot be used for comparisons with our data, because these authors sequenced a different *cox*1 fragment than that allowing molecular identification of species/lineages available on GenBank (e.g. Detwiler et al., 2010; Georgieva et al., 2014; Zikmundová et al., 2014; our study).

Taken together, these data help infer 165 host-trematode associations: 22 with the first intermediate mollusc hosts, 26 with the second intermediate hosts and 117 with the definitive fish and bird hosts (Table 3). Of these, 47 life-cycle links are firm, i.e. based on matching sequences for cercarial, metacercarial and adult (for two *Crepidostomum* spp.) isolates from the lake. Sequencing representative isolates from the first intermediate hosts and phylogenetic analyses helped us identify two mollusc intermediate hosts (*Radix balthica* and *Pisidium casertanum*) to the species level and another (*Sphaerium* sp.) to the genus level. All but five of the genetic lineages use *R. balthica* as their first-intermediate host and all but five mature in birds (Table 3) even though Takvatn has more fish than bird abundance and biomass. Matching sequence data for different life-cycle stages allowed us to elucidate the life-cycle of *C. metoecus* and partly elucidate the life-cycles for another 13 species in the lake. Of these, 12 species are trophically transmitted and only two species (*T. franki* haplotype·"peregra" and *Notocotylus* sp.) do not require a second-intermediate host (Table 3). Life-cycle data for *Crepidostomum* spp., the only assemblage using fishes as definitive hosts among the digeneans identified at Takvatn, indicate that both salmonids (*S. trutta* and *S. alpinus*) might act as definitive hosts, and Kuhn et al. (2015) found eight specimens of *Crepidostomum* sp. (assumed to be either *C. metoecus* or *C. farionis*) in *G. aculeatus* in the lake. Therefore, all three fish species present at Takvatn might host both *Crepidostomum* spp. (Table 3). Inferring definitive bird hosts based on either records at the species (*C. cornutus* and *E. recurvatum*; 15 host-parasite associations) or genus level (*Apatemon* spp., *Diplostomum* spp., *Plagiorchis* spp., *Notocotylus* sp. and *T. franki* haplotype "peregra"; 90 host-parasite associations) is plausible, considering the trophic behaviour of the potential bird hosts and host-parasite compatibility based on records for congeneric digeneans at the NHM Host-Parasite Database (Gibson et al., 2005). Our data, therefore, help complete the Takvatn host-parasite interaction network adding the benthic component, which is characterised by a 3-fold higher diversity of macroparasites (24 *vs* 8 species) and twice as many host-parasite links (165 *vs* 75) than the network in the pelagic zone (see Amundsen et al., 2009).

In conclusion, our study adds to the sequence database (Georgieva et al., 2013; Blasco-Costa et al., 2014; Georgieva et al., 2014; Zikmundová et al., 2014) on digeneans in freshwater ecosystems that will allow a direct and taxonomically consistent way to identify host-parasite interaction networks in future large-scale network and/or food web studies in Arctic lakes. With our approach, partitioning interactions with novel species/genetic lineages can now be achieved without having to complete life-cycles in the laboratory.

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Figure legends

Fig. 1. Phylogram from Bayesian inference (BI) analysis of the 28S rDNA sequence alignment (Alignment 1, 721 nt, 71 sequences) for 27 species/lineages within the Allocreadiidae. Outgroup: *Polylekithum ictaluri*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values > 0.95 (BI) and > 70 (ML) are shown. Isolates from Takvatn are coded as in Table 2 with indication of host name and life-cycle stage (R, redia; C, cercaria; M, metacercaria; A, adult). The scale-bar indicates the expected number of substitutions per site. Sequence identification is as in GenBank, followed by a letter: At, Atopkin and Shedko (2014); B, Bray et al. (2012); Ch, Choudhury et al. (2007), Choudhury and León-Règagnon (2005); Cu, Curran et al. (2006, 2011); Pe, Petkevičiūtė et al. (2010); Pl, Platta and Choudhury (2006); PP, Pérez-Ponce de León et al. (2007, 2015); R-M, Razo-Mendivil et al. (2014a,b); T, Tkach et al. (2013).

Fig. 2. Phylogram from Bayesian inference (BI) analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) sequence alignment (Alignment 3, 407 nt, 65 sequences) for 22 species/lineages of the Strigeidae. Outgroup: *Diplostomum spathaceum*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values > 0.95 (BI) and > 70 (ML) are shown. Isolates from Takvatn are coded as in Table 2 with indication of host name and life-cycle stage (S, sporocyst; C, cercaria; M, metacercaria). The scale-bar indicates the expected number of substitutions per site. Sequence identification is as in GenBank, followed by a letter: B-C, Blasco-Costa et al. (2016); H-M, Hernández-Mena et al. (2014); K, Kuhn et al. (2015); L, Locke et al. (2010b, 2011); Mo, Moszczynska et al. (2009); PDO, Pérez-del-Olmo et al. (2014).

Fig. 3. Phylogram from Bayesian inference (BI) analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) sequence alignment (Alignment 5, 407 nt, 112 sequences) for 36 species/lineages of *Diplostomum*. Outgroup: *Tylodelphys clavata*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values > 0.95 (BI) and > 70 (ML) are shown. Isolates from Takvatn are coded as in Table 2 with indication of host name and life-cycle stage (C, cercaria; M, metacercaria). The scale-bar indicates the expected number of substitutions per site. Sequence identification is as in GenBank, followed by a letter: B-C, Blasco-Costa et al. (2014); B-G, Behrmann-Godel (2013); Ch, Chibwana et al. (2013); G, Georgieva et al. (2013b); K, Kuhn et al. (2015); L, Locke et al. (2010a,b, 2015); Mo, Moszczynska et al. (2009); PDO, Pérez-del-Olmo et al. (2014); Se, Selbach et al. (2015).

Fig. 4. Phylogram from Bayesian inference (BI) analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) sequence alignment (Alignment 6, 407 nt, 39 sequences) for 15 species/lineages of *Tylodelphys*. Outgroup: *Diplostomum spathaceum*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values > 0.95 (BI) and > 70 (ML) are shown. Isolates from Lake Takvatn are coded as in Table 2 with indication of host name and life-cycle stage (M, metacercaria). The scale-bar indicates the expected number of substitutions per site. Sequence identification is as in GenBank, followed by a letter: Ch, Chibwana et al. (2013); G, Georgieva et al. (2013b); G-V, García-Varela et al. (2015); L, Locke et al. (2015); O, Otachi et al. (2015); PDO, Pérez-del-Olmo et al. (2014).

Fig. 5. Phylograms from Bayesian inference (BI) analyses for *Plagiorchis* spp. A, Analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) sequence alignment (Alignment 7, 423 nt, 41 sequences) for 13 species/lineages. Outgroup: *Choledocystus hepaticus*. B, Analysis of the 28 rDNA sequence alignment (Alignment 8, 1,171 nt, 27 sequences) for 14 species/lineages. Outgroup: *Neoglyphe sobolevi*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values > 0.95 (BI) and > 70 (ML) are shown. Isolates from Takvatn are coded as in Table 2 with indication of host name and life-cycle stage (S, sporocyst; C, cercaria; M, metacercaria). The scale-bar indicates the expected number of substitutions per site. Sequence identification is as in GenBank, followed by a letter: B, Boyce et al. (2014); L, Lee et al. (2004); R-M, Razo-Mendivil and Pérez-Ponce de Léon (2011); T, Tkach et al. (1999, 2000, 2001a,b); Z, Zikmundová et al. (2014).

Fig. 6. Phylograms from Bayesian inference (BI) analyses for *Echinoparyphium* spp. A, Analysis of the nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) sequence alignment (Alignment 9, 472 nt, 21 sequences) for 7 species/lineages. B, Analysis of the 28 rDNA sequence alignment (Alignment 10, 1,190 nt, 11 sequences) for 7 species/lineages. Outgroup: *Echinostoma revolutum*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values > 0.95 (BI) and > 70 (ML) are shown. Isolates from Takvatn are coded as in Table 2 with indication of host name and life-cycle stage (R, redia; M, metacercaria). The scale-bar indicates the expected number of substitutions per site. Sequence identification is as in GenBank, followed by a letter: K, Kostadinova et al. (2003); D, Detwiler et al. (2010); M, Morgan and Blair (1998a,b); G, Georgieva et al. (2014); T, Tkach et al. (2001a, 2012, 2016); P, Pulis et al. (2011); S, Stanevičiūtė et al. (2015).

Fig. 7. Phylogram from Bayesian inference (BI) analysis of the concatenated ITS1 and ITS2 alignment (Alignment 11, 1,297 nt, 43 sequences) for 16 species/lineages of *Trichobilharzia* spp. from the analysis of the concatenated ITS1 and ITS2 gene data set. Outgroup: *Anserobilharzia brantae.* Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values > 0.95 (BI) and > 70 (ML) are shown. Isolates from Takvatn are coded as in Table 2 with indication of host name and life-cycle stage (S, sporocyst; C, cercaria). The scale-bar indicates the expected number of substitutions per site. Sequence identification is as in GenBank, followed by a letter: Al, Aldhoun et al. (2009a,b, unpublished); B, Brant and Loker (2009), Brant et al. (2013); Ch, Christiansen et al. (2016); J, Jouet et al. (2010); P, Pinto et al. (2014); R, Rudolfová et al. (2005, 2007).

Table 1 Details for the alignments used in the phylogenetic analyses

Trematode group	Gene/ region	Alignment	No. of newly-generated sequences	No. of sequences retrieved from GenBank[a]	No. of species[a,b]	Outgroup	Alignment length	Model
Family Allocreadiidae	28S rRNA	1	23	48	26	*Polylekithum ictaluri*	721	GTR+I+Γ
Genus *Crepidostomum* (Allocreadiidae)	28S rRNA	2	11	18	9	*Allocreadium lobatum*	714	GTR+I
Family Strigeidae	*cox*1	3	21	44	22	*Diplostomum spathaceum*	407	GTR+I+Γ
	28S rRNA	4	8	10	8	*Diplostomum phoxini*	975	GTR+I+Γ
Genus *Diplostomum* (Diplostomidae)	*cox*1	5	29	83	35	*Tylodelphys clavata*	407	HKY+I+Γ
Genus *Tylodelphys* (Diplostomidae)	*cox*1	6	2	37	14	*Diplostomum spathaceum*	407	GTR+I+Γ
Genus *Plagiorchis* (Plagiorchiidae)	*cox*1	7	28	13	6	*Choledocystus hepaticus*	423	GTR+I+Γ
	28S rRNA	8	16	11	7	*Neoglyphe sobolevi*	1,171	GTR+I+Γ
Genus *Echinoparyphium* (Echinostomatidae)	*nad*1	9	5	16	7	*Echinostoma revolutum*	472	GTR+I+Γ
	28S rRNA	10	3	8	7	*Echinostoma revolutum*	1,190	GTR+I
Genus *Trichobilharzia* (Schistosomatidae)	ITS1-ITS2	11	6	37	16	*Anserobilharzia brantae*	1,297	GTR+I+ Γ & HKY+I
Radix spp. (Lymnaeidae)	ITS2	12	4	26	13	*Lymnaea stagnalis*	367	GTR+I+Γ
Pisidium spp. and *Sphaerium* spp. (Sphaeriidae)	28S rRNA	13	2	15	10	*Eupera platensis*	745	GTR+I+Γ

[a] Ingroup

[b] Sequences retrieved from GenBank

Table 2 Summary data for the isolates from Lake Takvatn used for generation of the new *cox*1, *nad*1, 28S rDNA and ITS1-5.8S-ITS2/ITS2 sequences.

Species	**Host species**	**Host family**	**Life-cycle stage**[a]	**Isolate**	**Gene**	**GenBank accession number***
Family Allocreadiidae Looss, 1902						
Allocreadium neotenicum Peters, 1957	*Oreodytes sanmarkii*	Dytiscidae	M	ANTAK1, 2	28S	G203; G204
Crepidostomum farionis (Müller, 1780)	*Pisidium casertanum*	Sphaeriidae	R	CFTAK1, 2	28S	S529; S530
	Sphaerium sp.	Sphaeriidae	R	CFTAK3, 4	28S	G185; G186
	Pisidium casertanum	Sphaeriidae	C	CFTAK5, 6	28S	G190; G191
Crepidostomum metoecus (Braun, 1900)	*Pisidium casertanum*	Sphaeriidae	R	CMTAK1	28S	G189
	Gammarus lacustris	Gammaridae	M	CMTAK2-8	28S	S491; S570; G195; G196; G197; G198; G199
	Salmo trutta	Salmonidae	A	CMTAK9	28S	G193
Crepidostomum sp. 1	*Sphaerium* sp.	Sphaeriidae	C	CSP1TAK1	28S	S526
	Siphlonurus lacustris	Siphlonuridae	M	CSP1TAK2	28S	G202
Crepidostomum sp. 2	*Siphlonurus lacustris*	Siphlonuridae	M	CSP2TAK1	28S	S486
	Diura bicaudata	Perlodidae	M	CSP2TAK2, 3	28S	G200; G201
	Salmo trutta	Salmonidae	A	CSP2TAK4	28S	G194
Family Diplostomidae Poirier, 1886						
Diplostomum phoxini (Faust, 1918)	*Radix balthica*	Lymnaeidae	C	DPTAK1	*cox*1	S853
	Phoxinus phoxinus[d]	Cyprinidae	M	DPTAK2	*cox*1	S845
Diplostomum sp. 'Lineage 3'[b]	*Salmo trutta*	Salmonidae	M	DLIN3TAK1-3	*cox*1	S837; S839; S840
	Salvelinus alpinus	Salmonidae	M	DLIN3TAK4	*cox*1	S847
Diplostomum sp. 'Lineage 4'[b]	*Radix balthica*	Lymnaeidae	C	DLIN4TAK1-3	*cox*1	S851; S852; S856
	Gasterosteus aculeatus	Gasterosteidae	M	DLIN4TAK4, 5	*cox*1	S831; S834
Diplostomum sp. 'Lineage 5'[b]	*Gasterosteus aculeatus*	Gasterosteidae	M	DLIN5TAK1	*cox*1	S829
	Salmo trutta	Salmonidae	M	DLIN5TAK2	*cox*1	S836
	Salvelinus alpinus	Salmonidae	M	DLIN5TAK3-9	*cox*1	S842; S843; S844; S846; S848; S849; S850
Diplostomum sp. 'Lineage 6'[b]	*Radix balthica*	Lymnaeidae	C	DLIN6TAK1-5	*cox*1	S854; S855; S857; S858; S859
	Gasterosteus aculeatus	Gasterosteidae	M	DLIN6TAK6	*cox*1	S828; S830; S832; S835

				-9		
Tylodelphys sp.	*Salmo trutta*	Salmonidae	M	TSPTAK1	*cox*1	S838
	Salvelinus alpinus	Salmonidae	M	TSPTAK2	*cox*1	S841
Family Echinostomatidae Looss, 1899						
Echinoparyphium recurvatum (Linstow, 1873)	*Radix balthica*	Lymnaeidae	R	ERTAK1	*nad*1/28S	Ge621/S508
	Sphaerium sp.	Sphaeriidae	M	ERTAK2	*nad*1/28S	S960/Ge591
	Pisidium casertanum	Sphaeriidae	M	ERTAK3	*nad*1	S961
	Sphaerium sp.	Sphaeriidae	M	ERTAK4,5	*nad*1/28S	S962; Ge622/S528
Family Notocotylidae Lühe, 1909						
Notocotylus sp.	*Radix balthica*	Lymnaeidae	C	NSPTAK1	28S	G205
Family Plagiorchiidae Lühe, 1901						
Plagiorchis sp. 1	*Radix balthica*	Lymnaeidae	S	PSP1TAK1,2	*cox*1	S881; S882
	Radix balthica	Lymnaeidae	C	PSP1TAK3-12	*cox*1/28S	S992; S940; S872; S873; S874; S875; S876; S877; S941; S942/S485; S533; S1005
	Tipula salicetorum	Tipulidae	M	PSP1TAK13	28S	S487
Plagiorchis sp. 2	*Radix balthica*	Lymnaeidae	S	PSP2TAK1	*cox*1/28S	Ge756/S568
	Radix balthica	Lymnaeidae	C	PSP2TAK2,3	*cox*1/28S	S867; S869/S1003
	Radix balthica	Lymnaeidae	M	PSP2TAK4	*cox*1	S871
	Gammarus lacustris	Gammaridae	M	PSP2TAK5,6	*cox*1/28S	Ge754; Ge755/S489
Plagiorchis sp. 3	*Radix balthica*	Lymnaeidae	C	PSP3TAK1-3	*cox*1/28S	S879; S880; S939/S995
	Tipula salicetorum	Tipulidae	M	PSP3TAK4	*cox*1/28S	Ge757/S510
	Oreodytes alpinus	Dytiscidae	M	PSP3TAK5	28S	S514
Plagiorchis sp. 4	*Radix balthica*	Lymnaeidae	C	PSP4TAK1	*cox*1/28S	S868/S1004
	Radix balthica	Lymnaeidae	M	PSP4TAK2	*cox*1	S878
Plagiorchis sp. 5	*Radix balthica*	Lymnaeidae	C	PSP5TAK1,2	*cox*1/28S	S866; S870/S1001
	Sialis lutaria	Sialidae	M	PSP5TAK3	28S	S511
	Oreodytes alpinus	Dytiscidae	M	PSP5TAK4	28S	S490

Plagiorchis sp. 6	*Radix balthica*	Lymnaeidae	C	PSP6TAK1	*cox*1/28S	S943/S1002
Plagiorchis sp. 7	*Radix balthica*	Lymnaeidae	C	PSP7TAK1	*cox*1/28S	S991/S532
Family Schistosomatidae Stiles & Hassall, 1898						
Trichobilharzia franki haplotype "peregra"[c]	*Radix balthica*	Lymnaeidae	C	TFPTAK1-6	ITS1-5.8S-ITS2	G160; G161; G162; G163; G164; GeM1
Family Strigeidae Railliet, 1919						
Apatemon gracilis (Rudolphi, 1819)	*Radix balthica*	Lymnaeidae	S	AGTAK1-3	*cox*1	S550; S551; S919
	Radix balthica	Lymnaeidae	C	AGTAK4-10	*cox*1/28S	S552; S553; S554; S917; S860; S861; Ge584/Ge586; S512
	Gasterosteus aculeatus	Gasterosteidae	M	AGTAK11-13	*cox*1/28S	S833; G178; G177/Ge585
Apatemon sp.	*Gasterosteus aculeatus*	Gasterosteidae	M	ASPTAK1, 2	*cox*1/28S	G179; G180/Ge587; Ge588
Cotylurus cornutus (Rudolphi, 1808)	*Radix balthica*	Lymnaeidae	S	CCTAK1	*cox*1	S920
	Radix balthica	Lymnaeidae	M	CCTAK2-5	*cox*1/28S	S862; S863; S864; S865/Ge590
	Gyraulus acronicus	Planorbidae	M	CCTAK6, 7	*cox*1/28S	S555/Ge589; G28
Family Lymnaeidae Rafinesque, 1815						
Radix balthica (Linnaeus, 1758)	–	–	A	RBTAK1-4	ITS2	CS15-CS18
Family Sphaeriidae Deshayes, 1855						
Sphaerim sp.	–	–	A	SSPTAK1	28S	G208
Pisidium casertanum (Poli, 1791)	–	–	A	PCTAK1	28S	G207

[a] Life-cycle stages: A, adult; C, cercaria; R, redia; M, metacercaria/progenetic metacercaria of *A. neotenicum*.

[b] Lineages discovered in Iceland and characterised molecularly and morphologically by Blasco-Costa et al. (2014) and Faltýnková et al. (2014), respectively.

[c] *sensu* Jouet et al. (2010).

[d] Metacercaria ex *Phoxinus phoxinus* sampled at Lake Øvre Heimdalsvatnet, Norway.

*Sequence codes in this column will be replaced with sequence IDs

Table 3 Summary data for the intermediate hosts of the molecularly identified isolates and the possible definitive hosts of the trematodes completing their life-cycles in Takvatn. Possible fish definitive hosts are inferred from life-cycle data available for congeneric parasites; possible bird definitive hosts at Takvatn are inferred based on the records of congeneric digeneans in the Host-Parasite Database of the Natural History Museum, London (Gibson et al., 2005); only bird species breeding at the lake are considered as possible hosts.

Species	First intermediate host	Second intermediate host	Definitive hosts
Family Allocreadiidae			
Allocreadium neotenicum		*Oreodytes sanmarkii*	?
Crepidostomum farionis	*Pisidium casertanum*; *Sphaerium* sp.		*Gasterosteus aculeatus*; *Salmo trutta*; *Salvelinus alpinus*
Crepidostomum metoecus	*Pisidium casertanum*	*Gammarus lacustris*	*Gasterosteus aculeatus*; *Salmo trutta**; *Salvelinus alpinus*
Crepidostomum sp. 1[a]	*Sphaerium* sp.	*Siphlonurus lacustris*	*Gasterosteus aculeatus*; *Salmo trutta*; *Salvelinus alpinus*
Crepidostomum sp. 2[a]		*Siphlonurus lacustris*; *Diura bicaudata*	*Gasterosteus aculeatus*; *Salmo trutta**; *Salvelinus alpinus*
Family Diplostomidae			
Diplostomum phoxini	*Radix balthica*		*Aythya fuligula*; *Bucephala clangula*; *Gavia arctica*; *Larus canus*; *Mergus serrator*; *Sterna paradisaea*
Diplostomum sp. 'Lineage 3'[a,b]		*Salmo trutta*; *Salvelinus alpinus*	
Diplostomum sp. 'Lineage 4'[a,b]	*Radix balthica*	*Gasterosteus aculeatus*	
Diplostomum sp. 'Lineage 5'[a,b]		*Gasterosteus aculeatus*; *Salmo trutta*; *Salvelinus alpinus*	
Diplostomum sp. 'Lineage 6'[a,b]	*Radix balthica*	*Gasterosteus aculeatus*	
Tylodelphys sp.[a]		*Salmo trutta*; *Salvelinus alpinus*	*Gavia arctica*
Family Echinostomatidae			
Echinoparyphium recurvatum	*Radix balthica*	*Sphaerium* sp.; *Pisidium casertanum*	*Anas penelope*; *Anas platyrhynchos*; *Aythya fuligula*; *Bucephala clangula*; *Larus canus*; *Melanitta fusca*; *Melanitta nigra*; *Tringa totanus*
Family Notocotylidae			
Notocotylus sp.[d]	*Radix balthica*	–	*Anas penelope*; *Anas platyrhynchos*; *Aythya fuligula*; *Bucephala clangula*; *Larus canus*; *Melanitta fusca*
Family Plagiorchiidae			
Plagiorchis sp. 1[a]	*Radix balthica*	*Tipula salicetorum*	*Anas platyrhynchos*; *Aythya fuligula*; *Larus canus*; *Tringa hypoleucos*; *Tringa totanus*
Plagiorchis sp. 2[a]	*Radix balthica*	*Gammarus lacustris*	
Plagiorchis sp. 3[a]	*Radix balthica*	*Tipula salicetorum*; *Oreodytes alpinus*	
Plagiorchis sp. 4[a]	*Radix balthica*		
Plagiorchis sp. 5[a]	*Radix balthica*	*Sialis lutaria*; *Oreodytes alpinus*	
Plagiorchis sp. 6[a]	*Radix balthica*		
Plagiorchis sp. 7[a]	*Radix balthica*		
Family Schistosomatidae			
Trichobilharzia franki haplotype "peregra" [a, c, d]	*Radix balthica*	–	*Anas penelope*; *Anas platyrhynchos*; *Aythya fuligula*; *Bucephala clangula*
Family Strigeidae			
Apatemon gracilis	*Radix balthica*	*Gasterosteus aculeatus*	*Anas penelope*; *Anas platyrhynchos*; *Aythya fuligula*; *Bucephala clangula*; *Melanitta fusca*; *Melanitta nigra*; *Mergus serrator*
Apatemon sp.[a]		*Gasterosteus aculeatus*	
Cotylurus cornutus	*Radix balthica*	*Radix balthica*; *Gyraulus acronicus*	*Anas penelope*; *Anas platyrhynchos*; *Aythya fuligula*; *Bucephala clangula*; *Melanitta*

nigra; *Sterna paradisaea*; *Tringa totanus*

[a] Putative new species

[b] Lineages discovered in Iceland and characterised molecularly and morphologically by Blasco-Costa et al. (2014) and Faltýnková et al. (2014)

[c] Lineage discovered in Iceland by Jouet et al. (2010) based on molecular data

[d] No second intermediate host in the life-cycle

* Hosts of adult isolates sequenced

Figure 1

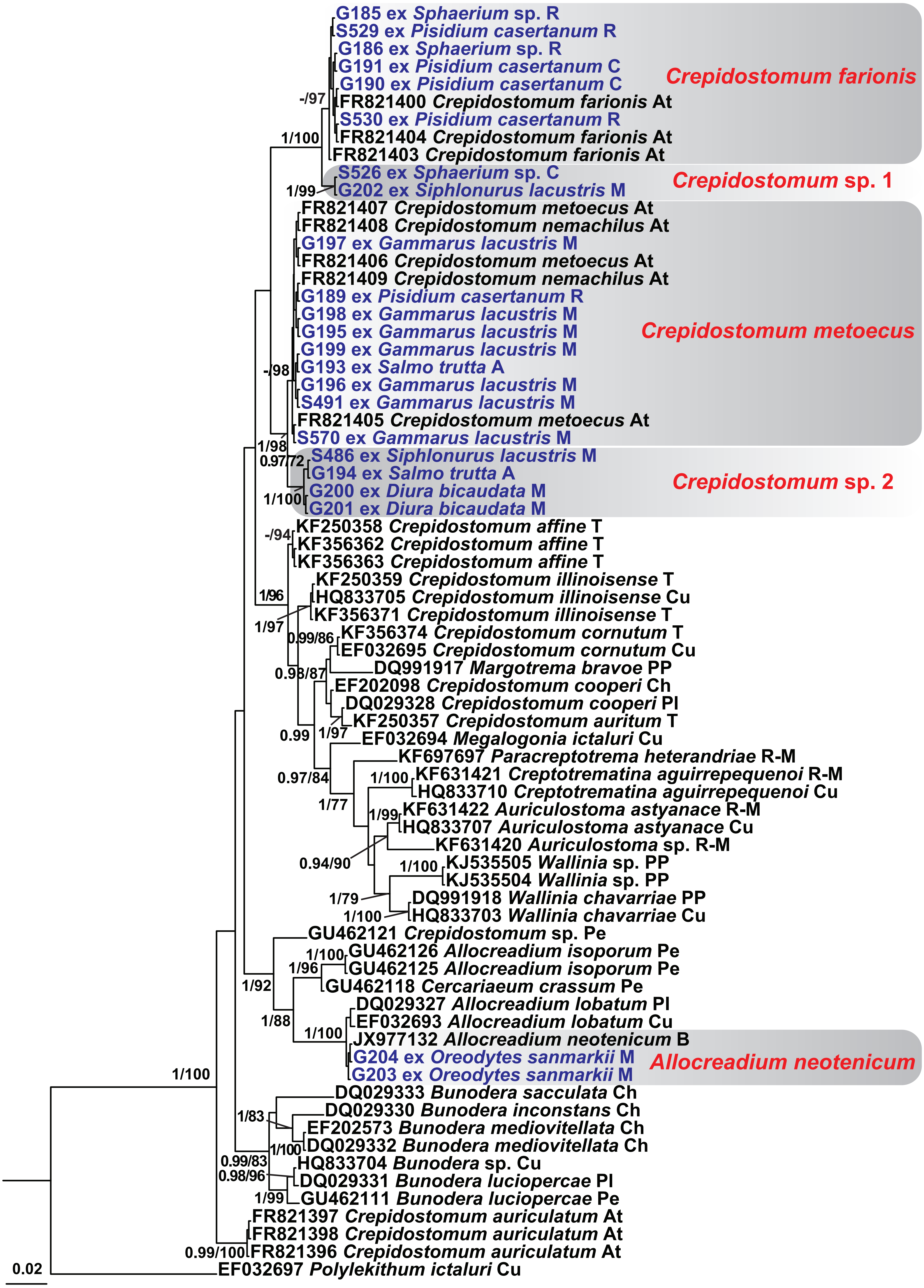
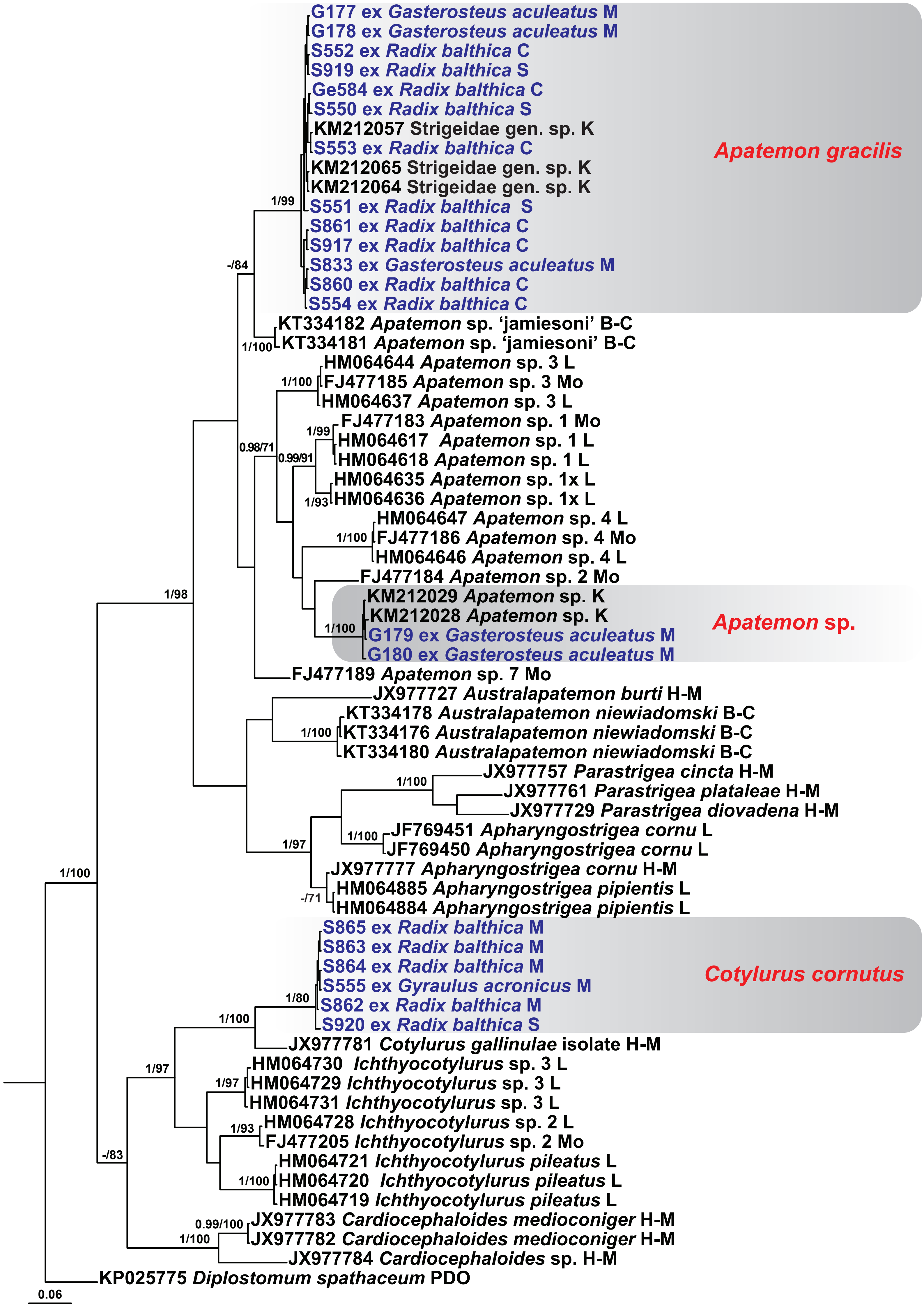

G185 ex Sphaerium sp. R
S529 ex Pisidium casertanum R
G186 ex Sphaerium sp. R
G191 ex Pisidium casertanum C
G190 ex Pisidium casertanum C
FR821400 Crepidostomum farionis At
S530 ex Pisidium casertanum R
FR821404 Crepidostomum farionis At
FR821403 Crepidostomum farionis At
Crepidostomum farionis
-/97
1/100
S526 ex Sphaerium sp. C
G202 ex Siphlonurus lacustris M
Crepidostomum sp. 1
1/99
FR821407 Crepidostomum metoecus At
FR821408 Crepidostomum nemachilus At
G197 ex Gammarus lacustris M
FR821406 Crepidostomum metoecus At
FR821409 Crepidostomum nemachilus At
G189 ex Pisidium casertanum R
G198 ex Gammarus lacustris M
G195 ex Gammarus lacustris M
G199 ex Gammarus lacustris M
G193 ex Salmo trutta A
G196 ex Gammarus lacustris M
S491 ex Gammarus lacustris M
FR821405 Crepidostomum metoecus At
S570 ex Gammarus lacustris M
Crepidostomum metoecus
-/98
1/98
0.97/72
S486 ex Siphlonurus lacustris M
G194 ex Salmo trutta A
G200 ex Diura bicaudata M
G201 ex Diura bicaudata M
Crepidostomum sp. 2
1/100
KF250358 Crepidostomum affine T
KF356362 Crepidostomum affine T
KF356363 Crepidostomum affine T
-/94
KF250359 Crepidostomum illinoisense T
HQ833705 Crepidostomum illinoisense Cu
KF356371 Crepidostomum illinoisense T
1/96
1/97
KF356374 Crepidostomum cornutum T
EF032695 Crepidostomum cornutum Cu
0.99/86
DQ991917 Margotrema bravoe PP
0.98/87
EF202098 Crepidostomum cooperi Ch
DQ029328 Crepidostomum cooperi Pl
KF250357 Crepidostomum auritum T
0.99
1/97
EF032694 Megalogonia ictaluri Cu
KF697697 Paracreptotrema heterandriae R-M
0.97/84
KF631421 Creptotrematina aguirrepequenoi R-M
HQ833710 Creptotrematina aguirrepequenoi Cu
1/100
1/77
KF631422 Auriculostoma astyanace R-M
HQ833707 Auriculostoma astyanace Cu
1/99
KF631420 Auriculostoma sp. R-M
0.94/90
KJ535505 Wallinia sp. PP
KJ535504 Wallinia sp. PP
1/100
DQ991918 Wallinia chavarriae PP
HQ833703 Wallinia chavarriae Cu
1/79
1/100
GU462121 Crepidostomum sp. Pe
GU462126 Allocreadium isoporum Pe
GU462125 Allocreadium isoporum Pe
1/100
1/96
GU462118 Cercariaeum crassum Pe
1/92
DQ029327 Allocreadium lobatum Pl
EF032693 Allocreadium lobatum Cu
JX977132 Allocreadium neotenicum B
G204 ex Oreodytes sanmarkii M
G203 ex Oreodytes sanmarkii M
Allocreadium neotenicum
1/88
1/100
1/100
DQ029333 Bunodera sacculata Ch
DQ029330 Bunodera inconstans Ch
EF202573 Bunodera mediovitellata Ch
DQ029332 Bunodera mediovitellata Ch
1/83
1/100
HQ833704 Bunodera sp. Cu
DQ029331 Bunodera luciopercae Pl
GU462111 Bunodera luciopercae Pe
0.99/83
0.98/96
1/99
FR821397 Crepidostomum auriculatum At
FR821398 Crepidostomum auriculatum At
FR821396 Crepidostomum auriculatum At
0.99/100
EF032697 Polylekithum ictaluri Cu
0.02

Figure 2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G178 ex Gasterosteus aculeatus M
S552 ex Radix balthica C
S919 ex Radix balthica S
Ge584 ex Radix balthica C
S550 ex Radix balthica S
KM212057 Strigeidae gen. sp. K
S553 ex Radix balthica C
KM212065 Strigeidae gen. sp. K
KM212064 Strigeidae gen. sp. K
S551 ex Radix balthica S
S861 ex Radix balthica C
S917 ex Radix balthica C
S833 ex Gasterosteus aculeatus M
S860 ex Radix balthica C
S554 ex Radix balthica C
Apatemon gracilis
1/99
-/84
KT334182 Apatemon sp. 'jamiesoni' B-C
KT334181 Apatemon sp. 'jamiesoni' B-C
1/100
HM064644 Apatemon sp. 3 L
FJ477185 Apatemon sp. 3 Mo
HM064637 Apatemon sp. 3 L
1/100
FJ477183 Apatemon sp. 1 Mo
HM064617 Apatemon sp. 1 L
HM064618 Apatemon sp. 1 L
1/99
0.98/71
0.99/91
HM064635 Apatemon sp. 1x L
HM064636 Apatemon sp. 1x L
1/93
HM064647 Apatemon sp. 4 L
FJ477186 Apatemon sp. 4 Mo
HM064646 Apatemon sp. 4 L
1/100
FJ477184 Apatemon sp. 2 Mo
KM212029 Apatemon sp. K
KM212028 Apatemon sp. K
G179 ex Gasterosteus aculeatus M
G180 ex Gasterosteus aculeatus M
1/100
Apatemon sp.
1/98
FJ477189 Apatemon sp. 7 Mo
JX977727 Australapatemon burti H-M
KT334178 Australapatemon niewiadomski B-C
KT334176 Australapatemon niewiadomski B-C
KT334180 Australapatemon niewiadomski B-C
1/100
JX977757 Parastrigea cincta H-M
JX977761 Parastrigea plataleae H-M
JX977729 Parastrigea diovadena H-M
1/100
JF769451 Apharyngostrigea cornu L
JF769450 Apharyngostrigea cornu L
1/100
1/97
JX977777 Apharyngostrigea cornu H-M
HM064885 Apharyngostrigea pipientis L
HM064884 Apharyngostrigea pipientis L
-/71
1/100
S865 ex Radix balthica M
S863 ex Radix balthica M
S864 ex Radix balthica M
S555 ex Gyraulus acronicus M
S862 ex Radix balthica M
S920 ex Radix balthica S
Cotylurus cornutus
1/80
1/100
JX977781 Cotylurus gallinulae isolate H-M
1/97
HM064730 Ichthyocotylurus sp. 3 L
HM064729 Ichthyocotylurus sp. 3 L
HM064731 Ichthyocotylurus sp. 3 L
1/97
HM064728 Ichthyocotylurus sp. 2 L
FJ477205 Ichthyocotylurus sp. 2 Mo
1/93
HM064721 Ichthyocotylurus pileatus L
HM064720 Ichthyocotylurus pileatus L
HM064719 Ichthyocotylurus pileatus L
1/100
-/83
JX977783 Cardiocephaloides medioconiger H-M
JX977782 Cardiocephaloides medioconiger H-M
0.99/100
1/100
JX977784 Cardiocephaloides sp. H-M
KP025775 Diplostomum spathaceum PDO
0.06

Figure 3

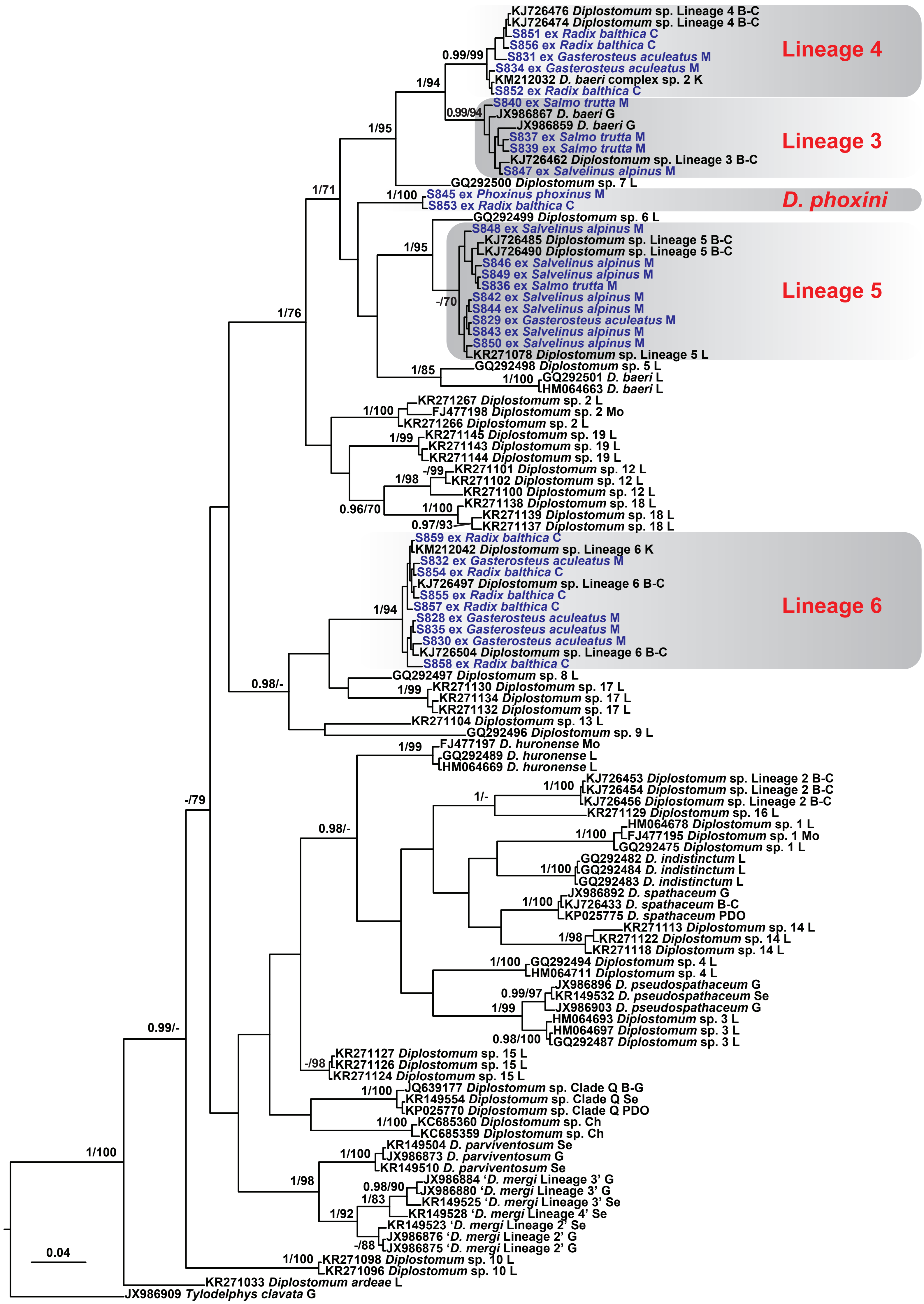

KJ726476 *Diplostomum* sp. Lineage 4 B-C
KJ726474 *Diplostomum* sp. Lineage 4 B-C
S851 ex *Radix balthica* C
S856 ex *Radix balthica* C
0.99/99
S831 ex *Gasterosteus aculeatus* M
S834 ex *Gasterosteus aculeatus* M
KM212032 *D. baeri* complex sp. 2 K
1/94
S852 ex *Radix balthica* C
Lineage 4
S840 ex *Salmo trutta* M
0.99/94
JX986867 *D. baeri* G
1/95
JX986859 *D. baeri* G
S837 ex *Salmo trutta* M
S839 ex *Salmo trutta* M
Lineage 3
KJ726462 *Diplostomum* sp. Lineage 3 B-C
S847 ex *Salvelinus alpinus* M
GQ292500 *Diplostomum* sp. 7 L
1/71
1/100
S845 ex *Phoxinus phoxinus* M
S853 ex *Radix balthica* C
D. phoxini
GQ292499 *Diplostomum* sp. 6 L
S848 ex *Salvelinus alpinus* M
KJ726485 *Diplostomum* sp. Lineage 5 B-C
1/95
KJ726490 *Diplostomum* sp. Lineage 5 B-C
S846 ex *Salvelinus alpinus* M
S849 ex *Salvelinus alpinus* M
S836 ex *Salmo trutta* M
Lineage 5
-/70
S842 ex *Salvelinus alpinus* M
S844 ex *Salvelinus alpinus* M
1/76
S829 ex *Gasterosteus aculeatus* M
S843 ex *Salvelinus alpinus* M
S850 ex *Salvelinus alpinus* M
KR271078 *Diplostomum* sp. Lineage 5 L
GQ292498 *Diplostomum* sp. 5 L
1/85
1/100
GQ292501 *D. baeri* L
HM064663 *D. baeri* L
KR271267 *Diplostomum* sp. 2 L
1/100
FJ477198 *Diplostomum* sp. 2 Mo
KR271266 *Diplostomum* sp. 2 L
KR271145 *Diplostomum* sp. 19 L
1/99
KR271143 *Diplostomum* sp. 19 L
KR271144 *Diplostomum* sp. 19 L
-/99
KR271101 *Diplostomum* sp. 12 L
1/98
KR271102 *Diplostomum* sp. 12 L
KR271100 *Diplostomum* sp. 12 L
1/100
KR271138 *Diplostomum* sp. 18 L
0.96/70
KR271139 *Diplostomum* sp. 18 L
0.97/93
KR271137 *Diplostomum* sp. 18 L
S859 ex *Radix balthica* C
KM212042 *Diplostomum* sp. Lineage 6 K
S832 ex *Gasterosteus aculeatus* M
S854 ex *Radix balthica* C
KJ726497 *Diplostomum* sp. Lineage 6 B-C
S855 ex *Radix balthica* C
S857 ex *Radix balthica* C
1/94
Lineage 6
S828 ex *Gasterosteus aculeatus* M
S835 ex *Gasterosteus aculeatus* M
S830 ex *Gasterosteus aculeatus* M
KJ726504 *Diplostomum* sp. Lineage 6 B-C
S858 ex *Radix balthica* C
GQ292497 *Diplostomum* sp. 8 L
0.98/-
KR271130 *Diplostomum* sp. 17 L
1/99
KR271134 *Diplostomum* sp. 17 L
KR271132 *Diplostomum* sp. 17 L
KR271104 *Diplostomum* sp. 13 L
GQ292496 *Diplostomum* sp. 9 L
1/99
FJ477197 *D. huronense* Mo
GQ292489 *D. huronense* L
HM064669 *D. huronense* L
KJ726453 *Diplostomum* sp. Lineage 2 B-C
1/100
KJ726454 *Diplostomum* sp. Lineage 2 B-C
-/79
1/-
KJ726456 *Diplostomum* sp. Lineage 2 B-C
KR271129 *Diplostomum* sp. 16 L
HM064678 *Diplostomum* sp. 1 L
0.98/-
1/100
FJ477195 *Diplostomum* sp. 1 Mo
GQ292475 *Diplostomum* sp. 1 L
GQ292482 *D. indistinctum* L
1/100
GQ292484 *D. indistinctum* L
GQ292483 *D. indistinctum* L
JX986892 *D. spathaceum* G
1/100
KJ726433 *D. spathaceum* B-C
KP025775 *D. spathaceum* PDO
KR271113 *Diplostomum* sp. 14 L
1/98
KR271122 *Diplostomum* sp. 14 L
KR271118 *Diplostomum* sp. 14 L
1/100
GQ292494 *Diplostomum* sp. 4 L
HM064711 *Diplostomum* sp. 4 L
JX986896 *D. pseudospathaceum* G
0.99/97
KR149532 *D. pseudospathaceum* Se
1/99
JX986903 *D. pseudospathaceum* G
HM064693 *Diplostomum* sp. 3 L
HM064697 *Diplostomum* sp. 3 L
0.99/-
0.98/100
GQ292487 *Diplostomum* sp. 3 L
KR271127 *Diplostomum* sp. 15 L
-/98
KR271126 *Diplostomum* sp. 15 L
KR271124 *Diplostomum* sp. 15 L
1/100
JQ639177 *Diplostomum* sp. Clade Q B-G
KR149554 *Diplostomum* sp. Clade Q Se
KP025770 *Diplostomum* sp. Clade Q PDO
1/100
KC685360 *Diplostomum* sp. Ch
KC685359 *Diplostomum* sp. Ch
KR149504 *D. parviventosum* Se
1/100
JX986873 *D. parviventosum* G
KR149510 *D. parviventosum* Se
1/98
0.98/90
JX986884 ‘*D. mergi* Lineage 3’ G
JX986880 ‘*D. mergi* Lineage 3’ G
1/83
KR149525 ‘*D. mergi* Lineage 3’ Se
1/92
KR149528 ‘*D. mergi* Lineage 4’ Se
KR149523 ‘*D. mergi* Lineage 2’ Se
-/88
JX986876 ‘*D. mergi* Lineage 2’ G
JX986875 ‘*D. mergi* Lineage 2’ G
1/100
KR271098 *Diplostomum* sp. 10 L
1/100
KR271096 *Diplostomum* sp. 10 L
KR271033 *Diplostomum ardeae* L
0.04
JX986909 *Tylodelphys clavata* G

Figure 4

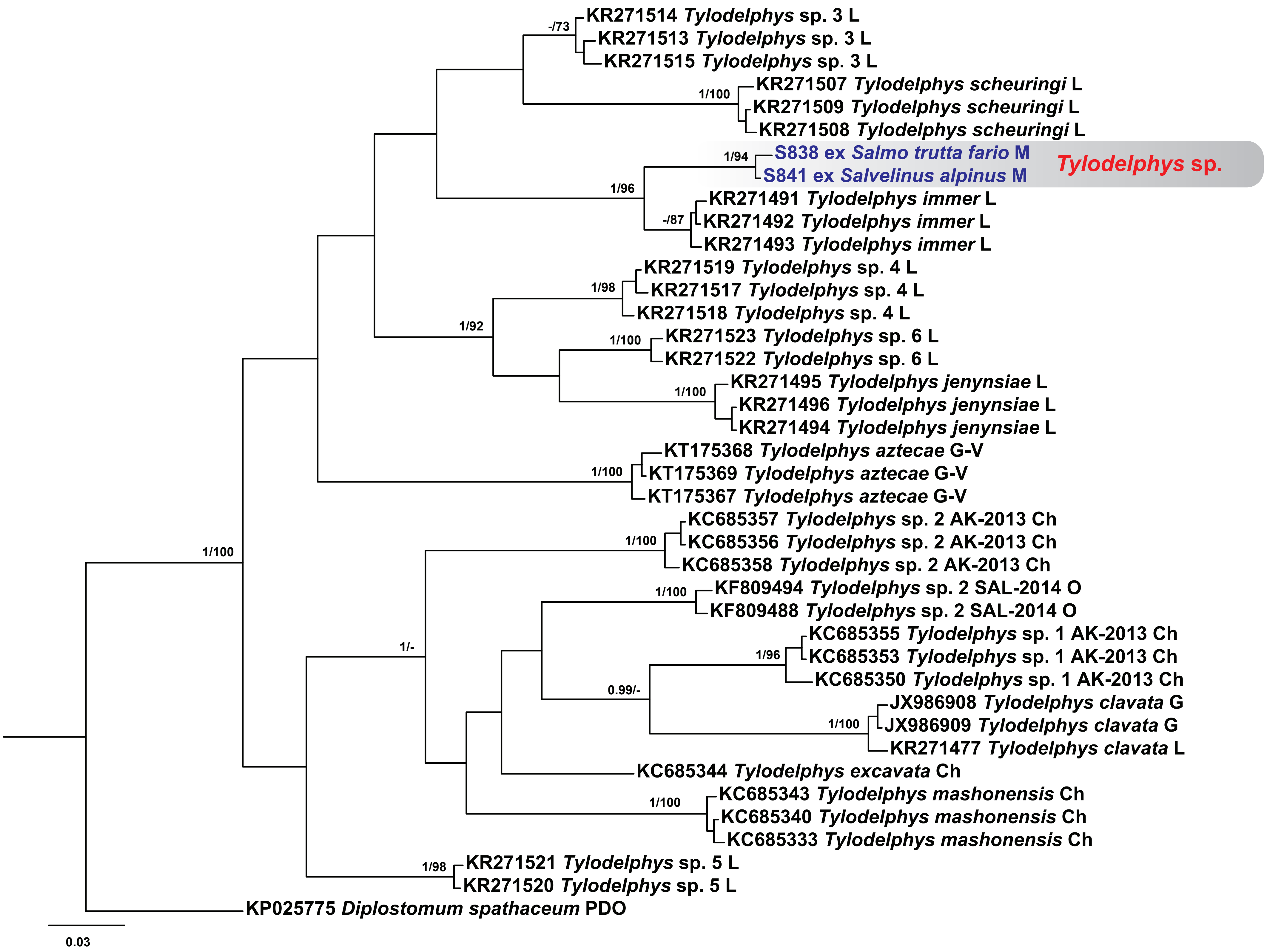

-/73
KR271514 Tylodelphys sp. 3 L
KR271513 Tylodelphys sp. 3 L
KR271515 Tylodelphys sp. 3 L
1/100
KR271507 Tylodelphys scheuringi L
KR271509 Tylodelphys scheuringi L
KR271508 Tylodelphys scheuringi L
1/94
S838 ex Salmo trutta fario M
S841 ex Salvelinus alpinus M
Tylodelphys sp.
1/96
KR271491 Tylodelphys immer L
-/87
KR271492 Tylodelphys immer L
KR271493 Tylodelphys immer L
KR271519 Tylodelphys sp. 4 L
1/98
KR271517 Tylodelphys sp. 4 L
KR271518 Tylodelphys sp. 4 L
1/92
1/100
KR271523 Tylodelphys sp. 6 L
KR271522 Tylodelphys sp. 6 L
KR271495 Tylodelphys jenynsiae L
1/100
KR271496 Tylodelphys jenynsiae L
KR271494 Tylodelphys jenynsiae L
KT175368 Tylodelphys aztecae G-V
1/100
KT175369 Tylodelphys aztecae G-V
KT175367 Tylodelphys aztecae G-V
KC685357 Tylodelphys sp. 2 AK-2013 Ch
1/100
KC685356 Tylodelphys sp. 2 AK-2013 Ch
1/100
KC685358 Tylodelphys sp. 2 AK-2013 Ch
1/100
KF809494 Tylodelphys sp. 2 SAL-2014 O
KF809488 Tylodelphys sp. 2 SAL-2014 O
1/-
KC685355 Tylodelphys sp. 1 AK-2013 Ch
1/96
KC685353 Tylodelphys sp. 1 AK-2013 Ch
0.99/-
KC685350 Tylodelphys sp. 1 AK-2013 Ch
JX986908 Tylodelphys clavata G
1/100
JX986909 Tylodelphys clavata G
KR271477 Tylodelphys clavata L
KC685344 Tylodelphys excavata Ch
KC685343 Tylodelphys mashonensis Ch
1/100
KC685340 Tylodelphys mashonensis Ch
KC685333 Tylodelphys mashonensis Ch
1/98
KR271521 Tylodelphys sp. 5 L
KR271520 Tylodelphys sp. 5 L
KP025775 Diplostomum spathaceum PDO
0.03

Figure 5

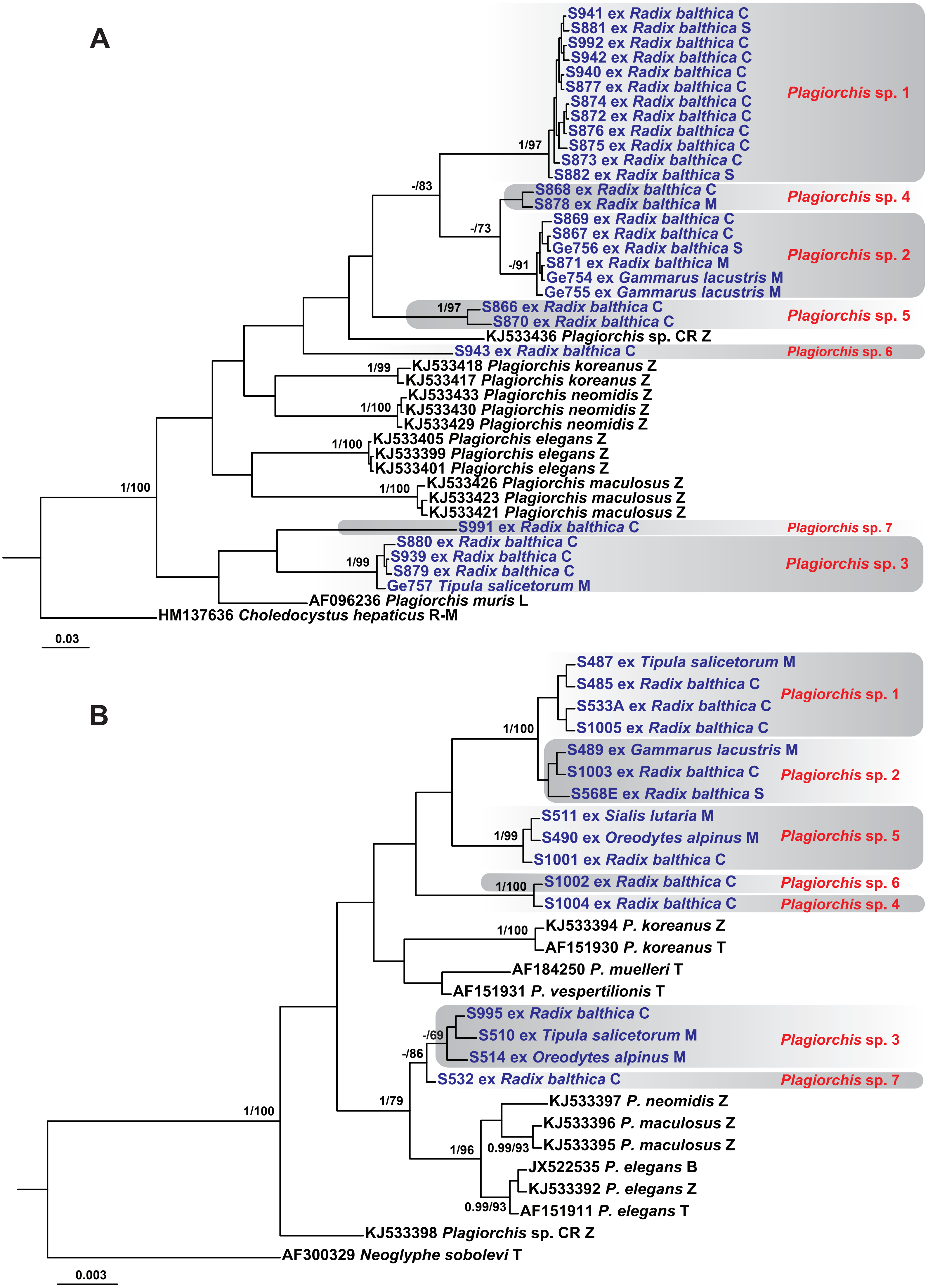

A
S941 ex Radix balthica C
S881 ex Radix balthica S
S992 ex Radix balthica C
S942 ex Radix balthica C
S940 ex Radix balthica C
S877 ex Radix balthica C
S874 ex Radix balthica C
S872 ex Radix balthica C
S876 ex Radix balthica C
S875 ex Radix balthica C
S873 ex Radix balthica C
S882 ex Radix balthica S
Plagiorchis sp. 1
1/97
-/83
S868 ex Radix balthica C
S878 ex Radix balthica M
Plagiorchis sp. 4
-/73
S869 ex Radix balthica C
S867 ex Radix balthica C
Ge756 ex Radix balthica S
S871 ex Radix balthica M
Ge754 ex Gammarus lacustris M
Ge755 ex Gammarus lacustris M
-/91
Plagiorchis sp. 2
1/97
S866 ex Radix balthica C
S870 ex Radix balthica C
Plagiorchis sp. 5
KJ533436 Plagiorchis sp. CR Z
S943 ex Radix balthica C
Plagiorchis sp. 6
1/99
KJ533418 Plagiorchis koreanus Z
KJ533417 Plagiorchis koreanus Z
KJ533433 Plagiorchis neomidis Z
1/100
KJ533430 Plagiorchis neomidis Z
KJ533429 Plagiorchis neomidis Z
1/100
KJ533405 Plagiorchis elegans Z
KJ533399 Plagiorchis elegans Z
KJ533401 Plagiorchis elegans Z
1/100
1/100
KJ533426 Plagiorchis maculosus Z
KJ533423 Plagiorchis maculosus Z
KJ533421 Plagiorchis maculosus Z
S991 ex Radix balthica C
Plagiorchis sp. 7
S880 ex Radix balthica C
1/99
S939 ex Radix balthica C
S879 ex Radix balthica C
Ge757 Tipula salicetorum M
Plagiorchis sp. 3
AF096236 Plagiorchis muris L
HM137636 Choledocystus hepaticus R-M
0.03
B
S487 ex Tipula salicetorum M
S485 ex Radix balthica C
Plagiorchis sp. 1
S533A ex Radix balthica C
1/100
S1005 ex Radix balthica C
S489 ex Gammarus lacustris M
S1003 ex Radix balthica C
Plagiorchis sp. 2
S568E ex Radix balthica S
S511 ex Sialis lutaria M
1/99
S490 ex Oreodytes alpinus M
Plagiorchis sp. 5
S1001 ex Radix balthica C
S1002 ex Radix balthica C
1/100
Plagiorchis sp. 6
S1004 ex Radix balthica C
Plagiorchis sp. 4
1/100
KJ533394 P. koreanus Z
AF151930 P. koreanus T
AF184250 P. muelleri T
AF151931 P. vespertilionis T
S995 ex Radix balthica C
-/69
S510 ex Tipula salicetorum M
Plagiorchis sp. 3
-/86
S514 ex Oreodytes alpinus M
S532 ex Radix balthica C
Plagiorchis sp. 7
1/79
KJ533397 P. neomidis Z
KJ533396 P. maculosus Z
1/96
0.99/93
KJ533395 P. maculosus Z
JX522535 P. elegans B
KJ533392 P. elegans Z
0.99/93
AF151911 P. elegans T
1/100
KJ533398 Plagiorchis sp. CR Z
AF300329 Neoglyphe sobolevi T
0.003

Figure 6

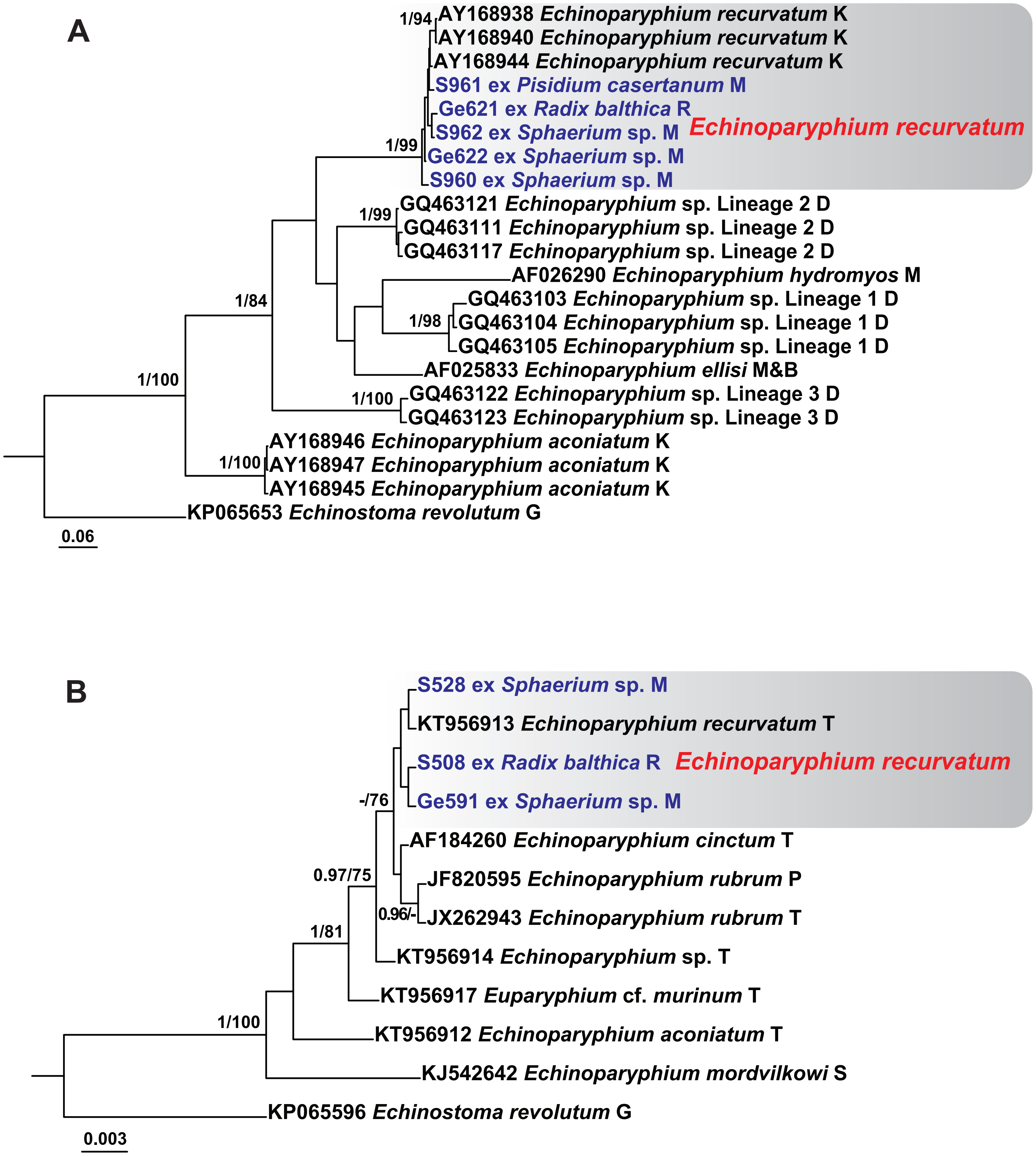
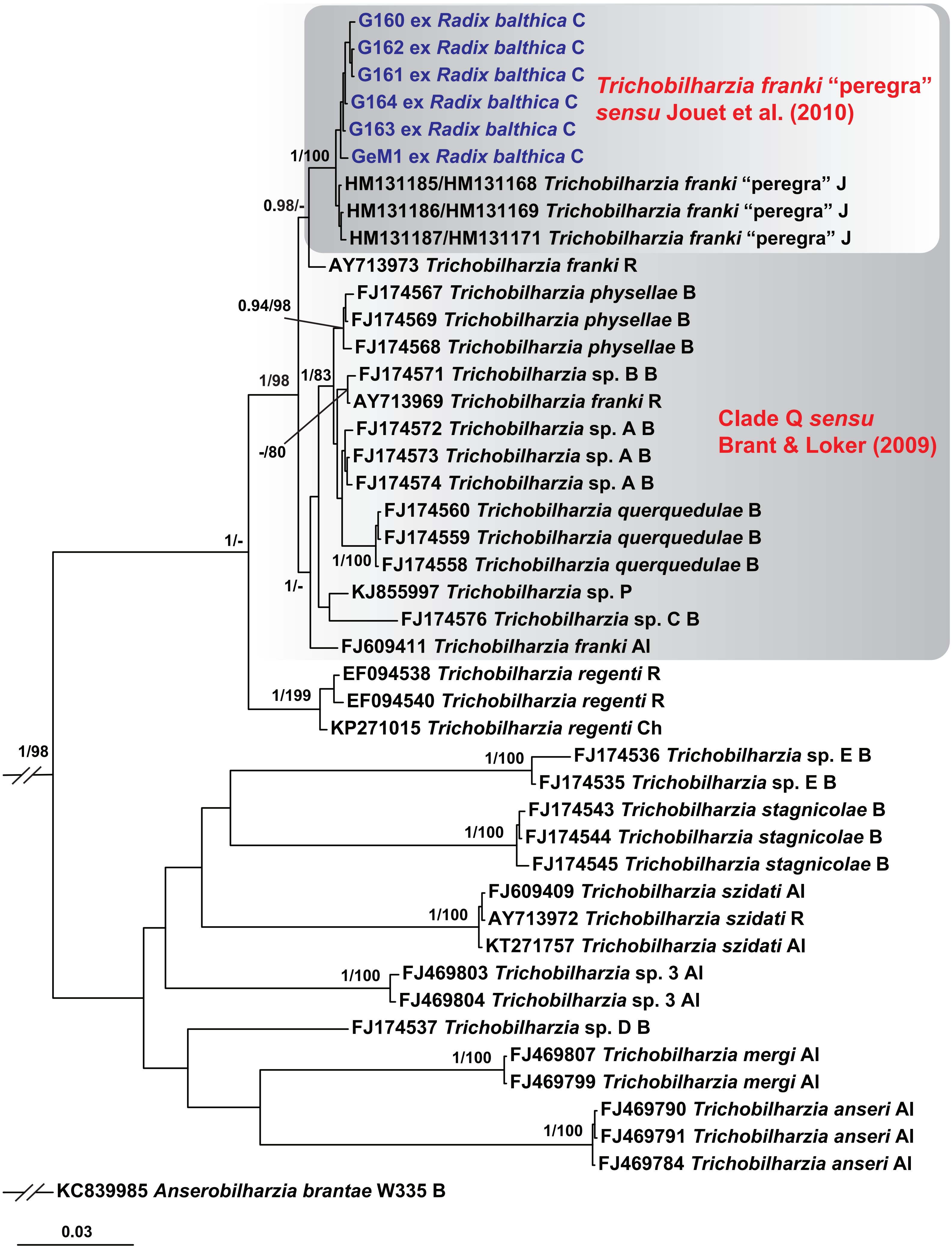

A
1/94
AY168938 Echinoparyphium recurvatum K
AY168940 Echinoparyphium recurvatum K
AY168944 Echinoparyphium recurvatum K
S961 ex Pisidium casertanum M
Ge621 ex Radix balthica R
S962 ex Sphaerium sp. M
Echinoparyphium recurvatum
1/99
Ge622 ex Sphaerium sp. M
S960 ex Sphaerium sp. M
1/99
GQ463121 Echinoparyphium sp. Lineage 2 D
GQ463111 Echinoparyphium sp. Lineage 2 D
GQ463117 Echinoparyphium sp. Lineage 2 D
AF026290 Echinoparyphium hydromyos M
1/84
GQ463103 Echinoparyphium sp. Lineage 1 D
1/98
GQ463104 Echinoparyphium sp. Lineage 1 D
GQ463105 Echinoparyphium sp. Lineage 1 D
AF025833 Echinoparyphium ellisi M&B
1/100
1/100
GQ463122 Echinoparyphium sp. Lineage 3 D
GQ463123 Echinoparyphium sp. Lineage 3 D
AY168946 Echinoparyphium aconiatum K
1/100
AY168947 Echinoparyphium aconiatum K
AY168945 Echinoparyphium aconiatum K
KP065653 Echinostoma revolutum G
0.06
B
S528 ex Sphaerium sp. M
KT956913 Echinoparyphium recurvatum T
S508 ex Radix balthica R
Echinoparyphium recurvatum
-/76
Ge591 ex Sphaerium sp. M
AF184260 Echinoparyphium cinctum T
0.97/75
JF820595 Echinoparyphium rubrum P
0.96/-
JX262943 Echinoparyphium rubrum T
1/81
KT956914 Echinoparyphium sp. T
KT956917 Euparyphium cf. murinum T
1/100
KT956912 Echinoparyphium aconiatum T
KJ542642 Echinoparyphium mordvilkowi S
KP065596 Echinostoma revolutum G
0.003

Figure 7


G160 ex *Radix balthica* C
G162 ex *Radix balthica* C
G161 ex *Radix balthica* C
G164 ex *Radix balthica* C
G163 ex *Radix balthica* C
GeM1 ex *Radix balthica* C
Trichobilharzia franki "peregra" *sensu* Jouet et al. (2010)
1/100
HM131185/HM131168 *Trichobilharzia franki* "peregra" J
0.98/-
HM131186/HM131169 *Trichobilharzia franki* "peregra" J
HM131187/HM131171 *Trichobilharzia franki* "peregra" J
AY713973 *Trichobilharzia franki* R
FJ174567 *Trichobilharzia physellae* B
0.94/98
FJ174569 *Trichobilharzia physellae* B
FJ174568 *Trichobilharzia physellae* B
1/98
1/83
FJ174571 *Trichobilharzia* sp. B B
AY713969 *Trichobilharzia franki* R
Clade Q *sensu* Brant & Loker (2009)
FJ174572 *Trichobilharzia* sp. A B
-/80
FJ174573 *Trichobilharzia* sp. A B
FJ174574 *Trichobilharzia* sp. A B
FJ174560 *Trichobilharzia querquedulae* B
1/-
FJ174559 *Trichobilharzia querquedulae* B
1/100
FJ174558 *Trichobilharzia querquedulae* B
1/-
KJ855997 *Trichobilharzia* sp. P
FJ174576 *Trichobilharzia* sp. C B
FJ609411 *Trichobilharzia franki* AI
EF094538 *Trichobilharzia regenti* R
1/199
EF094540 *Trichobilharzia regenti* R
KP271015 *Trichobilharzia regenti* Ch
1/98
1/100
FJ174536 *Trichobilharzia* sp. E B
FJ174535 *Trichobilharzia* sp. E B
FJ174543 *Trichobilharzia stagnicolae* B
1/100
FJ174544 *Trichobilharzia stagnicolae* B
FJ174545 *Trichobilharzia stagnicolae* B
FJ609409 *Trichobilharzia szidati* AI
1/100
AY713972 *Trichobilharzia szidati* R
KT271757 *Trichobilharzia szidati* AI
1/100
FJ469803 *Trichobilharzia* sp. 3 AI
FJ469804 *Trichobilharzia* sp. 3 AI
FJ174537 *Trichobilharzia* sp. D B
1/100
FJ469807 *Trichobilharzia mergi* AI
FJ469799 *Trichobilharzia mergi* AI
FJ469790 *Trichobilharzia anseri* AI
1/100
FJ469791 *Trichobilharzia anseri* AI
FJ469784 *Trichobilharzia anseri* AI
KC839985 *Anserobilharzia brantae* W335 B
0.03