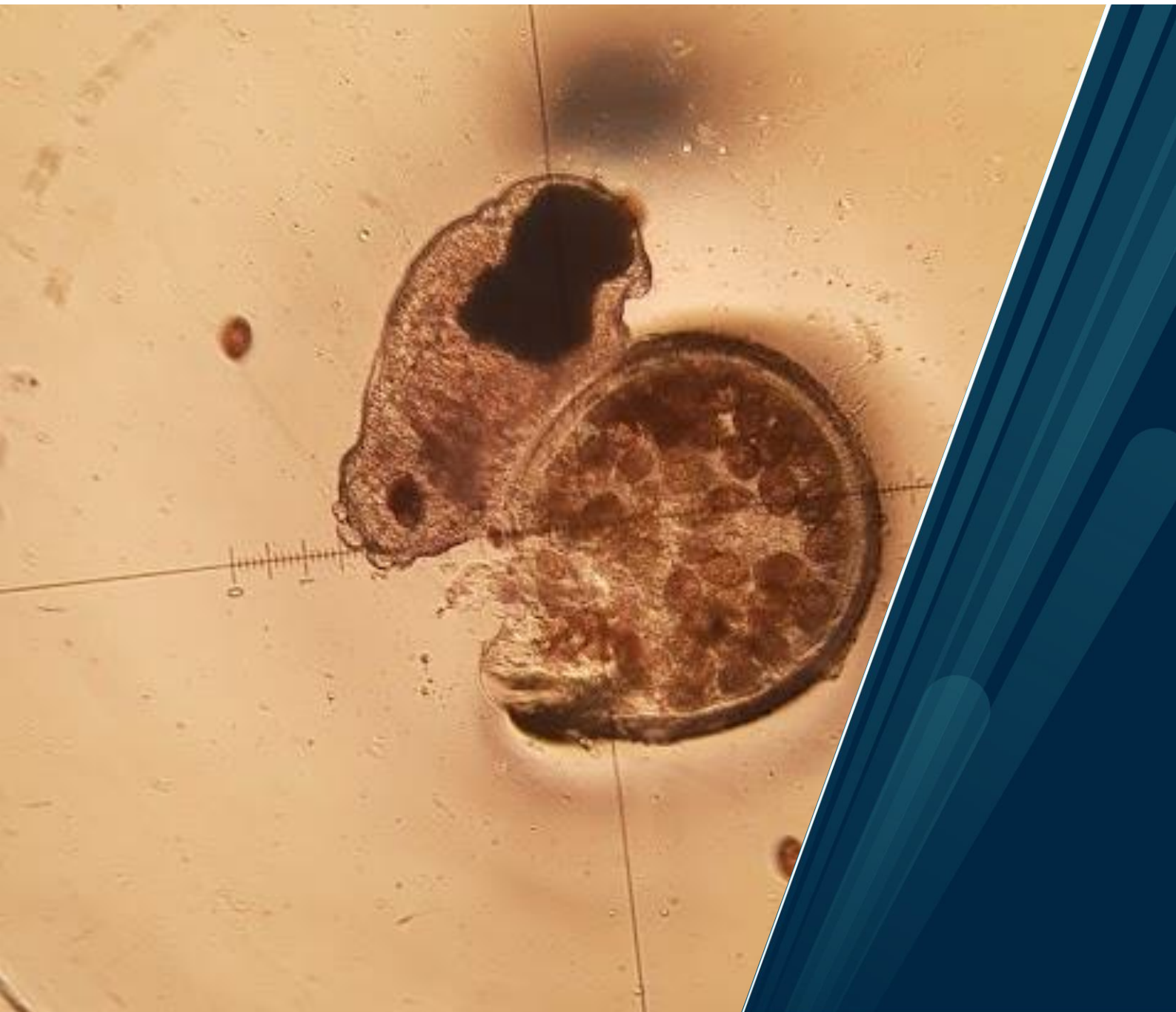![UiT The Arctic University of Norway]

Faculty of Biosciences, Fisheries, and Economics

Department of Arctic and Marine Biology

Molecular study of digenean diversity in aquatic organisms in northern Norway, with a focus on the seasonality of *Crepidostomum* [Braun, 1900]

Sigurd Slåteng

Master's thesis in Biology BIO-3950 June 2022

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Master of Science in Biology – Freshwater ecology

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Cover photo:
The metacercarial stage of the trematode *Crepidostomum metoecus*, removed from its cyst.
Photo by Sigurd Slåteng.

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Takk til alle som har bidratt.

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1 Abstract

This study aimed to elucidate cryptic trematode diversity across multiple life stages in aquatic organisms. I collected samples from first intermediate hosts (bivalves), second intermediate hosts (amphipods and insect nymphs), and definitive hosts (fish), and identified them by use of molecular methods. Phylograms based on molecular markers from 28S and COI genes were used to identify nine species from three families of trematodes. One of the species, *Bunodera vytautasi*, is new to Europe, and three of the species were found in putative new intermediate hosts. Phylograms were also made for the bivalve hosts of these trematodes, which permitted the identification of sphaeriid clams. My findings give further insights on the diversity of trematodes and their distributions in northern aquatic systems, as well as information on the macroinvertebrate hosts of trematodes.

Further, I observed some preliminary patterns on the seasonality of *Crepidostomum* in Arctic char and brown trout by looking at the species distribution from two sampling seasons. I observed a pattern where *C. farionis* and *C. metoecus* seem to infect and mature in fish at different seasons. Juvenile *C. farionis* stages were more common in the summer samples, and adults in the late autumn sample. *C. metoecus* were proportionally scarce compared to *C. farionis*, apart from the Arctic char autumn sample, where multiple juveniles were found. This pattern is likely to be attributed to fish predation on the second intermediate host where *C. farionis* infects insect larva, a prey item commonly consumed during the summer. *C. metoecus* on the other hand is the only species of *Crepidostomum* found to parasitise the amphipod *Gammarus lacustris*, a prey item Arctic char consumes more of during late autumn and winter. These findings give preliminary information on the seasonal variation closely related trematode species can have regarding their timing of trophic transmissions.

2 Preface

This thesis was originally titled "The developmental gene expression profiles of the freshwater digeneans *Crepidostomum metoecus* and *Phyllodistomum umblae*", with the goal of creating gene-expression profiles for at least one life stage in each host of these two parasites throughout their life cycles. After spending many weeks sampling and dissecting stoneflies in search of the metacercarial stages of *P. umblae* I eventually had to give up one of my two study animals as they remained absent. The title was then edited to "The developmental gene expression profiles of the freshwater digenean *Crepidostomum metoecus*". After many more months of sampling and dissecting I had managed to obtained *C. metoecus* specimens of every life stage I needed. I travelled to Switzerland to conduct RNA-extractions, but after multiple trials and errors I had to conclude that the number of samples required to create the gene expression profiles was a lot larger than anticipated. In the end, the main goal of the project had to be dropped. As such, the remnants of the sampling strategy targeting *P. umblae* and *C. metoecus* are still visible throughout the thesis, although a lot of the theory surrounding it has been excluded. All specimens had to be sampled alive and immediately stored in RNA later, which is part of the reason why the trematode identity is tied only to molecular data and not morphology. I think outlining the original intention of the project is necessary to justify many of the decisions that were made regarding the methodology, such as the sampling.

3 Introduction

High latitude freshwater systems represent understudied environments for parasite diversity. Studies over the last decade have uncovered multiple novel parasite lineages, as well as new information on parasite-host interactions for already known parasites from such systems (e.g., Georgieva et al., 2013; Faltýnková et al., 2014; Soldánová et al., 2017; Faltýnková et al., 2020). However, a large gap in information stems from the lack of data on parasite communities of macroinvertebrates, such as bivalves and aquatic arthropods. These groups serve as intermediate hosts for multiple parasite taxa and are important for trophic transmissions of parasites to vertebrates, such as fish, birds, or mammals (Amundsen et al., 2003). Studying the parasite communities of these macroinvertebrates is not unproblematic. Their parasites are often tiny, morphologically difficult to distinguish and occur in low prevalence. Even the host itself can prove difficult to identify without taxonomic expertise, as is the case with for example freshwater bivalves (Schultheiß et al., 2008; Prié et al., 2021). Fish on the other hand represent a well-studied freshwater host assemblage (Poulin, et al., 2020a), where cryptic parasites are being discovered regularly (e.g., Moszczynska et al., 2009; Faltýnková et al., 2014; Atopkin et al., 2018; Petkevičiute et al., 2018; Faltýnková et al., 2020). However, information on parasite patterns such as seasonal variance is lacking (Poulin, 2020b), especially in dimictic lakes with long ice-cover periods. Charting these seasonal patterns, along with the unveiling of cryptic diversity is important to obtain a complete understanding of an ecosystem and its functions.

Cryptic species can be defined as "morphologically indistinguishable species that can only be separated by molecular information", (but see Pérez-ponce De León & Nadler, 2010; Korshunova et al., 2019 for reviews on the problems with this term). These species pose challenges for researchers in multiple aspects. If the cryptic species are undetected, studies relying solely on morphology for species identification may overestimate the occurrence of the species that is believed to be, while simultaneously neglecting its cryptic counterpart. This can lead to erroneous estimates of, for example, population size, distribution patterns, or issues with conservation efforts. Even if the study site/host is known to harbour cryptic species, a significant number of resources is still required to conduct the work to molecularly distinguish them. Cryptic species is especially a problem when it comes to accurately and

practically identifying heteroxenous parasites with distinct morphological forms tied to different hosts, such as adult and cercarial stages of trematodes.

Trematoda is a class of strictly parasitic flatworms, characterised by having complex lifecycles with a minimum of two hosts, but often more. In almost all cases a mollusc (i.e., gastropod, bivalve or scaphopod) will be the first intermediate host (Kearn, 1998) where asexual reproduction from the miracidial (ciliated larva) to the cercarial (infective larva) life stage takes place. A vertebrate serves as the final host where the parasite matures. When more than two hosts are involved, amphipods, insect larva or fish are often the preferred second intermediate host where the parasite will enter and develop into a metacercarial (quiescent) stage. Because trematodes have these distinct forms throughout their lifecycles they can be challenging, if not impossible, to identify down to a species level when only considering morphology. Most studies describe the parasite only in its final host (Poulin & Presswell, 2016), and as such there are multiple gaps in the knowledge of the intermediate hosts, and the links between different parasite life stages (Blasco-Costa & Poulin, 2017). Therefore, the use of molecular method has become the preferred practice to identify cryptic trematodes and other parasites (Nadler & Pérez-ponce De León, 2011; Blasco-Costa et al., 2016). It should be noted that the reliability of reference sequences is very much reliant on isolates that are taxonomically correctly identified, a task highly dependent on thorough morphological identification on these vouchers (Faltýnková et al., 2022).

The necessity of molecular data to correctly identify cryptic species is portrayed well in Lake Takvatn, Norway (see Figure 1). This lake is currently known to house four cryptic species of trematodes belonging to genus *Crepidostomum* [Braun, 1900]. These include *C. farionis* [Müller, 1780], *C. pseudofarionis* [Faltýnková, Pantoja, Skírnisson, Kudlai, 2020]*, C. metoecus* [Braun, 1900] and *C. brinkmanni* [Faltýnková, Pantoja, Skírnisson, Kudlai, 2020]. However, prior to an extensive molecular study to reveal trematode diversity of invertebrates by Soldánová et al., (2017), *C. farionis* and *C. metoecus* were the only species of *Crepidostomum* believed to inhabit the lake. During the aforementioned study, the two previously undescribed species were found, along with 14 other species-level lineages belonging to undescribed trematodes in the cercarial and metacercarial stages. Sequences of

Crepidostomum were later used as reference in a molecular diversity study on trematodes in fish in Iceland (Faltýnková et al., 2020), where matching sequences of both species were found as adults in salmonids, and subsequently the species were formally described. Since their discovery both *C. brinkmanni* and *C. pseudofarionis* have been found in several places across Europe in the relatively short time span they have been known. In addition to the studies in Norway (Soldánová et al., 2017; Petkevičiute et al., 2018), and Iceland (Faltýnková et al., 2020), *C. brinkmanni* has been identified from brown trout (*Salmo trutta* L.) in Switzerland (Rochat, et al., 2021) and freshwater bivalves in Crimea, Ukraine (Petkevičiute et al., 2018), while *C. pseudofarionis* is confirmed from Arctic char (*Salvelinus alpinus* L.) from Scotland (Rochat et al., Under Review (2022)).

Based on available molecular data, the four species of *Crepidostomum* seem to exclusively parasitise sphaeriid clams as first intermediate hosts. Potential clam preferences have so far not been thoroughly detailed, but from the available data, European species of *Crepidostomum* seem to be generalists within the family Sphaeriidae (Soldánová et al., 2017; Petkevičiute et al., 2018). Aquatic insect larvae and amphipods are known to function as the second intermediate host for multiple trematode species, although major studies on their parasite communities are lacking (but see e.g., Grabner, 2017; Soldánová et al., 2017). It is possible that the metacercarial stage is a more specialised stage for some trematodes, seeing as only *C. metoecus* has so far been confirmed from the amphipod *Gammarus lacustris* [Sars, 1863] (Soldánová et al., 2017). This is also apparent for the different lineages of *Plagiorchis* in Takvatn. Of the 7 distinct lineages of *Plagiorchis*, they are all associated with the same first intermediate host, the snail *Radix balthica* L. Only one of them has been found to parasitise *G. lacustris* as second intermediate host, and the other lineages seem to have quite a diverse range of second intermediate hosts, including insect larvae such as *Tipula salicetorum* [Siebke, 1870], *Oreodytes alpinus* [Paykull, 1798] and *Sialis lutaria* L. (Soldánová et al., 2017). However, it is worth noting that most *Plagiorchis* lineages still have unresolved lifecycles, meaning that they could potentially be even more host specific in the second intermediate host, or less if they are found to overlap in host usage.

The objective of this study is to unveil hidden trematode diversity from intermediate bivalve and arthropod hosts, as well as to document the distribution of *Crepidostomum* spp. in Arctic char and brown trout. I hypothesise that cryptic trematodes still remain undiscovered,

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especially in macroinvertebrates whose parasites have not been studied on a large scale, nor identified with molecular data. In this study, I will achieve by using molecular methods to generate sequences from larval trematode stages from intermediate hosts to create phylograms with the new sequences coupled with sequences from earlier studies. My second hypothesis is that *Crepidostomum* spp. will display dissimilarities in their lifecycle, habitat (hosts) and/or seasonal rhythms. I will look for preliminary patterns by using molecular data to identify parts of the community of *Crepidostomum* from brown trout and Arctic char, to then examine the species proportions in each fish host from two seasonal samplings.

4 Material and methods

4.1 Study sites

Samples were collected from two lakes: Lake Takvatn (69°06'47.8"N 19°04'39.3"E) and Lake Skogsfjordvatn (69°56'24.9"N 19°10'12.3"E) (hereinafter Takvatn and Skogsfjordvatn respectively). Takvatn is a lake that has been utilised for numerous scientific projects and continuous monitoring by UiT - The Arctic University of Norway since 1979/80 (e.g., Amundsen et al., 2009; Amundsen et al., 2015). The majority of this research is dedicated to population dynamics of lake species, food web ecology, and parasite ecology of the fish community (e.g. Klemetsen et al., 1989; Knudsen et al., 1996; Klemetsen et al., 2002; Amundsen et al., 2015; Prati et al., 2020), but there are also studies focusing on the parasite community of invertebrates, zooplankton and phytoplankton communities, as well as other biogeochemical processes (e.g. Walseng & Halvorsen, 1993; Dahl-Hansen, 1995; Primicerio, 2000; Frainer et al., 2016; Soldánová et al., 2017; Shaw et al., 2020). Takvatn was selected as a study site because of the available molecular data on trematodes, *Crepidostomum* and *Plagiorchis* in particular, from invertebrates (Soldánová et al., 2017). Skogsfjordvatn was originally chosen for the collection of the trematode *Phyllodistomum umblae*, a parasite not present in Takvatn, but attempts at finding the intermediate life stages were unsuccessful. Skogsfjordvatn also harbours species of *Crepidostomum*, as well as a similar array of hosts as in Takvatn, and was thus also included in the study.

Takvatn and Skogsfjordvatn are both dimictic, oligotrophic, subarctic lakes situated in Northern Norway. Takvatn has a surface area of approximately 15 km^2, a maximum depth of 80 m and lies at 214 min altitude (Klemetsen et al., 1989; Amundsen et al., 2009; Frainer et al., 2016). Skogsfjordvatn is situated on the island Ringvassøya in Karlsøy municipality. It has a surface area of approximately 13.6 km^2, a maximum depth of approximately 100 min and lies at an altitude of 20 min above sea level (Smalås et al., 2017). Both lakes typically experience an ice-free period from early June till December. The rest of the year the lakes are covered by a thick sheet of ice (Smalås et al., 2013; Frainer et al., 2016; Smalås et al., 2017). The lakes are both situated more than 300 km north of the Polar circle, and as such experience a period of 24 h sunlight from when the ice melts till late July. From there, the sunlight decreases until it is no longer present in mid-November (Smalås et al., 2013; Frainer et al., 2016; Smalås et al., 2017). What separates the lakes is that Skogsfjordvatn has a more coastal environment with populations of anadromous Arctic char, brown trout, and Atlantic salmon

(*Salmo salar* L.), as well as three-spined sticklebacks (*Gasterosteus aculeatus* L.) and European eel (*Anguilla anguilla* L.) (Smalås et al., 2013). Takvatn on the other hand is a closed inland lake with a resident population of Arctic char, brown trout, and three-spined sticklebacks (Klemetsen et al., 2002). Little work has been done to assess the clam communities in these lakes down to a species level. From the molecular work of Soldánová et al., 2017 the presence of *Euglesa casertana* [Poli, 1791] and a species of *Sphaerium* was confirmed to inhabit Takvatn. In Skogsfjordvatn, no molecular work has been done on clams, and as such little is known about them at a species level.
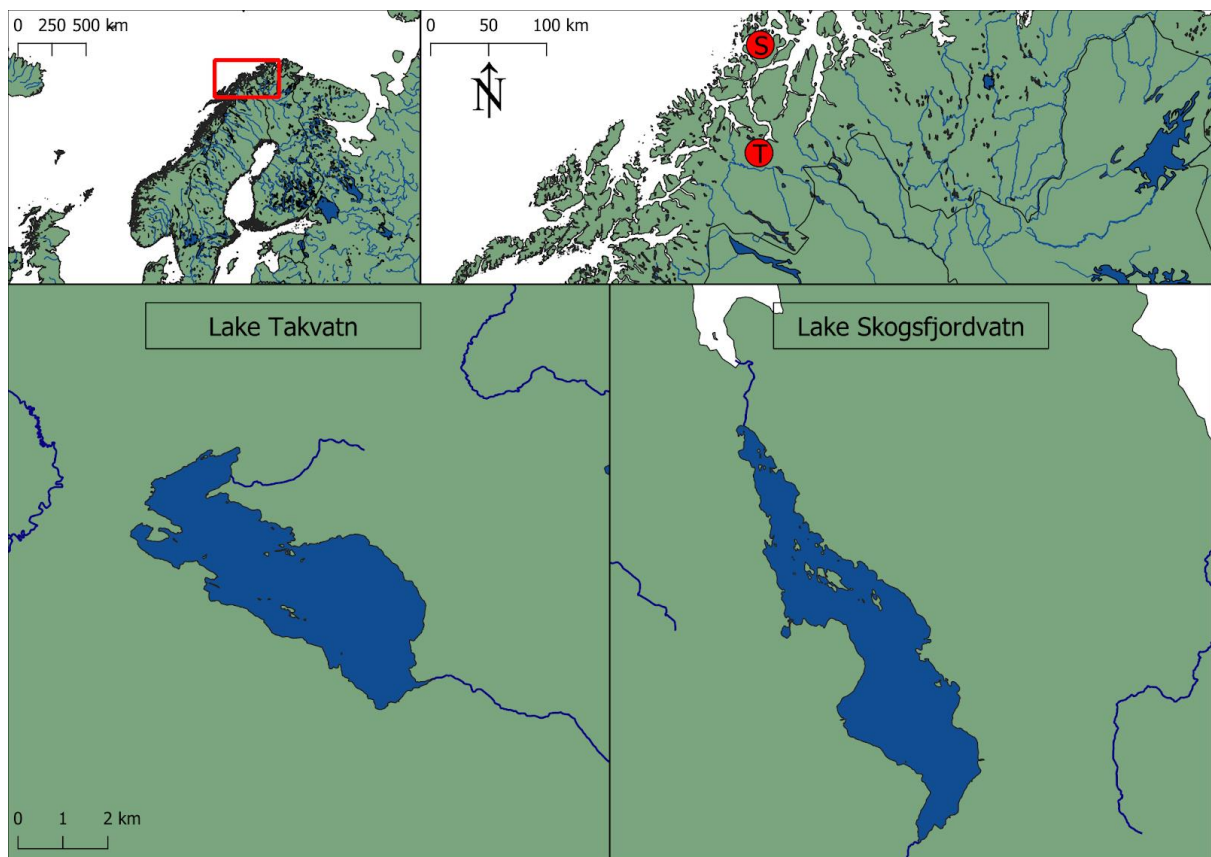

Figure 1: Map of the two study lakes, Takvatn (T) and Skogsfjordvatn (S).

4.2 Host sampling

The sampling for the amphipod *Gammarus lacustris*, stoneflies, and freshwater clams took place between the 4th of June and the 15th of September using mixed mesh size dredges that were scraped along the lake floor, either by boat or by utilising a kick-net technique. In Takvatn *G. lacustris* and sphaeriid clams were sampled from the dense *Nitella* belts in

Hauglibukta. In Skogsfjordvatn, stoneflies were sampled along with *G. lacustris* and sphaeriid clams, just outside Skogsfjord Grendehus in a small bay with dense algae growth.

The brown trout and Arctic char used in this study were caught during mid-August - beginning of September, and a second sampling in November, using mixed mesh size gillnets placed in littoral parts (1-10 m depth) of the lakes for approximately 24 h. Prior to parasitological examination, the fish weight and length- to the fork (FL) were measured, the otoliths collected, and the sex determined (not used in this study). The processing of fresh fish, including parasite collection, was completed no later than 48 h after they were caught, as all *Crepidostomum* specimens had to be sampled while still alive.

4.3 Parasite sampling

The amphipods and stoneflies were dissected by squishing them between two glass plates under a dissection microscope to examine the hosts' internal structures for the presence of metacercarial stages (Henriksen, personal communication, 2021). Once extracted, the metacercarial cyst of *Crepidostomum* was perforated with two needles, and the metacercarial larvae excysted, and rinsed in saline water prior to storage in RNAlater™. Some metacercariae were progenetic, i.e., they displayed precocious sexual reproduction while still in the cyst. These were also stored in RNAlater™, but the eggs were removed and kept in separate tubes. Metacercariae of *Plagiorchis* were also counted, and some of them excysted and preserved in RNAlater™ for molecular identification.

Crepidostomum life stages from fish included juveniles (defined here as recently established individuals that have yet to produce eggs), adults, and eggs containing miracidia. Juveniles and adult stages were extracted from the fishes' intestine and pyloric caeca, following standard dissection protocols (Cribb & Bray, 2010; Justine et al., 2012). Adults often displayed a behaviour where they discharged their eggs from the uterus without mechanical provocation. This was not limited to the terminal eggs, but also the non-terminal eggs. If this behaviour did not occur, the eggs were mechanically extracted. Both juveniles, adults and eggs were rinsed in saline water prior to storage in RNAlater™. All individuals from each life stage were stored separately at -20 °C.

4.4 Experimental infections of clams

Cercaria were attempted obtained by experimental infections of freshwater clams. Glass tubes, each containing a single clam, and lake-water, were exposed to a single dosage of 8-12 eggs from *Crepidostomum* spp. This event marked day 1 of the experimental infections. The eggs were extracted either from adult *Crepidostomum* spp. found in the final host or from progenetic metacercaria from *G. lacustris*. The clams were kept in a cooling unit at 8 °C and under constant light for a total of 6 weeks. The tubes were observed for cercarial swarming every second day by visually examining them against a light source. The lake-water in the tubes was replaced every 3-4 days after the first week had passed. This delay in water-substitution was deliberate to avoid removing any swimming miracidia and/or eggs that had yet to hatch. Concurrent to the water-substitution, the clams were examined to determine their health status, i.e., dead, or alive. Dead clams were immediately dissected to look for sporocysts and cercaria, following a similar dissection protocol as described earlier for *G. lacustris*. At the end of the 6-week period all remaining clams were dissected. The cercariae from individual clams were rinsed in saline water before being stored in 2 mL Eppendorf tubes containing RNAlater™ at - 20 °C for later molecular analysis. The same protocol was applied for the collection of sporocysts from the clam tissue.

4.5 DNA extraction procedure

Specimens primed for later RNA-extraction were briefly removed from RNAlater™ storage tubes onto a piece of aluminium foil where a tiny fragment of tissue was removed for subsequent DNA extraction. The specimens were covered in RNAlater™ throughout the entire procedure. For specimens not subjected to RNA-extraction, the whole individual was used for the DNA extraction. DNA extraction was performed using a Chelex®-solution of 5% Chelex® (Bio-Rad, Life Science Group) in 50 mL MQwater. 200 µL of this solution was added to each tube containing tissue. Two µL Proteinase K solution (10 mg/ml) was added to each tube before they were incubated in an Eppendorf Thermomixer® C (Eppendorf Smartblock™, 1,5 mL) at 56 °C overnight (minimum 2 h) with shaking at 850 rpm. Following incubation, samples were vortexed at low shake for homogenization and boiled at 90°C for 8 min in a Thermoblock, then vortexed again at low shake and centrifuged at 15,000 RCF for 10 min. Samples were stored at 4 °C for a minimum of 1 hour prior to PCR, or at -20 °C for long term storage.

Partial 28S rDNA sequences of flatworm parasite specimens, as well as bivalves, and amphipod hosts were amplified by using the primers U178 (forward; 5′-GCA CCC GCT GAA YTT AAG-3′) and L1642R (reverse; 5′-CCA GCG CCA TCC ATT TTC A-3′) (Lockyer et al., 2003). Partial CO1 sequences of flatworms belonging to the genus *Plagiorchis* were amplified by using the primers PlagDipCO1hF (forward; 5′-ACG TTG GAT CAY AAG CG-3′) (Blasco-Costa, unpublished) and CO1R-Trema (reverse; 5′-CAA CAA ATC ATG ATG CAA AAG G-3′) (Miura, et al, 2005). JB3 (forward; 5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) (Bowles et al., 1993) was used in some cases where the sequences had to be redone. PCR amplifications were carried out using a total volume of 15 µL per reaction consisting of 1,8 µL MQwater, 7,5 µL 2X MyFi™ Mix, 0,6 µL of each primer (10 pmol/µL) and 4.5 µL genomic DNA. The thermocycling profile used for 28S rDNA amplification was as follows: Initial denaturation of DNA (95 °C for 3 min); 38 cycles of amplification (94 °C for 50 s, 54 °C for 30 s and 72 °C for 1 min 20 s); final extension (72 °C for 4 min); and cooling (15 °C for 5 min). The same profile but with an annealing temperature of 50 °C was used for COI rDNA amplification.

Presence of DNA was confirmed with gel-electrophoresis. The gel was prepared using a molecular grade 1,5 % agar - tris borate EDTA (TBE) mixture, with 1 µL ethidium bromide (EtBr) added. 1 µL loading dye mixed with 2 µL PCR-DNA was added to the wells for each specimen. 1 µL DNA ladder peqGOLD 100 bp plus was used each run. If DNA was confirmed, 1 µL of Exo1-Fast AP, consisting of 0,1 µL Exo1 and 1 µL Fast AP, was added to the tube containing non-purified DNA. The tubes were shaken and spun to mix and remove any bubbles before undergoing an exofast thermocycling clean-up. The thermocycling profile used for the exofast PCR cleanup was as follows: incubation (37 °C for 40 min); and deactivation (94°C for 15 min). Tubes were then stored at -20 °C as purified DNA until they were prepared for sequence analysis.

Purified DNA was retrieved from storage and diluted with 10 µL MQwater if the DNA concentration was deemed too high, judging by the strength of the gel-bands. 5 µL of the corresponding primer was added to each well in the Macrogen EZ-seq -plate, followed by the addition of 5 µL purified DNA. Purified amplicons were sent to Macrogen Europe for sequencing with the same PCR primers used for amplification. An additional internal primer was also used for CO1: JB4.5 (reverse; 5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′)

(Bowles et al., 1993), and for 28S: L1200R (5′-GCA TAG TTC ACC ATC TTT CGG-3′) (Littlewood et al., 2000).

4.6 Sequence alignment

Sequences were assembled and scanned for errors using Geneious Prime® v.2022.0.2. Reference sequences used for phylogenetic construction were downloaded from GenBank and aligned together with the sequences from this study to confirm species epithets for trematode parasites and bivalve hosts. Two outgroups were used in most phylogenetic constructions to root the individual trees. The sequences were aligned using the MAFFT Multiple Alignment plugin v.1.4.0 (Katoh et al., 2002) with the default settings before being trimmed to a uniform length. COI sequences were checked for the presence of pseudogenes by examining all 6 reading frames for stop codons, using the "translate" feature on the alignments with the genetic code set to "Echinoderm mitochondrial". If one of the frames contained no stop codons, or only at the end, the sequences were considered to be the true COI gene.

The phylogenetic analyses were carried out using both maximum likelihood (ML) and Bayesian inference (BI) criteria on each dataset using the free web software CIPRES (Miller et al., 2010). ML analyses were conducted using RAxML-HPC2 on XSEDE version 8.2.12 (Stamatakis, 2014) with GTRGAMMA as the model for bootstrapping phase, and the number of bootstrap iterations set to 1000. BI analysis was conducted by using MrBayes on XSEDE version 3.2.7a (Ronquist et al., 2012) running 2 independent Markov chain Monte Carlo (MCMC) runs with 4 chains for 10^7 generations, with a sample tree frequency every 1000th generation and a burn-in frac set to 0.25. Phylograms were constructed in FigTree v.1.4.4 and further edited in Inkscape v1.0 for visualisation purposes.

5 Results

A total of 473 macroinvertebrates were dissected for this study; 62 stoneflies from Skogsfjordvatn, 382 Gammarus (237 Takvatn; 145 Skogsfjordvatn) and 29 sphaeriid bivalves (8 Takvatn; 21 Skogsfjordvatn). Trematode infections were reported in all invertebrate groups, and fell into four genera; *Crepidostomum*, *Plagiorchis*, *Bunodera* and *Phyllodistomum*, although the latter two are only represented with a single individual infection from freshwater clams.

5.1 Trematode infection data in *Gammarus lacustris*

The total trematode prevalence in *G. lacustris* was found to be 41,1 % (n = 157 infected). When split by study site, the prevalence was 15,2 % (n = 36 infected) in Takvatn and 83,4 % (n = 121 infected) in Skogsfjordvatn. Molecular identification of the parasite specimen revealed *C. metoecus* as sole *Crepidostomum* species using *G. lacustris* as host (see Figure 2), and *Plagiorchis* sp. 2 (sensus Soldánová et al., 2017) as sole *Plagiorchis* species infecting *G. lacustris* (see Figure 4). All instances of *C. metoecus* infections were accompanied by co-infections of *Plagiorchis* sp. 2. As such, the prevalence of *Plagiorchis* sp. 2 was the same as the total trematode prevalence in this host. The prevalence of *C. metoecus* was 2,6 % (n = 10) both lakes considered; 1,3 % (n = 3) in Takvatn and 4,8 % (n = 7) in Skogsfjordvatn. Of the 12 total *Crepidostomum* metacercaria, 83,3 % (n = 10) were progenetic, meaning that they had matured into adults while still in its cyst.

The mean intensity of *Plagiorchis* sp. 2 was 1,95 ± 2,12 (mean ± SD) in Takvatn, with a maximum of 13 individuals in a single host. In Skogsfjordvatn the mean intensity was 7,22 (±SD = 7,29) with 38 *Plagiorchis* sp. 2 as the highest intensity in a single individual. *C. metoecus* had a mean intensity of 1,0 (±SD = 0,0) in Takvatn, with no more than a single individual in a host. In Skogsfjordvatn the mean intensity was 1,29 (±SD = 0,45) with a maximum of two individuals in a single host.
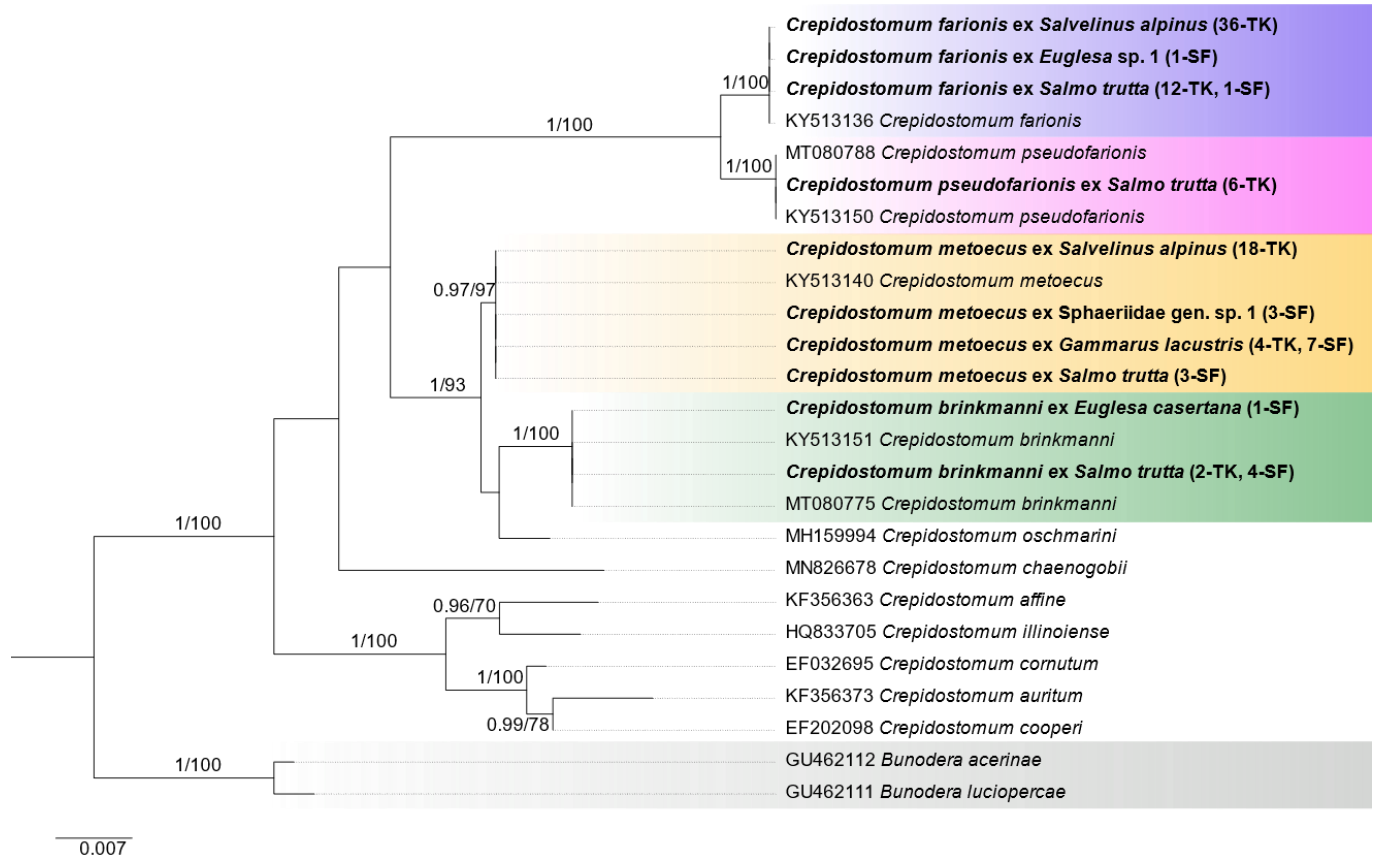
Figure 2: Phylogram built from a Maximum likelihood (ML) analysis of members of *Crepidostomum* based on a 28S sequence alignment (1125 nucleotides, 25 sequences). Nodal support is given above the branches as bootstrap support (BS) from the ML preceded by posterior probabilities (PP) from Bayesian Inference (BI) analysis. Only BS values > 70 and PP > 0.90 are shown. Taxa in bold represent lineages with new sequences from this study. Numbers in parenthesis indicates the number of identical sequences per site (TK = Takvatn, SF = Skogsfjordvatn). The scale bar indicates the number of expected substitutions per site. More information on hosts, life stages and geography for the newly generated sequences can be found in Table 2. Outgroup taxa are marked in grey.

5.2 Trematodes in Stoneflies

Plagiorchis was the only genus of trematodes found to infect stoneflies (see Figure 4 & Figure 5) and had a prevalence of 41,9 % (n = 26 infected) across all sampled Plecopterans. The mean intensity was 4,54 (±SD = 4,04) with the maximum infection registered of 21 *Plagiorchis* specimens in a single stonefly nymph. Only the stonefly *Diura bicaudata* was identified down to a species level, as this stonefly harboured the majority of metacercaria. 27 out of 63 stoneflies were identified as *Diura bicaudata* following the keys of Lillehammer, (1988) and (Hynes, 1993). The prevalence of *Plagiorchis* in *D. bicaudata* was found to be 88,9 % (n = 24 infected), with a mean intensity of 4,92 (±SD = 4,17). These infections

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belonged to two species of *Plagiorchis*, namely *Plagiorchis* sp. 3 and *Plagiorchis* sp. 6 (sensus Soldánová et al., 2017). To my knowledge this is the first record of these species in a plecopteran host. Of 22 molecularly identified *Plagiorchis* specimens, 7 were *Plagiorchis* sp. 3, and 15 *Plagiorchis* sp. 6. These were extracted from 14 individuals of *D. bicaudata*, with three instances of co-infections.

Figure 3: Non-sequenced metacercaria from the stonefly *Diura Bicaudata,* assumed to be *Plagiorchis* sp. 3 and/or *Plagiorchis* sp. 6. Compound scope 40x magnification.
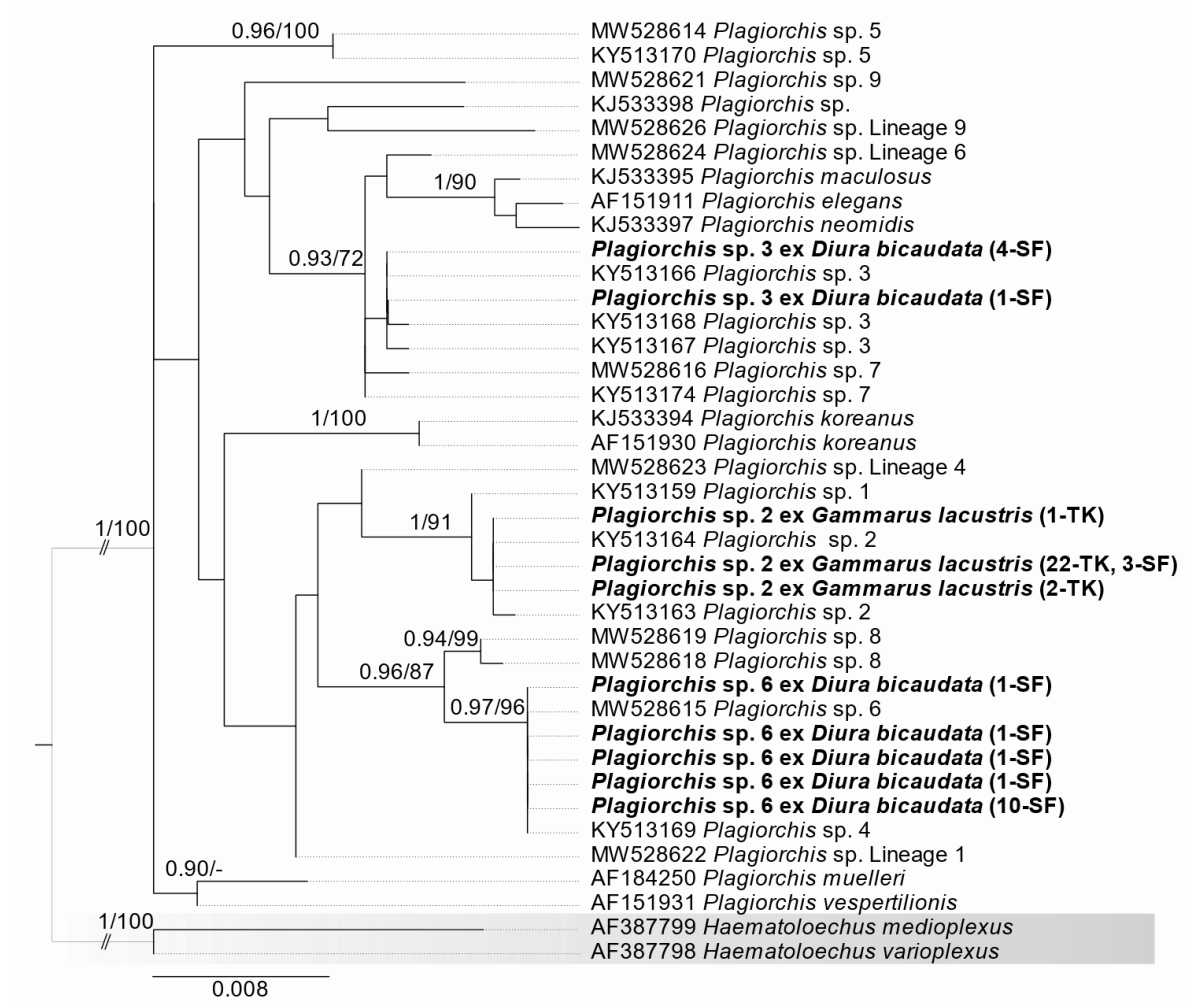
Figure 4: Phylogram built from a Maximum likelihood (ML) analysis of members of *Plagiorchis* based on a 28S sequence alignment (1119 nucleotides, 39 sequences). Nodal support is given as bootstrap from Maximum likelihood (ML) and posterior probabilities from Bayesian Inference (BI). ML values > 70 and BI > 0.90 are shown. Taxa in bold represent lineages with new sequences from this study. The scale bar indicates the number of expected substitutions per site. Numbers in parenthesis indicates the number of sequences per site (TK = Takvatn, SF = Skogsfjordvatn). The scale bar indicates the number of expected substitutions per site. More information on hosts, life stages and geography for the newly generated sequences can be found in Table 2. Outgroup taxa are marked in grey.
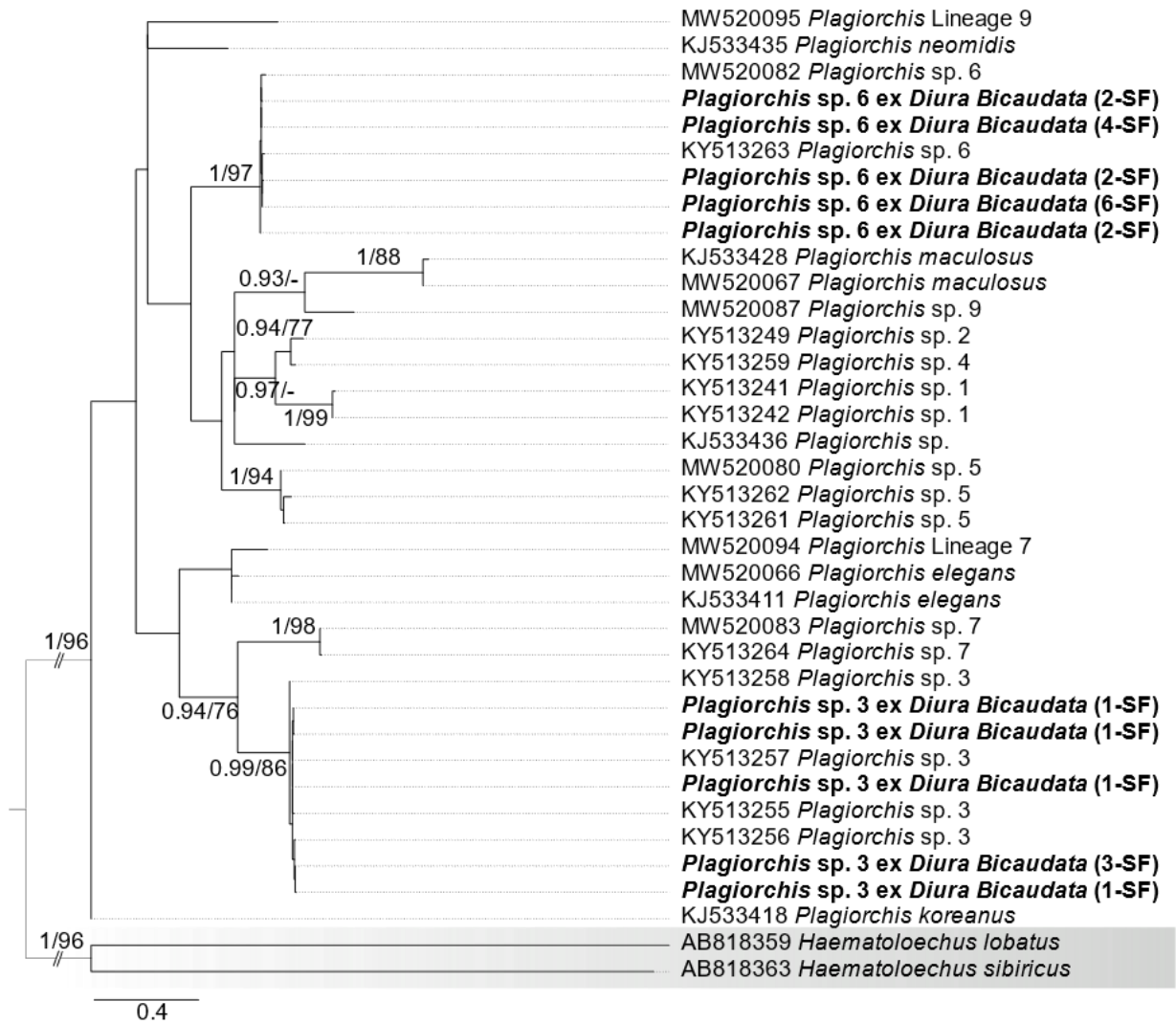
Figure 5: Phylogram built from a Maximum likelihood (ML) analysis of members of *Plagiorchis* based on a COI sequence alignment (362 nucleotides, 39 sequences). Nodal support is given as bootstrap from Maximum likelihood (ML) and posterior probabilities from Bayesian Inference (BI). ML values > 70 and BI > 0.90 are shown. Taxa in bold represent lineages with new sequences from this study. The scale bar indicates the number of expected substitutions per site. Numbers in parenthesis indicates the number of sequences per site (TK = Takvatn, SF = Skogsfjordvatn). The scale bar indicates the number of expected substitutions per site. More information on hosts, life stages and geography for the newly generated sequences can be found in Table 2. Outgroup taxa are marked in grey.

5.3 Sphaeriid clams and their trematode infections

We were able to molecularly confirm the presence of four sphaeriid clams from Skogsfjordvatn, namely *Euglesa casertana*, *E. lilljeborgii* [Clessin in Esmark & Hoyer, 1886], as well as a species closely related to *E. lilljeborgii* referred to as *Euglesa* sp. 1 (see Figure 6). Unfortunately, no reference sequences from 28S rDNA were available for the fourth species or to a close relative. Its position in the phylogram does not allow identification even to a genus level and is therefore referred to as Sphaeriidae gen. sp.1. The clams collected from Takvatn were not sequenced as no infections were found in them.
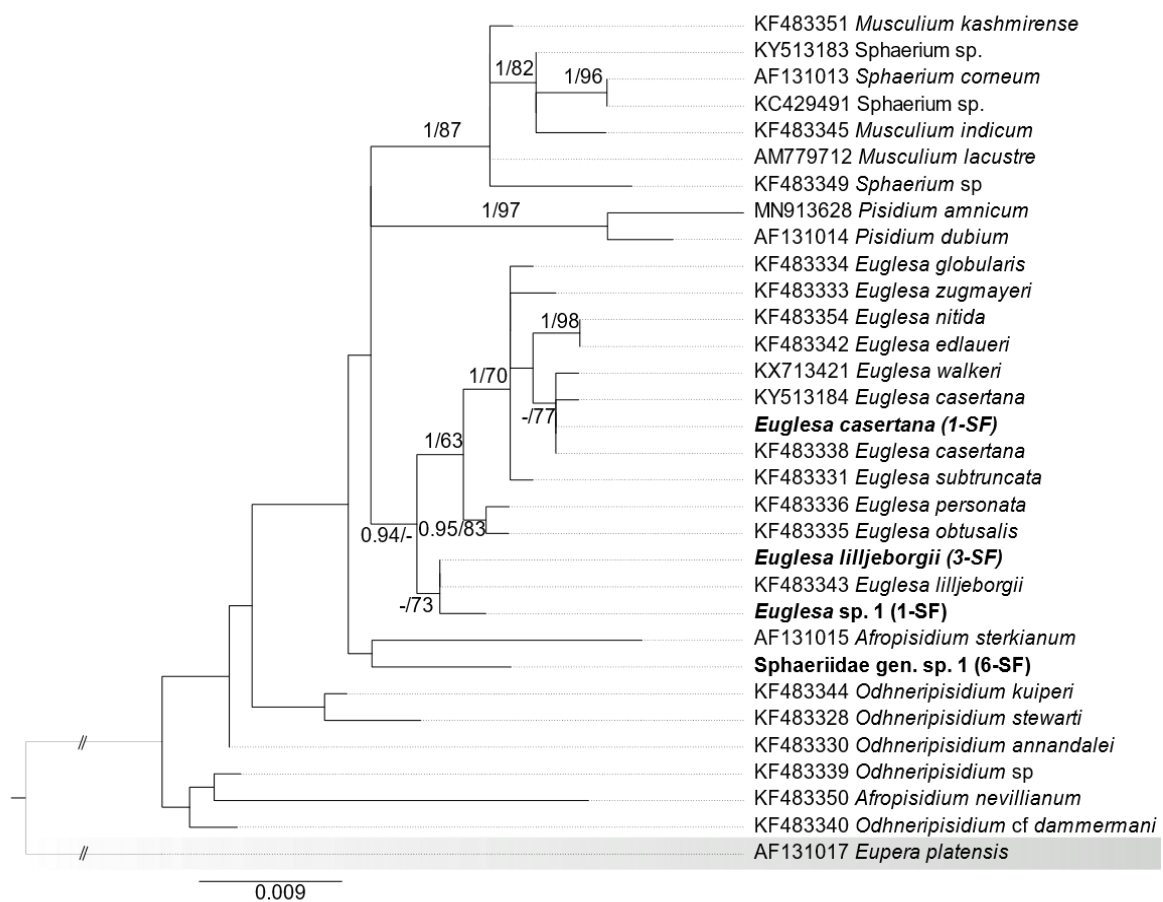

Figure 6: Phylogram built from a Maximum likelihood (ML) analysis of the Sphaeriidae family based on a 28S sequence alignment (725 nucleotides, 32 sequences). Nodal support is given as bootstrap from Maximum likelihood (ML) and posterior probabilities from Bayesian Inference (BI). ML values > 70 and BI > 0.90 are shown. Taxa in bold represent lineages with new sequences from this study. The scale bar indicates the number of expected substitutions per site. Numbers in parenthesis indicates the number of sequences per site (TK = Takvatn, SF = Skogsfjordvatn). The scale bar indicates the number of expected substitutions per site. More information on hosts, life stages and geography for the newly generated sequences can be found in Table 2. Outgroup taxa are marked in grey.

Trematodes had a 27,6 % (n = 8) prevalence in sphaeriid clams. Seven of these were molecularly identified as one did not amplify. Of the seven molecularly identified infections, five belonged to different species, from two families of trematodes (Allocreadiidae and Gorgoderidae). Cercaria and redia of *Bunodera vytautasi* [Atopkin, Sokolov, Shedko, Vainutis & Orlovskaya, 2018] were confirmed from the bivalve *Euglesa lilljeborgii* (see Figure 7), a new host for this parasite. *C. brinkmanni* was confirmed from *E. casertana* which is a known host (Petkevičiute et al., 2018). Cercaria and redia of *C. farionis* were found in *Euglesa* sp. 1, as no reference sequences were available for 28S. Cercaria and redia of *C. metoecus* were confirmed from three individuals of Sphaeriidae gen. sp.1 (see Figure 2). A single cercaria of *Phyllodistomum umblae* was found in a sphaeriid clam that did not amplify during PCR (see Figure 8). As such, no molecular information on this sphaeriid clam species is available from this study, but see Petkevičiūtė et al., (2015).
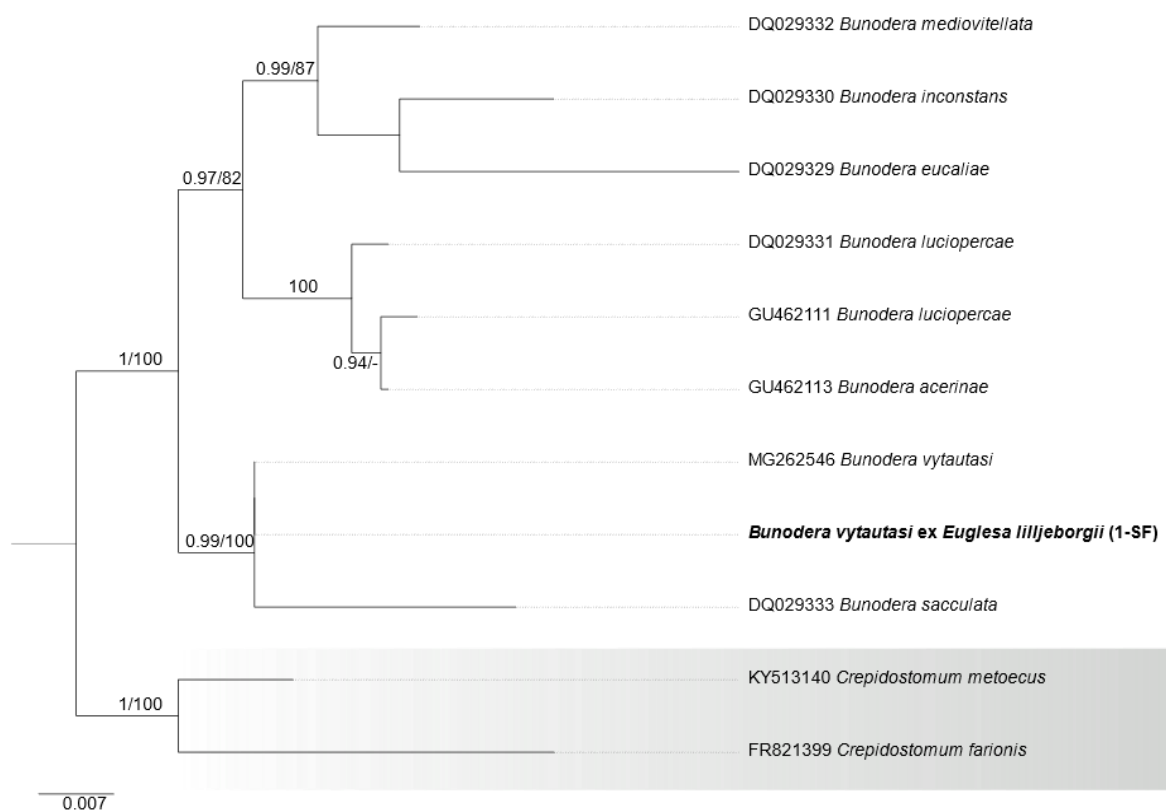
Figure 7: Phylogram built from a Maximum likelihood (ML) analysis based on a 28S sequence alignment of *Bunodera* species (752 nucleotides, 11 taxa). Nodal support is given as bootstrap values from Maximum likelihood and posterior probabilities from Bayesian Inference (BI). ML values > 70 and BI > 0.90 are shown. Taxa in bold represent lineages with new sequences from this study. The scale bar indicates the number of expected substitutions per site. Numbers in parenthesis indicates the number of sequences per site (TK = Takvatn, SF = Skogsfjordvatn). The scale bar indicates the number of expected substitutions per site. More

information on hosts, life stages and geography for the newly generated sequences can be found in Table 2. Outgroup taxa are marked in grey.
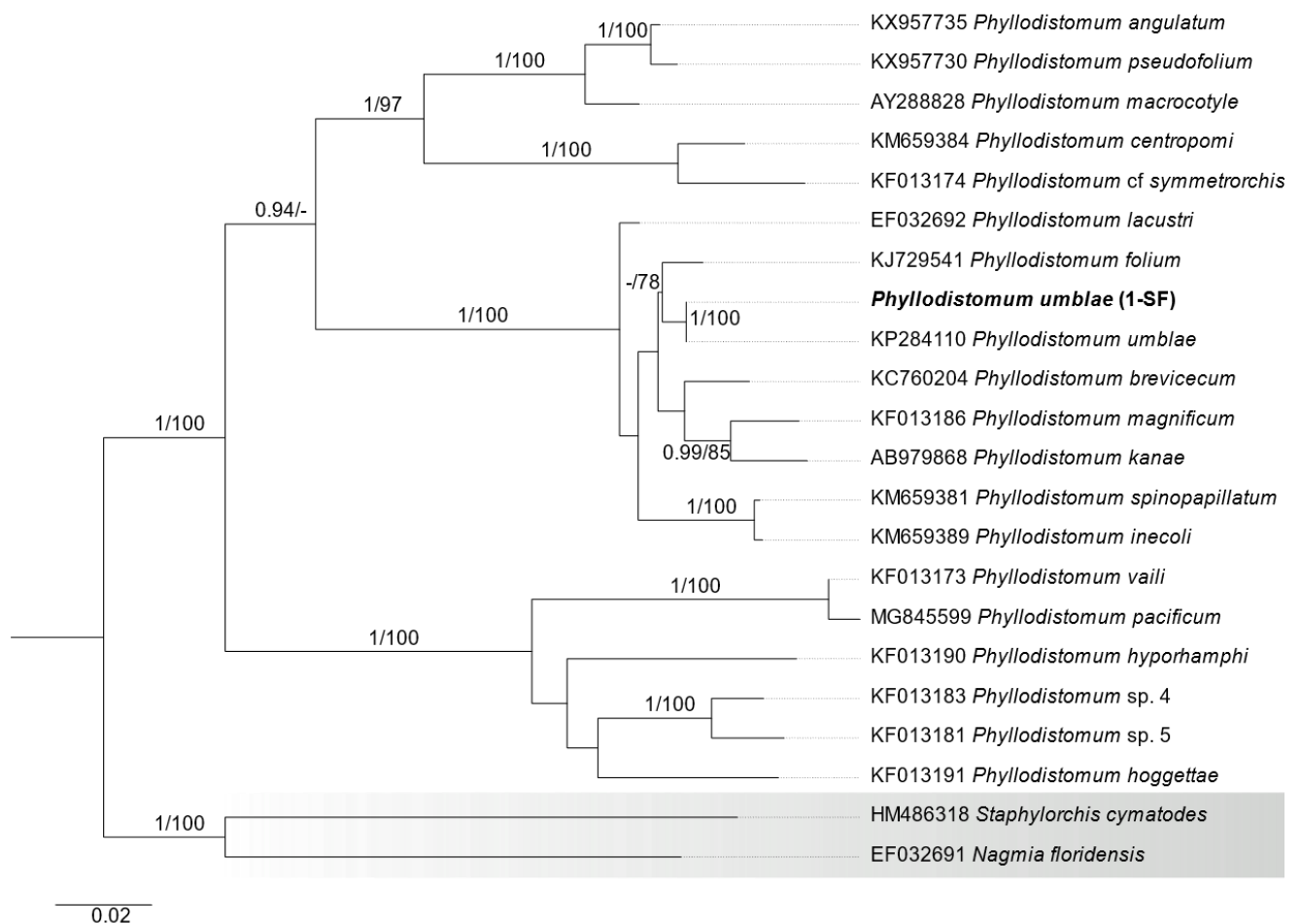
Figure 8: Phylogram built from a Maximum likelihood (ML) analysis of *Phyllodistomum* based on a 28S sequence alignment (1101 nucleotides, 22 sequences). Nodal support is given as bootstrap from Maximum likelihood and posterior probabilities from Bayesian Inference (BI). ML values > 70 and BI > 0.90 are shown. Taxa in bold represent lineages with new sequences from this study. The scale bar indicates the number of expected substitutions per site. Numbers in parenthesis indicates the number of sequences per site (TK = Takvatn, SF = Skogsfjordvatn). The scale bar indicates the number of expected substitutions per site. More information on hosts, life stages and geography for the newly generated sequences can be found in Table 2. Outgroup taxa are marked in grey.

5.4 *Crepidostomum* spp. in brown trout and Arctic char

A total of 34 fish were dissected for this study: 28 from Takvatn and 6 from Skogsfjordvatn. The fish from Takvatn was sampled in mid-August (n = 13) and November (n = 15), and the fish from Skogsfjordvatn in early September (n = 6). Because not every individual *Crepidostomum* was sampled and molecularly identified, the statistical parameters (i.e., prevalence and mean intensity) of the four species cannot be assessed. However, since I did not target a specific *Crepidostomum* spp. from the fish I was able to examine the proportion of specimens that belonged to each confirmed species via molecular identification in each fish host and season combination. In total, 86 isolates of *Crepidostomum* were molecularly analysed. Based on these sequences I confirmed that *C. metoecus*, *C. farionis*, *C. brinkmanni* and *C. pseudofarionis* resides in Takvatn, as well as *C. metoecus*, *C. farionis* and *C. brinkmanni* in Skogsfjordvatn. All four *Crepidostomum* species were present in *S. trutta*, but only *C. metoecus* and *C. farionis* were found in *S. alpinus* (see Figure 9). In both brown trout and Arctic char, the juvenile *C. farionis* were more numerous in August, and adults more numerous during November. *C. metoecus* were less numerous in both stages, except during November in Arctic char where juveniles constituted 41 % of the analysed specimens, and adults 10 % (see Figure 10).

Table 1: Number of fish caught by lake, month, and fish species. TK = Takvatn, SF = Skogsfjordvatn.

Species / Month + Lake	TK August	SF September	TK November	**Total fish**
Salmo trutta	3	6	5	**14**
Salvelinus alpinus	10	0	10	**20**
Total fish	**13**	**6**	**15**	**34**

Crepidostomum spp. host distibution

Arctic char

21; 36%

37; 64%

Brown trout

6; 21%

13; 47%

6; 21%

3; 11%

■ *C. farionis* ■ *C. pseudofarionis*
■ *C. metoecus* ■ *C. brinkmanni*
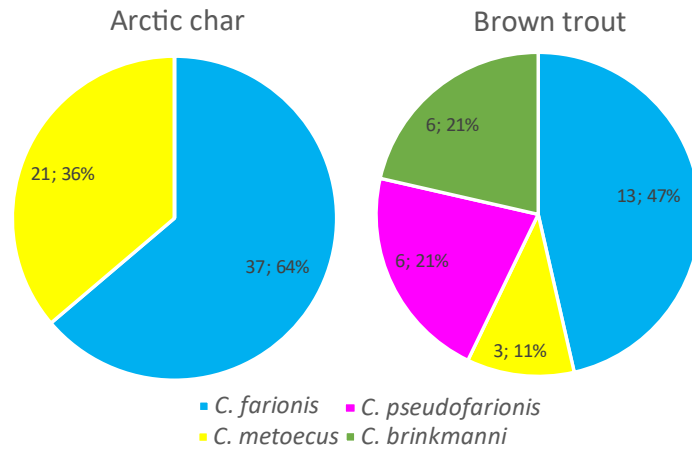
Figure 9: Pie charts showing the composition of the molecularly identified *Crepidostomum* spp. in Arctic char and brown trout in both lakes from all samplings. In each fraction, the absolute number of *Crepidostomum* specimens is given on the left of the semicolon, and the percentage of the total is indicated to the right.

C. metoecus & *C. farionis*
Seasonal & developmental distributions

Brown trout - Aug-Sep

2; 29%

1; 14%

4; 57%

Brown trout - Nov

1; 11%

8; 89%

Arctic char - Aug-Sep

1; 6%

8; 47%

8; 47%

Arctic char - Nov

17; 41%

20; 49%

4; 10%

■ *C. farionis* - Adult ■ *C. farionis* - Juvenile
■ *C. metoecus* - Adult ■ *C. metoecus* - Juvenile

Figure 10: Pie charts showing the distributions of adult and juvenile stages of *C. farionis* and *C. metoecus* in August/September and the November sampling. Each fraction shows the absolute number of *Crepidostomum* specimens on the left of the semicolon, and the percentage of the total is indicated to the right.
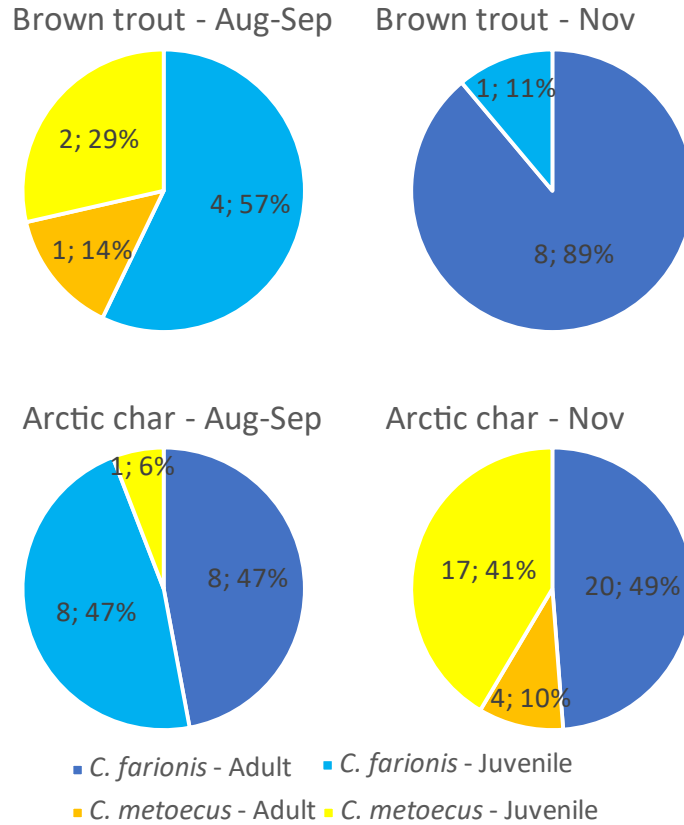
Table 2: Summary table for all isolates from this study that were used for obtaining new molecular data for 28S rDNA and COI (cytochrome c oxidase subunit 1) sequences. Lifecycle stages include adult (A), juvenile (J), metacercarial (M), redial (R), and cercarial (C) stages. SF = Skogsfjordvatn, TK = Takvatn. *Indicates a failed amplification.

Species	Host	Host family	Life stage	Locality	Gene
Family Allocreadiidae, [Loos 1902]					
Bunodera vytautasi [Atopkin, Sokolov, Shedko, Vainutis & Orlovskaya, 2018]	*Euglesa lilljeborgii*	Sphaeriidae [Deshayes, 1855 (1820)]	C, R	SF	28S
Crepidostomum brinkmanni [Faltýnková, Pantoja, Skírnisson, Kudlai, 2020]	*Euglesa casertana*	Sphaeriidae	C, R	SF	28S
	Salmo trutta	Salmonidae [Cuvier, 1816]	A, J	TK, SF	28S
Crepidostomum farionis [Müller, 1780]	*Euglesa* sp. 1	Sphaeriidae	C, R	SF	28S
	Salmo trutta	Salmonidae	A, J	TK, SF	28S
	Salvelinus alpinus	Salmonidae	A, J	TK	28S
Crepidostomum metoecus [Braun, 1900]	Sphaeriidae gen. sp.1	Sphaeriidae	C, R	SF	28S
	Gammarus lacustris	Gammaridae [Leach, 1813]	M	TK, SF	28S
	Salmo trutta	Salmonidae	A, J	SF	28S
	Salvelinus alpinus	Salmonidae	A, J	TK	28S
Crepidostomum pseudofarionis [Faltýnková, Pantoja, Skírnisson, Kudlai, 2020]	*Salmo trutta*	Salmonidae	A	TK	28S
Family Gorgoderidae [Looss, 1899]					
Phyllodistomum umblae [Fabricius, 1780 (Bakke, 1982)]	*Sphaeriidae gen. sp.	Sphaeriidae	C	SF	28S
Family Plagiorchiidae [Lühe, 1901]					
Plagiorchis sp. 2 (sensus Soldánová, 2017)	*Gammarus lacustris*	Gammaridae	M	TK, SF	28S
Plagiorchis sp. 3 (sensus Soldánová, 2017)	*Diura bicaudata*	Perlodidae [Klapálek, 1909]	M	SF	28S + COI
Plagiorchis sp. 6 (sensus Soldánová, 2017)	*Diura bicaudata*	Perlodidae	M	SF	28S + COI

6 Discussion

The macroinvertebrates examined in this study were found to be infected with a total of nine different species of trematodes, one which has never been recorded from Europe, and three which have never been recorded from the hosts they were extracted from. Considering that this study only looked at a very limited number of macroinvertebrates, it shows that there is still much to discover regarding cryptic trematodes in high latitudes and their host distributions. I also observed some preliminary patterns on the seasonality of *Crepidostomum* infections in *S. trutta* and *S. alpinus*, but a more focused and continuous sampling is required to statistically explore these patterns.

The infection parameters show some contrasting values with previous studies. The prevalence of *Plagiorchis* sp. 2 in *G. lacustris* in Takvatn was only 15,2 % in this study, while Shaw et al., (2020) found the prevalence to be 68,6 % from 474 specimens. Likewise, the prevalence of *C. metoecus* was 1,3 % in Takvatn in my study, and 13,3% in Shaw et al., (2020). This could be linked to the different sampling sites of the studies. While the sampling of Shaw et al., (2020) was split between 5 sites in Takvatn over multiple seasons with differences in prevalence, my study only utilised a single site during a single season. On the other hand, the prevalence of *Plagiorchis* sp. 2 in *G. lacustris* in Skogsfjordvatn was approximately 5,5 times higher than Takvatn, reaching 84,4 %, while the prevalence of *C. metoecus* remained low at 3,4 %. Unfortunately, no studies for comparison are available in Skogsfjordvatn. The huge difference between trematode infections in Skogsfjordvatn and Takvatn could be attributed to the sampling site, or the age class of *Gammarus*. While the length of *Gammarus* was not measured, it was noted that they generally appeared to be larger in Skogsfjordvatn than Takvatn. This could mean that older specimens, thus with a larger window to infection, were sampled from Skogsfjordvatn. The sampling site in Skogsfjordvatn was situated very shallowly in the water, right next to the shoreline where multiple species of birds, the putative definitive host of *Plagiorchis*, were observed. While the first intermediate host for *Plagiorchis*, the snail *Radix baltica,* was not sampled it could be that this sampling site constituted a well-suited place for *Plagiorchis* to complete its lifecycle, with aggregations of all its hosts.

It seems like COI, or possibly another rapidly mutating gene, is necessary for distinguishing closely related species. This is apparent when comparing *Plagiorchis* sp. 6 and *Plagiorchis* sp. 4 which are indistinguishable in their 28S gene, but different in their COI sequence. Without the use of COI and the sequences provided by Soldánová et al., (2017), I would not have known that there were in fact two lineages of *Plagiorchis* with similar 28S sequences. It is apparent that just like there is a gradient for the resolution of morphology (e.g., morphology by eye vs stereo microscope vs electron microscope), there is also a gradient for the resolution on molecular data, i.e., what genes are sequenced, as well as the length of the generated sequences.

The majority of *C. metoecus* metacercaria were found to be progenetic, an adaptation where the metacercarial worm matures while still in the cyst in *G. lacustris*. Although progenesis has been described from multiple trematode species from multiple families (e.g., Poulin & Cribb, 2002; Herrmann et al., 2014), the exact processes behind this adaptation have yet to be established, although multiple factors are believed to be involved in the promotion of this adaptation. The work of Lagrue & Poulin, (2009) found that progenesis in the trematode *Coitocaecum parvum* [Crowcroft, 1945] was linked the lack of chemical cues from its definitive host, and Herrmann & Poulin, (2012) found that host length (age) influenced the degree of progenesis. Looking into the relationship between *Gammarus* length and degree of progenesis could therefore be of interest.

The discovery of *B. vytautasi* in northern Norway increases the number of species of *Bunodera* [Railliet, 1896] in Europe to three. Only two other species, namely *B. luciopercae* [Müller, 1776; Lühe, 1908] and *B. acerinae* [Roitman & Sokolov, 1999], have previously been recorded from Europe. Till now, *B. vytautasi* was only known from the Russian far east, as adults in the intestine of *Pungitius pungitius* L. (nine-spine stickleback) (Atopkin et al., 2018). This means that this is also the first time this trematode has been identified from an intermediate host. However, *P. pungitius* is not present in Skogsfjordvatn, meaning that *B. vytautasi* is likely to infect other fish instead. While the parasite community of both *S. trutta* and *S. alpinus* in this lake has been studied, no studies have so far examined the parasite community of *Gasterosteus aculeatus* (three-spined stickleback) molecularly. It is therefore

possible that *G. aculeatus* serves as a definitive host of *B. vytautasi*. During a study on the parasite community of *G. aculeatus* in Takvatn by Kuhn et al., (2015), they found what was believed to be a species of *Crepidostomum*. Morphologically, *Crepidostomum* and *Bunodera* are relatively similar, and since no molecular identification was done, my findings and their result raise the question of whether this could in fact have been *B. vytautasi*. *Crepidostomum metoecus* has, however, on two occasions been recorded form *Pungitius thymensis* [Nikolskii, 1889] (Sakhalin stickleback) (Ayer & Katahira, 2015; Vainutis et al., 2021).

This study was, as stated in the preface, originally meant to explore the regulation of gene expression in *C. metoecus*. Because of this, only live specimens were collected. Dead *Crepidostomum* were registered, but not sampled. As such, if different *Crepidostomum* species display different survival-rates in a deceased host, they would be biased by sampling. This is, however, not likely to be the case when considering how similar the species are both taxonomically and, in their habitat/host usage. While I was only able to confirm *C. metoecus* and *C. farionis* in *S. alpinus*, former studies from Europe have confirmed both *C. brinkmanni* (Rochat et al., 2021) and *C. pseudofarionis* (Faltýnková et al., 2020; Rochat et al., Under Review (2022)) in this host. We were also able to confirm all four *Crepidostomum* species to infect *S. trutta*. All four *Crepidostomum* species were found in Takvatn, but *C. pseudofarionis* remained absent in Skogsfjordvatn. It is, however, very likely that *C. pseudofarionis* is present in Skogsfjordvatn, and its apparent absence explained by a low sampling effort in the lake. Both *C. metoecus* and *C. farionis* has been recorded from multiple other salmonid hosts, as summarised by Moravec, (2004). However, it is not unthinkable that some observations of *C. metoecus* and *C. farionis* that predates molecular identification, has in fact been *C. brinkmanni* and *C. pseudofarionis*. So far however, only *S. trutta* and *S. alpinus* are described as the principal definitive hosts of *C. brinkmanni* and *C. pseudofarionis*.

Seasonal patterns are difficult to affirm from only two samplings from two seasons. However, I was able to observe differences in the proportions of adults and juveniles of two co-infecting species, *C. metoecus* and *C. farionis* from the late summer and the late autumn samples. In both brown trout and Arctic char, the juvenile *C. farionis* were more numerous in August, and adults more numerous during November. In Arctic char, some adult *C. farionis*

were also found in the summer sample. This may be an indication of a seasonal pattern where fish are infected mainly during summer by *C. farionis,* and the parasite matures during autumn and winter. *Crepidostomum metoecus* on the other hand was only found in brown trout during the summer as a single adult and two juveniles, and no individuals during November. In Arctic char, *C. metoecus* were represented by four adults and 17 juveniles from the November sample. This could suggest that *C. metoecus* has a distinct autumn/winter population, where Arctic char is infected mainly during autumn and matures during winter. Unfortunately, as stated above the sampling size is too low to draw conclusions. A possible explanation for this trend is that *G. lacustris*, the intermediate host for *C. metoecus*, is more preyed upon by Arctic char during the late Autumn and winter periods (Knudsen et al., 2008), thus facilitating infection during that season. By contrast, insect larvae, where *C. farionis* are encysted, are more preyed upon during the summer period (Knudsen et al., 2008), making this the period where most infections of *C. farionis* occur. From Prati et al., (2020) we know that *Crepidostomum* spp. infections are prevalent the whole year, but with fluctuations. Prati et al., (2020) also found that the intensity of *Crepidostomum* spp. doubled from summer to early in winter in Arctic char but was steadily low in brown trout from summer to late winter. The prevalence of *Crepidostomum* spp. was consistent in both brown trout and Arctic char, with a small peak during early winter. To my knowledge, no studies have examined the difference in seasonal and developmental patterns between closely related *Crepidostomum* species. Some studies which predate the discovery of *C. brinkmanni* and *C. pseudofarionis* described distinct seasonal cycles for *C. metoecus* (Thomas, 1958; Awichie, 1968; Moravec, 1982) with the winter as the maximum and the summer as the minimum infection periods, although this probably varies depending on the species of *Crepidostomum* in question, and their longevity, of which little is known. Moravec, (1982), also notes that *S. trutta* acquire new infections of *C. metoecus* particularly during spring and autumn, the latter which is an observation that is also seen in this study.

No molecular large-scale studies have been conducted on the bivalves in either Takvatn, Skogsfjordvatn, or to my knowledge, anywhere else in Scandinavia. Soldánová et al., 2017 identified *E. casertana*, as well as a species of *Sphaerium* to reside in Takvatn, but only two isolates were sequenced in total. In my study I confirmed multiple species of freshwater bivalves to inhabit Skogsfjordvatn. The species I did not manage to identify down to species or even genus level, i.e., *Euglesa* sp. 1. and Sphaeriidae gen. sp.1 are likely already

described morphologically but they lack available 28S sequence data that would allow molecular matching. Seeing that molecular identification has become more common for the identification of cryptic clams (e.g. Lee & Foighil, 2003; Clewing et al., 2013; Bößneck et al., 2016; Mouthon & Forcellini, 2017; Prié et al., 2021; Clewing et al., 2022) these will undoubtedly be identified in the future. I should mention that taxonomy of the Sphaeriidae family is, as noted by Bogatov, (2017), subject to discussion. Here I chose a more liberal approach regarding the newly assigned names for the clams. This is in accordance with what is being used by databases such as MolluscaBase and other authors (e.g., Mouthon & Forcellini, 2017; Prié et al., 2021). That being said, Clewing et al., (2022) proposed that a complete taxonomic revision of the Sphaeriidae family, including all its sublevels, is required to fully determine the accuracy of the current taxonomic classification.

Insect larvae, stoneflies in particular, have been pinpointed as a potential intermediate host for the digenean *Phyllodistomum umblae* (e.g., Knudsen et al., 1997; Moccetti et al., 2019), although no studies detailing prevalence of infection, or the binomial ID of the stoneflies involved in *P. umblae* seem to be available. Other studies claim that species from this genus infects fish when the fish either eats clams directly (Anikieva et al., 1983 as in Rahkonen & Valtonen, 1987; Prozorova & Shed'ko, as in Busarova et al., 2017) or from cercaria penetrating the skin of the fish (Orecchia et al., 1975 (abstract only)). It is peculiar that in a lake with a reported prevalence of *P. umblae* exceeding 90 %, from Arctic char (Siwertsson et al., 2016), not a single metacercaria of this parasite has been reported from any intermediate hosts or from stomach samples. The lack of infections in stoneflies from my study further supports the notion that stoneflies may not be involved in the lifecycle of this parasite. However, more direct research on the life cycle of this trematode is required to draw concrete explanations.

The motives for resolving parasite life cycles can be varied, but Blasco-Costa & Poulin, (2017) highlights some specific scientific areas that would benefit from parasite life cycle elucidation. Applied ecology is a field heavily reliant on knowledge of parasite life stages to predict and mitigate transmission of parasites between hosts and to new sites. For instance, it would be of interest to know what parasites are expected to be co-introduced with

a potential new host in an area. This is particularly relevant in this time and age, where climate changes have been found to induce a northward migration in multiple species, and where transportation methods may facilitate migration of "hitchhiking" species. There is also an increased interest in the incorporation of parasites in food webs (e.g., Blasco-Costa & Poulin, (2017); Morton, & Lafferty, 2022), and the effect they have on stability and other metrics. Taxonomists may find that a parasite has some morphological characteristics that makes it more recognizable the intermediate hosts (Niewiadomska, 2002 as in Blasco-Costa & Poulin, (2017). It could also be that the final host of the parasite is involved in conservation efforts, or are otherwise elusive, thus making it hard to obtain data from them. In such cases, the data from intermediate hosts may be the best information contributor. The new data on parasite life cycles generated in this study, coupled with the newly generated sequence can therefore be of use to multiple scientific fields.

7 Conclusion

I discovered the presence of a species new to Europe, *Bunodera vytautasi*, as well as multiple new macroinvertebrate hosts in the life cycle pathways of trematodes, thus elucidating the diversity of the trematode community. Two lineages of *Plagiorchis* were new to *Diura bicaudata*, and the freshwater clam *Euglesa lilljeborgii* is a new host to *B. vytautasi*. I was able to expand the molecular knowledge on bivalves living in the northern lakes, in some cases down to a species level, thus increasing comprehension on not only the parasites themselves, but also their hosts. The sequences I generated have a large coverage and will be made available for future comparative studies. Some preliminary patterns were observed on the seasonal patterns of *C. metoecus* and *C. farionis*. Most notably, juvenile *C. farionis* were almost exclusively found in August/September, and by November almost all *C. farionis* specimens were adults. This could be an indication that infections happen during summer, and the parasite matures during the late Autumn. *Crepidostomum metoecus* was overall scarcely distributed, except in the Arctic char sample from November, where juveniles suddenly increased. This could indicate that *C. metoecus* infects Arctic char mainly during the late autumn and matures during winter. A more targeted and consistent seasonal sampling procedure is required to confirm patterns, but from these results there seems to be a difference in when *C. farionis* and *C. metoecus* infects their definitive hosts.

8 Future perspective

Looking to the future, I would suggest a new project collecting trematodes from *G. aculeatus* in both Takvatn, Skogsfjordvatn and possibly Lake Sagelvvatn (the lake where the Takvatn stickleback population is introduced from) and employing molecular methods as a mean to confirm the species identity of these trematodes. As of now, it is not known if three-spined sticklebacks are actually parasitized by *Crepidostomum* spp. or *Bunodera* spp. With the preliminary patterns observed in this study, I would also suggest looking deeper into the seasonal patterns of *Crepidostomum* in trout and Arctic char. Discovering differences on when fish are infected could cast new light on the evolution of this particular trematode genera.

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Appendix 1 - RNA extraction protocols

Multiple RNA extraction protocols were tested to determine the protocol best suited for the samples. The specimens, regardless of the life stage, contained small amounts of RNA and as such choosing an extraction method sensitive enough to measure them was paramount. All equipment was UV sterilised to minimise the risk of RNase contamination prior to starting any of the extractions. Filter tips, low-bind tubes and clean gloves were used for all protocols, as well as a fume hood for protocols utilising toxic chemicals.

First, an RNA extraction protocol utilising TRIzol™ was used. TRIzol™ is a commonly used reagent for both RNA, DNA, and protein isolation. A total of 8 *Crepidostomum* specimens were processed with this protocol: 5 juveniles and 3 eggs. The eggs were chosen because they represented the specimens with the lowest amount of RNA out of any of the samples, meaning that if RNA should be successfully quantified, the amount of RNA in all other samples should not be a concern. The juveniles were chosen because they represented the largest sample size, and as such some of them were expendable. Juveniles were also expected to contain the second highest amount of RNA, meaning that if they were found to not provide enough RNA, the metacercarial, cercarial, and sporocyst stages would likely not work with this protocol.

Whole specimens were removed from storage in RNAlater™ and immediately transferred to clean tubes, each containing 1 mL of TRIzol™. To homogenise the samples, 6 - 8 small ceramic beads were placed in each tube prior to placement in the TissueLyser (2 x 30 s at 20 x frequency). Because no insoluble material was detected for any of the samples, the homogenate was kept in the same tube to incubate for 5 min at room temperature (RT).Then, the samples were centrifuged at 12,000 rcf for 10 min at 4 °C, and subsequently transferred to new clean tubes. 100 µL BCP (Bathocuproine) was added to each tube, followed by a quick shake for 15 s, and incubation at RT for 10 min. Then, the tubes were centrifuged at 12,000 rcf for 15 min at 4 °C. The RNA containing aqueous phase was then transferred to a new clean tube, carefully so as not to mix the layers. 500 µL isopropanol was then added to the aqueous phase. This mixture was briefly vortexed, and incubated at RT for 10 min, or at - 20 °C overnight for some of the samples (for these samples the remaining steps were followed the next day) to see if the different incubation methods could affect the result. 1 µL glycogen was added to one sample to see if this could help locate the RNA pellet that forms after the

subsequent centrifugation at 12,000 rcf for 20 min at 4 °C. Post centrifugation, the supernatant was carefully discarded with the pellet left in the tube. Because the pellet was too small to be seen, the only way to confirm that it remained in the tube was to obtain positive results from RNA quantification. 1 mL of 75 % ethanol was added to each pellet-containing tube, and centrifuged at 12,000 rcf for 5 min at 4 °C. The ethanol was then removed, and the tubes centrifuged briefly to consolidate the remaining ethanol for removal. The tubes were then left open to air dry under a fume hood until all visible drops of ethanol had evaporated (5 - 10 min), and the pellets resuspended in 40 µL of MQwater. 6 µL of this solution was transferred into separate tubes for immediate Qubit fluorometric quantification, and later fragment analysis (FA). The remaining solution was placed in longtime storage at - 80 °C for later RNA analysis.

The second attempted protocol was the RNeasy® Micro extraction Kit (Qiagen). This protocol closely followed the provided recommendations in the handbook for "Purification of Total RNA from Animal and Human Cells". Because samples were limited, only two juveniles were used for this extraction, along with a control specimen, namely a piece of the cestode *Proteocephalus longicollis* [Zeder, 1800 (Benedict, 1900)]. This control had, like the *Crepidostomum* specimens, been subjected to storage in RNAlater™. The cestode piece used had an approximate size of 3 times that of the *Crepidostomum* specimens used here.

The specimens were removed from the RNAlater™ solution and placed onto a piece of aluminium foil, together with enough RNAlater™ to cover the sample. Here, a tiny piece of the parasite was removed for later DNA analysis. The dissections had to happen swiftly as the RNAlater™ crystallises quickly outside the tube, as well as the risk of contamination with RNases. The specimens were moved into tubes, each containing 350 µL RLT buffer and 6 ceramic beads for tissue disruption and homogenisation of the lysate. These tubes were placed in a TissueLyser for 2 x 30 s at 20 x frequency. The lysate was then centrifuged for 3 min at 15000 rcf, and carefully transferred to a new microcentrifuge tube. 350 µL 70 % ethanol was added to each tube and mixed into the lysate by repeated pipetting. After mixing, the samples were transferred to spin columns in 2 mL collection tubes, and subsequently centrifuged for 15 s at 8,000 rcf. Because no protein recovery was to be performed, the flow through was discarded. 350 µL RW1 buffer was added to each spin column and then centrifuged for 15 s at 8000 rcf to wash the column membrane. 10 µL DNase 1 solution was carefully mixed with 70 µL RDD buffer and added to the spin column membrane. These mixtures were then left to

incubate at RT for 25 min. Another 350 µL RW1 buffer was added and spun for 15 s at 8000 rcf. The spin columns were then moved over into new 2 mL collection tubes. Here, 500 µL RPE buffer was added before the tubes were spun for 15 s at 8000 rcf. 500 µL of 80 % ethanol was subsequently added, and the tubes once again spun for 15 s at 8000 rcf for a final rinse. The spin columns were then relocated to new collection tubes and spun at 15000 rcf for 5 min. After this, the spin columns were placed in new 1.5 mL collection tubes. 14 µL MQwater was added to the spin columns before they were centrifuged for 1 min at 15000 rcf to elute the RNA. 1 µL solution was transferred to a new tube and stored at 4 °C for 30 min and quantified using Qubit, following the standard protocol for RNA.

The third RNA extraction I attempted consisted of two TRIzol™ protocols with some extra rinsing steps of the specimens before transferring them into TRIzol™. These are referred to as protocol 3.1 and 3.2, respectively. In protocol 3.1, two juvenile specimens, together with a drop of RNAlater™, were individually transferred to a large petri dish. A drop containing 200 µL of Phosphate-buffered saline (PBS) was added to the petri dish, away from the RNAlater™. The specimens were then pipetted from the RNAlater™, into the drop of PBS, with as little RNAlater™ residual as possible. Repeated "up and down" pipetting was used to clean samples in the PBS. After rinsing, the samples were moved into 2 mL tubes containing ceramic beads. 1 mL TRIzol™ was added, and from here the classic TRIzol™ protocol, as described earlier, was followed. The sole deviation being that GlycoBlue™ (Invitrogen™) was utilised as a visual cue for the location of the RNA pellet.

For protocol 3.2, two samples of juveniles, together with the RNAlater™, were individually pipetted into new 2 mL tubes. 200 µL PBS was added to each tube and mixed by vortexing. Following this, the solutions were centrifuged at 7,600 rcf for 2 min at 4 °C. Post centrifugation, the liquid was carefully removed from the tube so as not to disturb the specimens which were supposed to stick to the tubes. Ceramic beads and 1 mL TRIzol™ were then added to the tubes. After this, the classic TRIzol™ protocol was followed. As with 3.1, the only deviation being that GlycoBlue™ was also utilised to see where the location of the RNA pellet.