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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, Norway, we characterised 120 trematode isolates from mollusc first intermediate hosts, metacercariae from second intermediate host fishes and invertebrates, and adults from fish and invertebrate definitive hosts, using molecular techniques. Phylogenies based on nuclear and/or mtDNA 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 of sequence data for different stages, thus achieving molecular elucidation of trematode life-cycles and exploration of host-parasite interactions. 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 indicating that sub-Arctic and Arctic ecosystems may be characterised by unique trematode assemblages.

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 in some vertebrate hosts at high latitudes (e.g. Storer, 2000, 2002; Muzzafar and Jones, 2004; Perdiguero-Alonso et al., 2008; Kutz et al., 2012; for a detailed review see Hoberg et al., 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.

Digenean 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; Kuris et al., 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, using these features of digenean systems is hampered by the notoriously difficult identification of the larval stages and problems in linking the life-cycle stages in intermediate hosts and sexually mature adults that require substantial taxonomic expertise (Nolan and Cribb, 2005; Faltýnková et al., 2016).

☆ *Note:* Nucleotide sequence data reported in this paper are available in GenBank under accession numbers KY513132–KY513184 (28S rDNA), KY513270–KY513275 (ITS1-5.8S-ITS2), KY513276–KY513279 (ITS2), KY513185–KY513264 (*cox1*) and KY513265–KY513269 (*nad1*).

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Molecular methods are being increasingly used in digenean research and sequence data have been accumulated that may provide rapid molecular identification in large-scale digenean surveys in North America (Brant and Loker, 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). Recent exploration of freshwater digenean diversity, using morphological and molecular genetic approaches, has 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 sub-Arctic lakes in Iceland. These data indicate unexpected digenean diversity in the high latitude ecosystems. 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 newly generated sequence data and the recently developed molecular framework and sequence datasets for Europe, 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 Lake Takvatn (Norway), we examined samples of several free-living animal taxa potentially acting as intermediate hosts for digeneans. Using coarse-grained morphological identification 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 that will allow consistent identification and exploration of host-parasite interactions, and food web studies in Arctic lakes.

2. Materials and methods

2.1. Study lake

Lake Takvatn (hereinafter 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 study on macroinvertebrate diversity in the rocky littoral zone demonstrated the presence of 25 taxa (18 insects and seven non-insects, see Klemetsen and Elliott, 2010 for details). Of these, the gastropod *Radix peregra* (identified here as *Radix balthica*), the amphipod *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) at 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*; *Melanitta nigra*; *Mergus serrator*; *Sterna paradisaea*; *Tringa hypoleucos* and *Tringa 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 detect more species and discern their 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 in the littoral of the lake (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 zone (depth 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 *R. balthica* occurred at 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 2 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 Czech Academy of Sciences, 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 (61.42248, 8.867512) were also used to generate molecular data. Foot tissue taken from infected *Radix* spp. and two morpho-

types 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. 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 the Qiagen QIAquick™ 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 3130×1 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 were submitted to the GenBank database under accession numbers KY513132–KY513279.

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 nucleotides (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

Table 1
Details for the alignments used in the phylogenetic analyses in this study.

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[c]
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

*cox*1, cytochrome *c* oxidase subunit 1; *nad*1, nicotinamide adenine dinucleotide dehydrogenase subunit 1; ITS, internal transcribed spacer region.
[a] Ingroup.
[b] Sequences retrieved from GenBank.
[c] GTR+I+Γ, general time reversible model including estimates of invariant sites and gamma distributed among-site variation; GTR+I, general time reversible model including estimates of invariant sites; HKY+I+Γ, Hasegawa-Kishino-Yano model including estimates of invariant sites and gamma distributed among-site rate variation; HKY +I, Hasegawa-Kishino-Yano model including estimates of invariant sites.

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 using 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 (http://beast.bio.ed.ac.uk/Tracer) 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/), 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 *R. 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 unexpectedly 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 has now added 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 the matching of 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 26 species available in the GenBank database; see Tables 1 and 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 basal 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 and 2 and Supplementary Table S4 for details) revealed similar patterns and support but with *C. auriculatum* clustering with *Crepidostomum 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 isolates from the dytiscid beetle *Oreodytes sanmarkii* clustered within the clade of *Allocreadium* spp. with maximum support. These results indicate that two pairs of genetically 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 *S. trutta* as definitive host) and the closely related sister species *Crepidostomum* sp. 2 (using nymphs of the mayfly *S. 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% and 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% and 4.5% (27–32 nt).

The sequences for the worms 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 *Allocreadium lobatum*, differed by only two nucleotide positions. Beetles infected with adult worms were filled with *A. neotenicum* eggs and lacked most internal organs, including digestive and reproductive systems. Worm eggs are presumably released upon the death of the host.

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 and 2 and Supplementary Table S4 for details) and 28S rDNA (Alignment 4 including data for eight species/lineages from GenBank; see Tables 1 and 2 and Supplementary Table S4 for details). Individual gene analyses yielded tree topologies with congruent sister-group relationships among the

Table 2
Summary data for isolates from Lake Takvatn, Norway, used for generation of new cytochrome c oxidase subunit 1 (*cox*1), nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1), 28S rDNA and the complete ribosomal internal transcribed spacer region 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	A	ANTAK1, 2	28S	KY513132; KY513133
Crepidostomum farionis (Müller, 1780)	*Pisidium casertanum*	Sphaeriidae	R	CFTAK1, 2	28S	KY513134; KY513135
	Sphaerium sp.	Sphaeriidae	R	CFTAK3, 4	28S	KY513136; KY513137
	Pisidium casertanum	Sphaeriidae	C	CFTAK5, 6	28S	KY513138; KY513139
Crepidostomum metoecus (Braun, 1900)	*Pisidium casertanum*	Sphaeriidae	R	CMTAK1	28S	KY513140
	Gammarus lacustris	Gammaridae	M	CMTAK2-8	28S	KY513141–KY513147
	Salmo trutta	Salmonidae	A	CMTAK9	28S	KY513148
Crepidostomum sp. 1	*Sphaerium* sp.	Sphaeriidae	C	CSP1TAK1	28S	KY513149
	Siphlonurus lacustris	Siphlonuridae	M	CSP1TAK2	28S	KY513150
Crepidostomum sp. 2	*Siphlonurus lacustris*	Siphlonuridae	M	CSP2TAK1	28S	KY513151
	Diura bicaudata	Perlodidae	M	CSP2TAK2, 3	28S	KY513152; KY513153
	Salmo trutta	Salmonidae	A	CSP2TAK4	28S	KY513154
Family Diplostomidae Poirier, 1886						
Diplostomum phoxini (Faust, 1918)	*Radix balthica*	Lymnaeidae	C	DPTAK1	*cox*1	KY513185
	Phoxinus phoxinus[d]	Cyprinidae	M	DPOH	*cox*1	KY513186
Diplostomum sp. 'Lineage 3'[b]	*Salmo trutta*	Salmonidae	M	DLIN3TAK1-3	*cox*1	KY513187–KY513189
	Salvelinus alpinus	Salmonidae	M	DLIN3TAK4	*cox*1	KY513190
Diplostomum sp. 'Lineage 4'[b]	*Radix balthica*	Lymnaeidae	C	DLIN4TAK1-3	*cox*1	KY513191–KY513193
	Gasterosteus aculeatus	Gasterosteidae	M	DLIN4TAK4, 5	*cox*1	KY513194; KY513195
Diplostomum sp. 'Lineage 5'[b]	*Gasterosteus aculeatus*	Gasterosteidae	M	DLIN5TAK1	*cox*1	KY513196
	Salmo trutta	Salmonidae	M	DLIN5TAK2	*cox*1	KY513197
	Salvelinus alpinus	Salmonidae	M	DLIN5TAK3-9	*cox*1	KY513198–KY513204
Diplostomum sp. 'Lineage 6'[b]	*Radix balthica*	Lymnaeidae	C	DLIN6TAK1-5	*cox*1	KY513205–KY513209
	Gasterosteus aculeatus	Gasterosteidae	M	DLIN6TAK6-9	*cox*1	KY513210–KY513213
Tylodelphys sp.	*Salmo trutta*	Salmonidae	M	TSPTAK1	*cox*1	KY513214
	Salvelinus alpinus	Salmonidae	M	TSPTAK2	*cox*1	KY513215
Family Echinostomatidae Looss, 1899						
Echinoparyphium recurvatum (von Linstow, 1873)	*Radix balthica*	Lymnaeidae	R	ERTAK1	*nad*1/28S	KY513265/KY513155
	Sphaerium sp.	Sphaeriidae	M	ERTAK2	*nad*1/28S	KY513266/KY513156
	Pisidium casertanum	Sphaeriidae	M	ERTAK3	*nad*1	KY513267
	Sphaerium sp.	Sphaeriidae	M	ERTAK4,5	*nad*1/28S	KY513268; KY513269/KY513157
Family Notocotylidae Lühe, 1909						
Notocotylus sp.	*Radix balthica*	Lymnaeidae	C	NSPTAK1	28S	KY513158
Family Plagiorchiidae Lühe, 1901						
Plagiorchis sp. 1	*Radix balthica*	Lymnaeidae	S	PSP1TAK1, 2	*cox*1	KY513237; KY513238
	Radix balthica	Lymnaeidae	C	PSP1TAK3-13	*cox*1/28S	KY513239–KY513248/KY513159–KY513161
	Tipula salicetorum	Tipulidae	M	PSP1TAK14	28S	KY513162
Plagiorchis sp. 2	*Radix balthica*	Lymnaeidae	S	PSP2TAK1	*cox*1/28S	KY513249/KY513163
	Radix balthica	Lymnaeidae	C	PSP2TAK2, 3	*cox*1/28S	KY513250; KY513251/KY513164
	Radix balthica	Lymnaeidae	M	PSP2TAK4	*cox*1	KY513252
	Gammarus lacustris	Gammaridae	M	PSP2TAK5, 6	*cox*1/28S	KY513253; KY513254/KY513165
Plagiorchis sp. 3	*Radix balthica*	Lymnaeidae	C	PSP3TAK1-3	*cox*1/28S	KY513255–KY513257/KY513166
	Tipula salicetorum	Tipulidae	M	PSP3TAK4	*cox*1/28S	KY513258/KY513167
	Oreodytes alpinus	Dytiscidae	M	PSP3TAK5	28S	KY513168
Plagiorchis sp. 4	*Radix balthica*	Lymnaeidae	C	PSP4TAK1	*cox*1/28S	KY513259/KY513169
	Radix balthica	Lymnaeidae	M	PSP4TAK2	*cox*1	KY513260
Plagiorchis sp. 5	*Radix balthica*	Lymnaeidae	C	PSP5TAK1, 2	*cox*1/28S	KY513261; KY513262/KY513170
	Sialis lutaria	Sialidae	M	PSP5TAK3	28S	KY513171
	Oreodytes alpinus	Dytiscidae	M	PSP5TAK4	28S	KY513172
Plagiorchis sp. 6	*Radix balthica*	Lymnaeidae	C	PSP6TAK1	*cox*1/28S	KY513263/KY513173

(*continued on next page*)

Table 2 (*continued*)

Species	Host species	Host family	Life-cycle stage[a]	Isolate	Gene	GenBank accession number
Plagiorchis sp. 7	*Radix balthica*	Lymnaeidae	C	PSP7TAK1	*cox*1/28S	KY513264/KY513174
Family Schistosomatidae Stiles & Hassall, 1898						
Trichobilharzia franki haplotype "peregra"[c]	*Radix balthica*	Lymnaeidae	C	TFPTAK1-6	ITS1-5.8S-ITS2	KY513270–KY513275
Family Strigeidae Railliet, 1919						
Apatemon gracilis (Rudolphi, 1819)	*Radix balthica*	Lymnaeidae	S	AGTAK1-3	*cox*1	KY513216–KY513218
	Radix balthica	Lymnaeidae	C	AGTAK4-10	*cox*1/28S	KY513219–KY513225/KY513175; KY513176
	Gasterosteus aculeatus	Gasterosteidae	M	AGTAK11-13	*cox*1/28S	KY513226–KY513228/KY513177
Apatemon sp.	*Gasterosteus aculeatus*	Gasterosteidae	M	ASPTAK1, 2	*cox*1/28S	KY513229; KY513230/KY513178; KY513179
Cotylurus cornutus (Rudolphi, 1808)	*Radix balthica*	Lymnaeidae	S	CCTAK1	*cox*1	KY513231
	Radix balthica	Lymnaeidae	M	CCTAK2-5	*cox*1/28S	KY513232–KY513235/KY513180
	Gyraulus acronicus	Planorbidae	M	CCTAK6, 7	*cox*1/28S	KY513236/KY513181; KY513182
Family Lymnaeidae Rafinesque, 1815						
Radix balthica (Linnaeus, 1758)	–	–	A	RBTAK1-4	ITS2	KY513276–KY513279
Family Sphaeriidae Deshayes, 1855						
Sphaerium sp.	–	–	A	SSPTAK1	28S	KY513183
Pisidium casertanum (Poli, 1791)	–	–	A	PCTAK1	28S	KY513184

[a] Life-cycle stages: A, adult; C, cercaria; R, redia; M, metacercaria; S, sporocyst.
[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).

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 *G. 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 *G. 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 and 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 and 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–

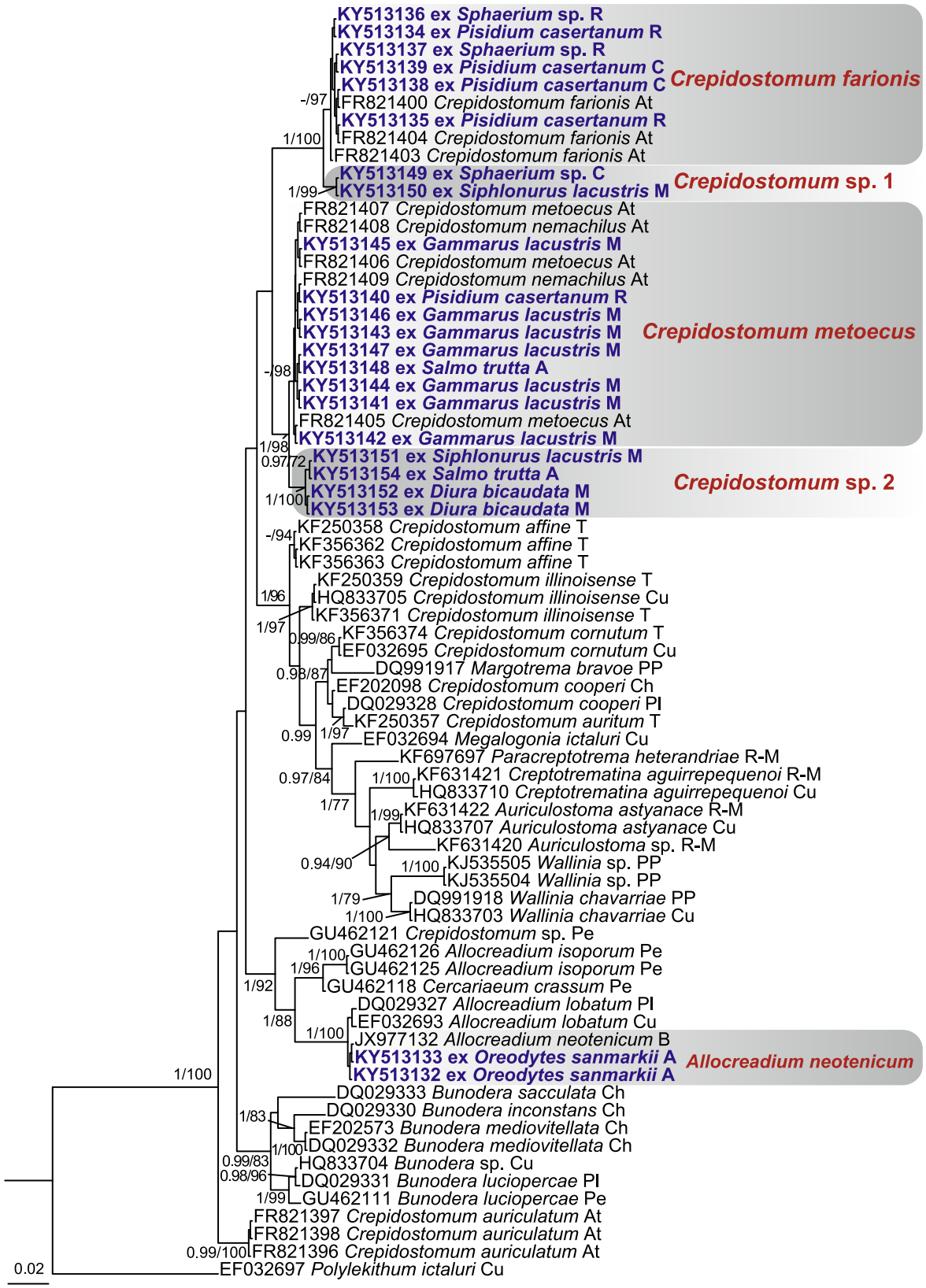

Fig. 1. Phylogram from Bayesian inference (BI) analysis of the 28S rDNA sequence alignment (Alignment 1, 721 nucleotides, 71 sequences) for 28 species/lineages within the Allocreadiidae. Outgroup: *Polylekithum ictaluri*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values >0.95 (BI) and >70 (ML) are shown. Host and life-cycle stage (R, redia; C, cercaria; M, metacercaria; A, adult) are indicated for isolates from Takvatn, Norway (in bold) (see Table 2 for details). 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). Shaded rectangles indicate species and species-level lineages identified in this study.

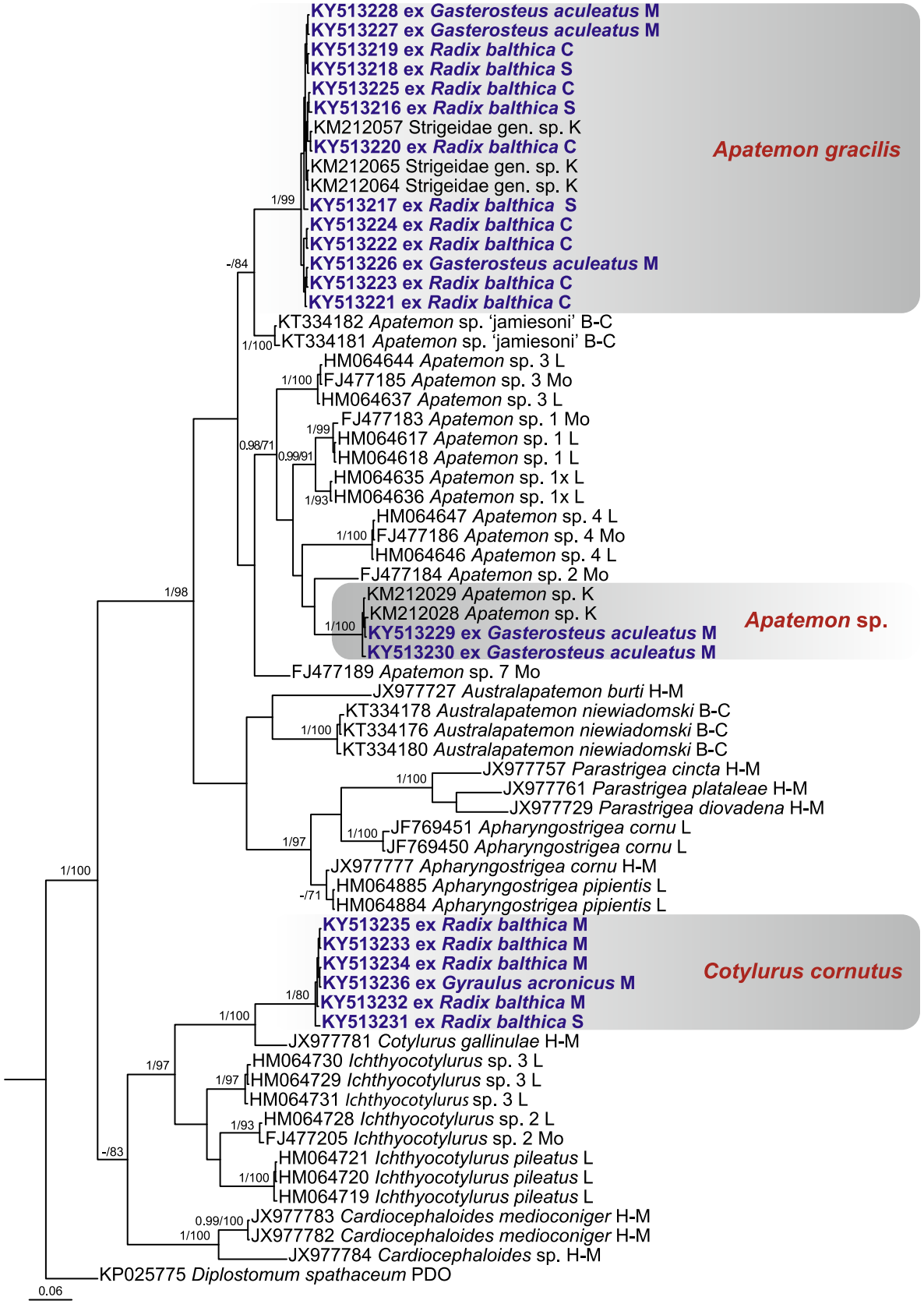

Fig. 2. Phylogram from Bayesian inference (BI) analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) sequence alignment (Alignment 3, 407 nucleotides, 65 sequences) for 22 species/lineages of the Strigeidae. Outgroup: *Diplostomum spathaceum*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values >0.95 (BI) and >70 (ML) are shown. Host and life-cycle stage (S, sporocyst; C, cercaria; M, metacercaria) are indicated for isolates from Takvatn, Norway (in bold) (see Table 2 for details). 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). Shaded rectangles indicate species and species-level lineages identified in this study.

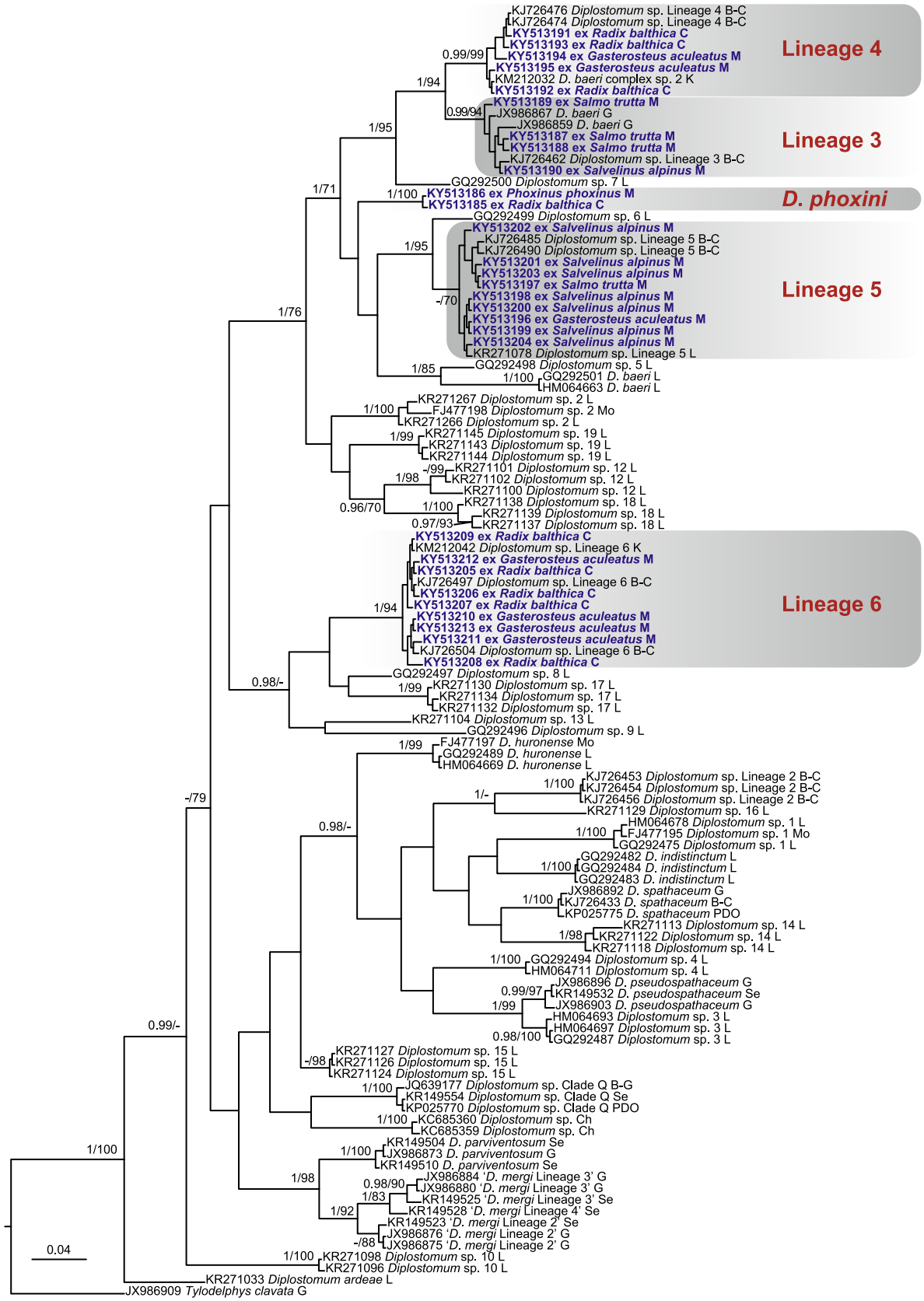

Fig. 3. Phylogram from Bayesian inference (BI) analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) sequence alignment (Alignment 5, 407 nucleotides, 112 sequences) for 36 species/lineages of *Diplostomum*. Outgroup: *Tylodelphys clavata*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values >0.95 (BI) and >70 (ML) are shown. Host and life-cycle stage (C, cercaria; M, metacercaria) are indicated for isolates from Takvatn, Norway (highlighted in bold) (see Table 2 for details). 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, 2010b, 2015); Mo, Moszczynska et al. (2009); PDO, Pérez-del-Olmo et al. (2014); Se, Selbach et al. (2015). Shaded rectangles indicate species and species-level lineages identified in this study.

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', one haplotype (KY513190) 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', one haplotype (KY513192) 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', one haplotype (KY513197) 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 KY513208 ex *R. balthica* and four isolates (KM212035, KM212036, KM212043 and KM212052); (ii) haplotype 2: isolates KY513210 and KY513213 ex *G. aculeatus* and four isolates (KM212037, KM212040, KM212041 and KM212047); (iii) haplotype 3: isolates KY513205 and KY513209 ex *R. balthica* and five isolates (KM212039, KM212042, KM212045, KM212046 and KM212051); and (iv) haplotype 4: isolate KY513212 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 and 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 *Tylodelphys 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%.

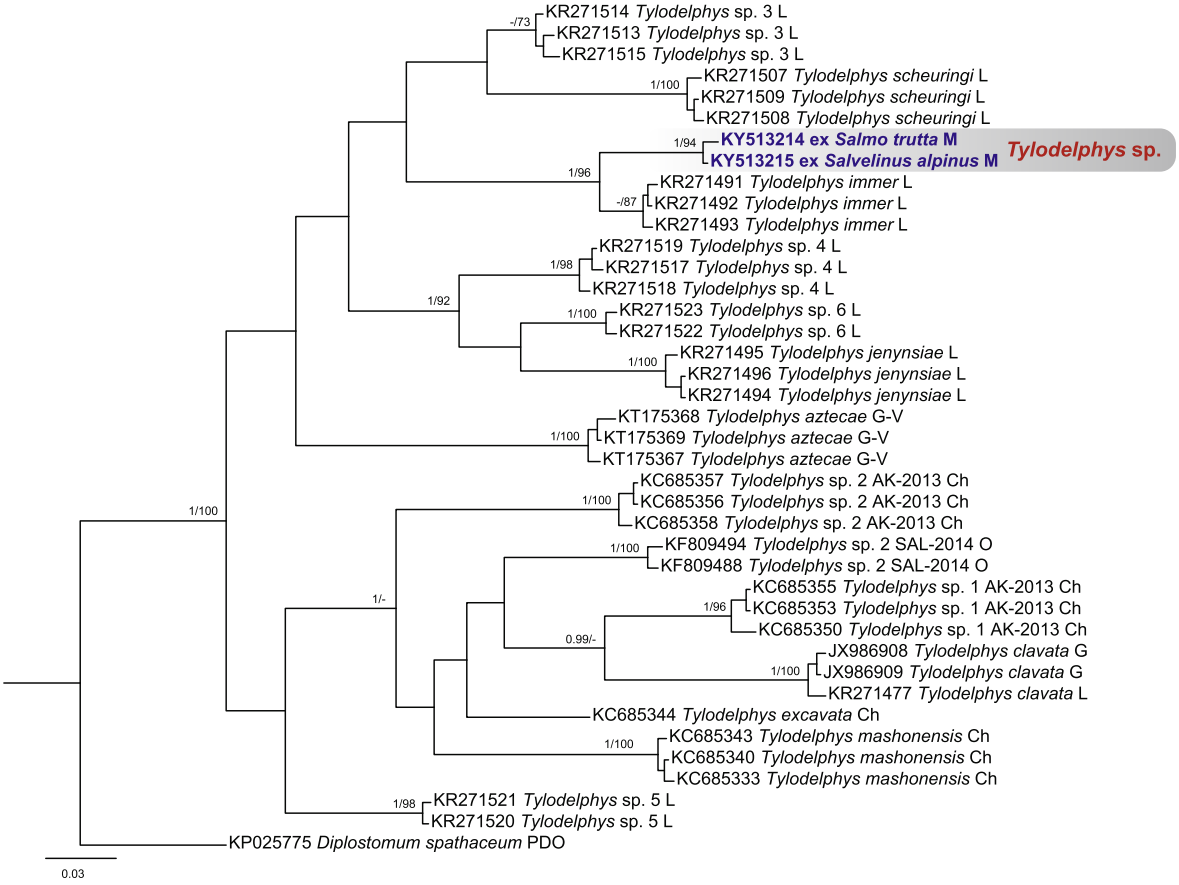

Fig. 4. Phylogram from Bayesian inference (BI) analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) sequence alignment (Alignment 6, 407 nucleotides, 39 sequences) for 15 species/lineages of *Tylodelphys*. Outgroup: *Diplostomum spathaceum*. Nodal support is given as posterior probabilities (BI) and bootstrap values resulting from maximum likelihood (ML) analysis; only values >0.95 (BI) and >70 (ML) are shown. Host and life-cycle stage (M, metacercaria) are indicated for isolates from Takvatn, Norway (in bold) (see Table 2 for details). 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). Shaded rectangles indicate species and species-level lineages identified in this study.

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 and 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 and 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 and 2 and Supplementary Table S4 for details) and 28S rDNA (Alignment 10; see Tables 1 and 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 and 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 and 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 *Trichobilharzia 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 and 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 1 nt from the Icelandic isolate of Huňová et al. (2012) and by 2 nt 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 and 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 *P. 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 10 genera and seven families, the latter being the most diverse and widely distributed supra-generic 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*)

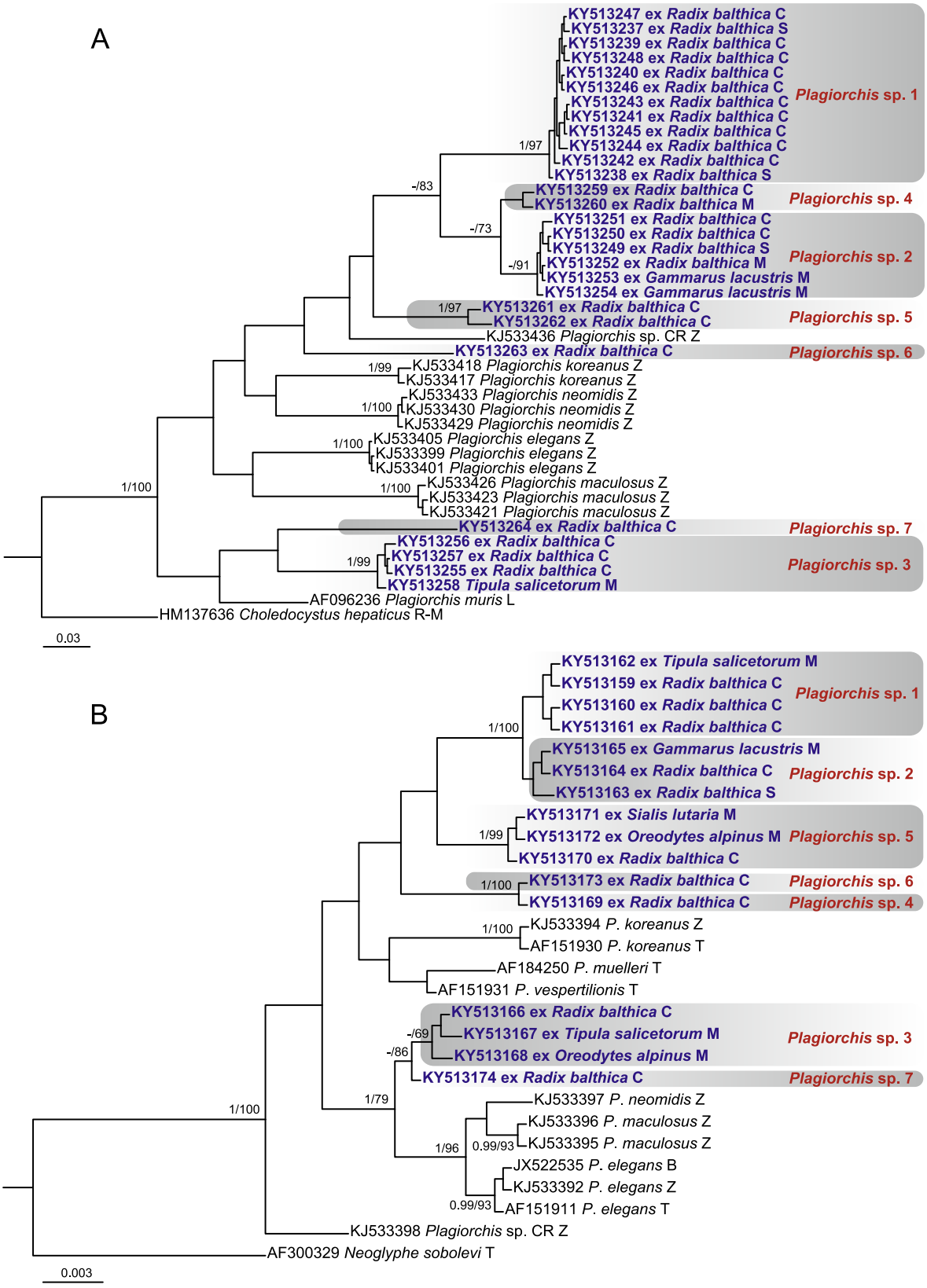

Fig. 5. Phylograms from Bayesian inference (BI) analyses for *Plagiorchis* spp. (A) Analysis of the cytochrome *c* oxidase subunit 1 (*cox*1) sequence alignment (Alignment 7, 423 nucleotides, 41 sequences) for 13 species/lineages. Outgroup: *Choledocystus hepaticus*. (B) Analysis of the 28 rDNA sequence alignment (Alignment 8, 1,171 nucleotides, 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. Host and life-cycle stage (S, sporocyst; C, cercaria; M, metacercaria) are indicated for isolates from Takvatn, Norway (in bold) (see Table 2 for details). 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). Shaded rectangles indicate species and species-level lineages identified in this study.

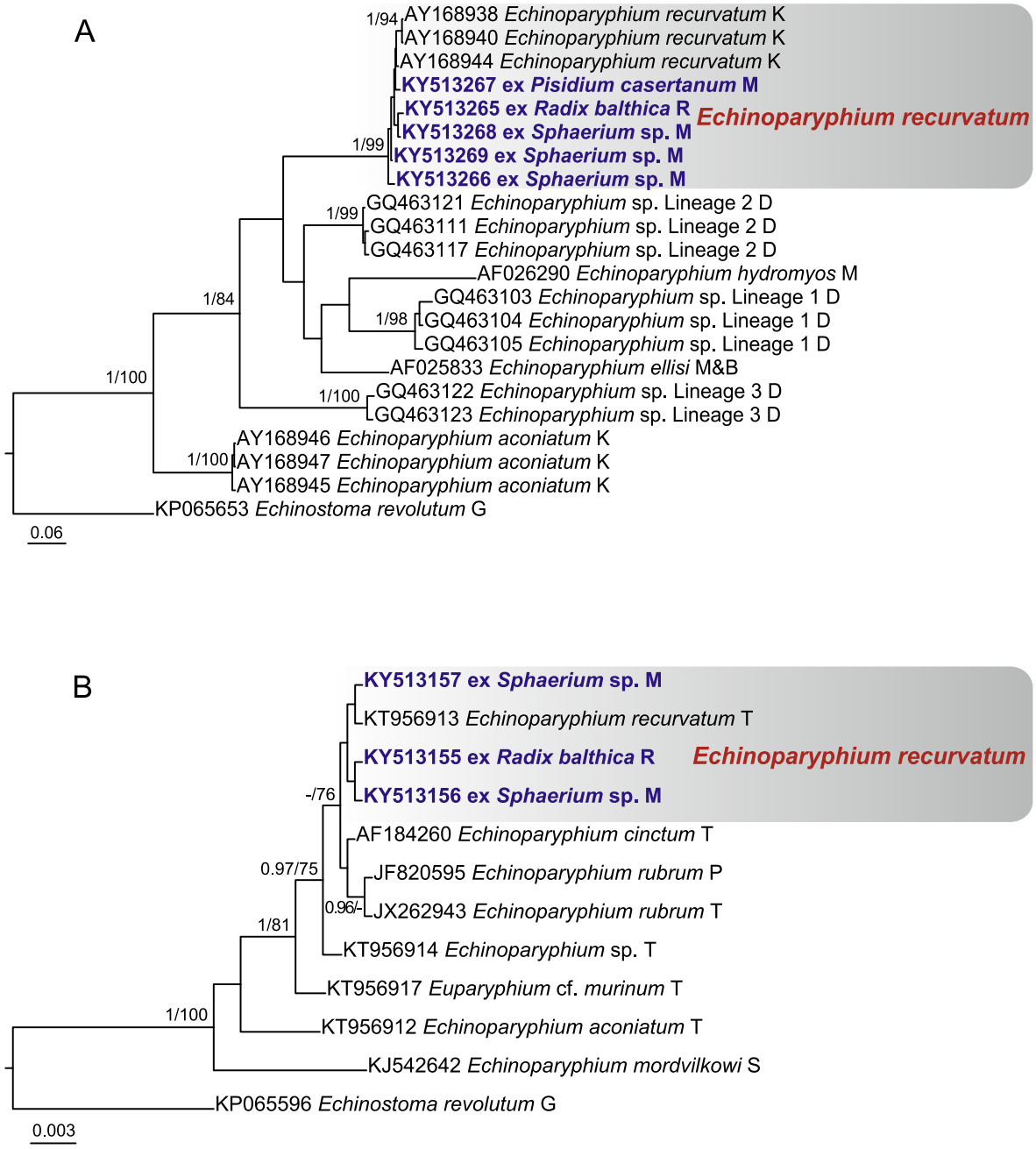

Fig. 6. Phylograms from Bayesian inference (BI) analyses for *Echinoparyphium* spp. (A) Analysis of the nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) sequence alignment (Alignment 9, 472 nucleotides, 21 sequences) for seven species/lineages. (B) Analysis of the 28 rDNA sequence alignment (Alignment 10, 1,190 nucleotides, 11 sequences) for seven 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. Host and life-cycle stage (R, redia; M, metacercaria) are indicated for isolates from Takvatn, Norway (in bold) (see Table 2 for details). The scale bar indicates the expected number of substitutions per site. Sequence identification is as in GenBank, followed by a letter: D, Detwiler et al. (2010); G, Georgieva et al. (2014); K, Kostadinova et al. (2003); M, Morgan and Blair (1998a,b); P, Pulis et al. (2011); S, Stanevičiūtė et al. (2015); T, Tkach et al. (2001a, 2012, 2016). Shaded rectangles indicate species and species-level lineages identified in this study.

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*, *Crepidostomum metoecus* and *Crepidostomum wikgreni* Gibson & Valtonen, 1988. Further molecular studies focused on the adult stages may reveal the actual diversity of *Crepidostomum* spp. in the sub-Arctic freshwater ecosystems.

It is worth noting that we sequenced few metacercariae from fishes. However, the detection of the novel species of *Apatemon* and *Tylodelphys*, *A. gracilis* and five species of *Diplostomum,* and the presence of similar or shared haplotypes with isolates from a previous extensive sampling of *G. aculeatus* in Takvatn (*A. 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 high diversity of fish parasites in Takvatn, revealed by the molecular and phylogenetic approaches applied here, challenges sub-Arctic diversity baselines compiled

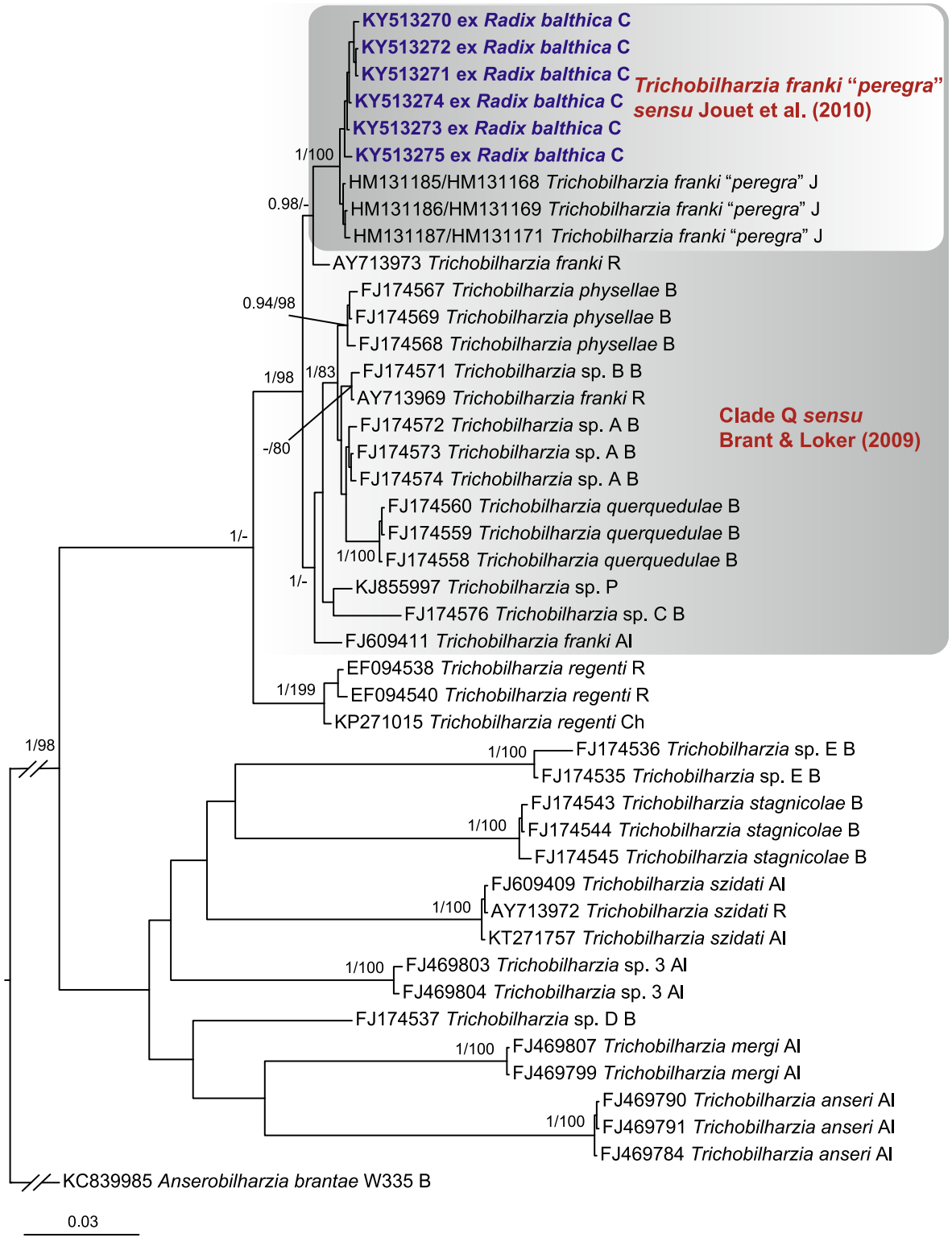

Fig. 7. Phylogram from Bayesian inference (BI) analysis of the concatenated ITS1 and ITS2 alignment (Alignment 11, 1,297 nucleotides, 43 sequences) for 16 species/lineages of *Trichobilharzia* spp. from the analysis of the concatenated ITS1 and ITS2 gene data set. Outgroup: *Anserobilharzia brantae*. 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. Host and life-cycle stage (S, sporocyst; C, cercaria) are indicated for isolates from Takvatn, Norway (in bold) (see Table 2 for details). 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). Shaded rectangles indicate species and species-level lineages identified in this study.

from studies relying on morphological identification (e.g. Poulin et al., 2011; Wrona et al., 2013). Thus, our study adds nine and seven species, respectively, to species richness estimates for parasites in *G. aculeatus* (1–11 species per ecosystem; Poulin et al., 2011) and salmonid and coregonid hosts (4–18 spp. per ecosystem; Wrona et al., 2013) in the sub-Arctic and Arctic ecosystems.

Table 3
Summary data for the intermediate hosts of molecularly identified isolates and the possible definitive hosts of the trematodes completing their life-cycles in Takvatn, Norway. 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 at the Natural History Museum (NHM, UK) Host-Parasite Database; www.nhm.ac.uk/research-curation/scientific-resources/taxonomy-systematics/host-parasites/index.html; 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[a]		–	*Oreodytes sanmarkii*
Crepidostomum farionis	*Pisidium casertanum*; *Sphaerium* sp.		*G. aculeatus*; *S. trutta*; *S. alpinus*
Crepidostomum metoecus	*P. casertanum*	*Gammarus lacustris*	*G. aculeatus*; *S. trutta*[e]; *S. alpinus*
Crepidostomum sp. 1[b]	*Sphaerium* sp.	*Siphlonurus lacustris*	*G. aculeatus*; *S. trutta*; *S. alpinus*
Crepidostomum sp. 2[b]		*S. lacustris*; *Diura bicaudata*	*G. aculeatus*; *S. trutta*[e]; *S. alpinus*
Family Diplostomidae			
Diplostomum phoxini	*Radix balthica*		*Aythya fuligula*; *Bucephala clangula*; *Gavia arctica*; *Larus canus*; *Mergus serrator*; *Sterna paradisaea*
Diplostomum sp. 'Lineage 3'[b,c]		*Salmo trutta*; *Salvelinus alpinus*	
Diplostomum sp. 'Lineage 4'[b,c]	*R. balthica*	*Gasterosteus aculeatus*	
Diplostomum sp. 'Lineage 5'[b,c]		*G. aculeatus*; *S. trutta*; *S. alpinus*	
Diplostomum sp. 'Lineage 6'[b,c]	*R. balthica*	*G. aculeatus*	
Tylodelphys sp.[b]		*S. trutta*; *S. alpinus*	*G. arctica*
Family Echinostomatidae			
Echinoparyphium recurvatum	*R. balthica*	*Sphaerium* sp.; *P. casertanum*	*Anas penelope*; *Anas platyrhynchos*; *A. fuligula*; *B. clangula*; *L. canus*; *Melanitta fusca*; *Melanitta nigra*; *Tringa totanus*
Family Notocotylidae			
Notocotylus sp.[a]	*R. balthica*	–	*A. penelope*; *A. platyrhynchos*; *A. fuligula*; *B. clangula*; *L. canus*; *M. fusca*
Family Plagiorchiidae			
Plagiorchis sp. 1[b]	*R. balthica*	*Tipula salicetorum*	*A. platyrhynchos*; *A. fuligula*; *L. canus*; *Tringa hypoleucos*; *T. totanus*
Plagiorchis sp. 2[b]	*R. balthica*	*G. lacustris*	
Plagiorchis sp. 3[b]	*R. balthica*	*T. salicetorum*; *Oreodytes alpinus*	
Plagiorchis sp. 4[b]	*R. balthica*		
Plagiorchis sp. 5[b]	*R. balthica*	*Sialis lutaria*; *O. alpinus*	
Plagiorchis sp. 6[b]	*R. balthica*		
Plagiorchis sp. 7[b]	*R. balthica*		
Family Schistosomatidae			
Trichobilharzia franki haplotype "peregra"[a,b,d]	*R. balthica*	–	*A. penelope*; *A. platyrhynchos*; *A. fuligula*; *B. clangula*
Family Strigeidae			
Apatemon gracilis	*R. balthica*	*G. aculeatus*	*A. penelope*; *A. platyrhynchos*; *A. fuligula*; *B. clangula*; *M. fusca*; *M. nigra*; *M. serrator*
Apatemon sp.[b]		*G. aculeatus*	
Cotylurus cornutus	*R balthica*	*R. balthica*; *Gyraulus acronicus*	*Anas penelope*; *A. platyrhynchos*; *A. fuligula*; *B. clangula*; *M. nigra*; *S. paradisaea*; *T. totanus*

[a] No second intermediate host in the life-cycle.
[b] Putative new species.
[c] Lineages discovered in Iceland and characterised molecularly and morphologically by Blasco-Costa et al. (2014) and Faltýnková et al. (2014).
[d] Lineage discovered in Iceland by Jouet et al. (2010) based on molecular data.
[e] Hosts of adult isolates sequenced.

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.), *Radix ovata* (syn. of *R. balthica*; 31 spp.) and *Radix 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 1–5 species, thus highlighting the extraordinary digenean diversity in a single snail in Takvatn. Diversity estimates vary locally (Faltýnková et al., 2016) but the digenean species richness (19 species) in *R. balthica* in Takvatn is high compared with 12 species (1–7 species per lake) in *R. auricularia* from 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, Canada (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 recently 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 description.

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 discovery of novel lineages of *Apatemon*, *Crepidostomum* and *Tylodelphys* and by the fact that two of the novel *Diplostomum* spp. lineages (lineages 5 and 6)

and the lineage *T. franki* haplotype "peregra" have to date 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 (*Trichobilharzia anseri* (FJ469790, FJ469791, FJ469784); *T. franki* haplotype "peregra" (HM131185/HM131168; HM131186/HM131169; HM131187/HM131171; present study); *Trichobilharzia mergi* (FJ469807, FJ469799); and *Trichobilharzia* sp. 3 of Aldhoun et al. (2009b) (FJ469803, FJ469804) (see Aldhoun et al., 2009a,b; Jouet et al., 2010) compared with only three species (i.e. *T. franki*, *T. regenti* and *Trichobilharzia szidati*) reported in central Europe despite a much higher sampling effort in this region. 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 geographical 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, 25 with the second intermediate hosts and 117 with the definitive fish and bird hosts, and one with a beetle definitive host (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 (*R. balthica* and *P. 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 greater 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 is plausible, considering the trophic behaviour of the potential bird hosts and host-parasite compatibility based on records for congeneric digeneans at the Natural History Museum (NHM, UK) Host-Parasite Database (Gibson, D.I., Bray, R.A., Harris, E.A. (Compilers) (2005). Host-Parasite Database of the Natural History Museum, London. Available at: www.nhm.ac.uk/research-curation/scientific-resources/taxonomy-systematics/host-parasites/index.html). We based this 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). Our data, therefore, extend the Takvatn host-parasite interaction network, adding the benthic component. This is characterised by a threefold higher diversity of macroparasites (24 versus eight species) and adds twice as many host-parasite links (165 versus 75) than did the network based solely on the pelagic zone (see Amundsen et al., 2009). The life-cycle linkages from first intermediate hosts, and many of the second intermediate hosts, dynamically meld the benthic and pelagic habitats of this, and likely other lacustrine ecosystems.

In conclusion, our study adds to the sequence database (Georgieva et al., 2013a,b, 2014; Blasco-Costa et al., 2014; Zikmundová et al., 2014) on digeneans in freshwater ecosystems. This will facilitate direct, taxonomically consistent recognition of host-parasite interaction networks in future food web analyses of Arctic lakes. Using this approach, partitioning of interactions with novel species/genetic lineages can now be achieved without having to complete life-cycles in the laboratory, nor surmise them based on extensive sampling followed by detailed morphological studies.

Disclaimer

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2016.12.008.

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