Humic-acid-driven escape from eye parasites revealed by RNA-seq and target-specific metabarcoding

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Abstract

Background Next generation sequencing (NGS) technologies are extensively used to dissect the molecular mechanisms of host-parasite interactions in human pathogens. However, ecological studies have yet to fully exploit the power of NGS as a rich source for formulating and testing new hypotheses.

Methods We studied Eurasian perch (*Perca fluviatilis*) and its eye parasite (Trematoda, *Diplostomidae*) communities in fourteen lakes that differed in humic content in order to explore host-parasite-environment interactions. We hypothesised that high humic content along with low pH would decrease the abundance of the intermediate hosts (gastropods), thus limiting the occurrence of diplostomid parasites in humic lakes. This hypothesis was initially invoked by whole eye RNA-seq data analysis and subsequently tested using PCR-based detection and a novel targeted metabarcoding approach.

Results Whole eye transcriptome results revealed over expression of immune-related genes and the presence of eye parasite sequences in RNA-seq data obtained from perch living in clear-water lakes. Both PCR-based and targeted-metabarcoding approach showed that perch from humic lakes were completely free from diplostomid parasites, while the prevalence of eye flukes in clear-water lakes that contain low amounts of humic substances was close to 100%, with the majority of NGS reads assigned to *Tylodelphys clavata*.

Conclusions High intraspecific diversity of *T. clavata* indicates that massively parallel sequencing of naturally pooled samples represents an efficient and powerful strategy for shedding light on cryptic diversity of eye parasites. Our results demonstrate that perch populations in clear-water lakes experience contrasting eye parasite pressure compared to those from humic lakes, which is reflected by prevalent differences in the expression of immune-related genes in the eye. This study highlights the utility of NGS to discover novel host-parasite-environment interactions and provide unprecedented power to characterize the molecular diversity of cryptic parasites.

Background

The evolutionary arms-race between host and parasites is of a key importance for maintaining species diversity and community composition. However, the pace of evolutionary change in host-parasite systems is modulated not only by co-interacting communities, but also by common components of their extrinsic environment [1–3]. Yet, the role of environment in shaping host-parasite interactions is much less understood [4]. The advancement of next-generation sequencing (NGS) technologies provides the opportunity to expand our understanding about such complex interactions at an unprecedented speed [5, 6].

During the last decade, high-throughput RNA sequencing (RNA-seq) has been increasingly used to explore infection, disease- and stress-related changes in gene expression of the host. Gene expression analyses at the whole transcriptome level have also shed light on fundamental aspects of host and parasite biology [e.g., 7, 8] and host-parasite interactions [9]. With increased sequencing depth of mixed host and parasite transcripts (i.e. dual-RNA-seq) it is possible to simultaneously observe gene expression changes in the interacting taxa [10–12]. In addition, novel insights into the dynamics of host-parasite interactions at the molecular level are increasingly gained also by analysing sequence data that were traditionally deemed to be invaluable and hence excluded [13]. For example, atypical bioinformatics analysis pipeline involves a step where reads from DNA or RNA sequencing are aligned to the target species genome; those that do not align are simply discarded. This principle is integrated into the majority of existing pipelines because unmapped reads could originate from library contamination and sequencing errors. As such, much effort has been put towards sorting out this type of nuisance information [13, 14]. However, there is growing awareness that some of the unmapped reads could actually harbour novel genetic and ecological information. Thus far, unmapped reads from RNA- or DNA-seq data have been used to discover symbionts, pathogens, and undescribed features of the target species genome, such as highly divergent regions or insertions of the reference genome that would have been missed otherwise [13, 15–18]. Given that parasite and pathogen RNA typically represents only a tiny proportion of the total RNA of the host, a very deep sequencing is necessary to obtain comprehensive understanding of the pathogen transcriptomes and genetic diversity. This means that by using untargeted sequencing of the host transcriptome it is rarely possible to obtain enough power for pathogen community composition analyses. As an alternative to sequencing of whole transcriptomes and genomes, a targeted amplicon-based high-throughput sequencing, known as metabarcoding, has become essential tool for monitoring biodiversity [19, 20] and also increasingly used for understandingparasite diversity in host tissues and environmental samples [e.g., 21, 22]. Community metabarcoding is a sensitive

technique that allows detection of rare and cryptic species and species associations [23, 24] as well as analyses of within species genetic variability and population structuring [25].

Diplostomidae is geographically widely distributed trematode parasite group that has a complex life-cycle which includes two intermediate hosts–lymnaeid snails and fishes–while a piscivorous bird usually serves as a definitive host. After infecting and completing its development in snail, metacercariae enters fish eye structures and sometimes neural tissues, which may lead to changes in host behaviour that may reduce general condition of the fish [26, but see 27]. *Diplostomidae* species are morphologically extremely difficult to distinguish and each fish may be infected by hundreds of parasites. As a result, estimating species diversity, community composition, host-parasite interaction and effects of environmental factors is challenging in *Diplostomidae* [28–30]. While the use of molecular approaches and especially COI fragment based species identification via Sanger sequencing [31] have advanced the field tremendously by revealing hidden species diversity, most of the studies have focused on describing species from single fluke isolates [30, 32, 33]. However, using single fluke sequencing is suboptimal for characterizing community composition and intraspecific genetic diversity. Therefore, massive parallel sequencing with whole tissue extracts from host represents a potentially powerful strategy to improve the throughput and efficiency, to characterize both inter- and intraspecific diversity of parasites [29]. Different NGS approaches potentially provide complementary information, however, few studies to date have successfully combined multiple massive sequencing methods to further understanding of host-parasites-environment interactions.

Here, we describe how initial transcriptome screening of fish eyes – where we used both host-specific and unmapped RNA-seq reads– invoked a novel hypothesis that humic-associated differences among lakes affect the prevalence of *Diplostomidae* eye parasites in Eurasian perch (*Perca fluviatilis*). In particular, by building on RNA-seq read data and expanding upon previous work on eye parasites in perch [34], we hypothesized that the elevated content of humic substances (often measured as dissolved organic carbon (DOC) concentrations and spectral parameters of the water) would have a negative effect on the abundance of the intermediate hosts of eye flukes, gastropods. Since high humic content is also associated with increased acidity of the water, we expect this would negatively affect calcium availability necessary for building shells, or/and decrease the light availability for the underwater plants that serve as an important food source for gastropods [35, 36]. We tested the potential link between humic substances and occurrence of *Diplostomidae* eye parasites by conducting extensive molecular screening of eye flukes and developing a targeted metabarcoding approach to efficiently screen intra- and interspecific genetic diversity of parasites from host eye tissue. Our work demonstrates how integrated use of NGS approaches can lead to the discovery of novel host-parasite-environment interactions and provide unprecedented power to characterize the molecular diversity of cryptic parasites.

Methods

Sample collection

Perch sampling was carried out in eight humic and six clear-water lakes in Estonia in 2016 and 2017 (Table S1 and Fig. S1 in Supplementary appendix). Fish were sacrificed by an overdose of tricainemethanesulfonate (MS-222), individually labelled, and their eyes were enucleated and snap frozen in liquid nitrogen. Surface water samples from each lake were collected during the sampling in 2016 and pH, DOC concentrations to characterize the humic content of lakes, and different spectral parameters were determined (Table 1, Table S6) as described previously [37]. The diversity of gastropods in ten out of the fourteen studied lakes was obtained from the Estonian Environmental Monitoring database (Table 1; https://www.keskkonnaagentuur.ee). The handling of fish during sampling adhered to the regulations of the Estonian Animal Protection Act. Fishing permits were issued by the Estonian Ministry of the Environment (permits no. 54/2016 and 37/2017). The details on sampling protocol and subsequent NGS analyses are provided in the Supplementary appendix.

Table 1
Study lake characteristics and gastropod occurrence data.

Lake	Type[1]	Geographic coordinates	Water chemistry				Gastropod species occurence[2]		
			Dissolved organic carbon (DOC, mg/l)	Freshness index	Fluorescence index	pH	Number of sampling visits	Sampling year	Gastropod species[3]
Holvandi Kivijärv	H	N58.0410°; E27.1965°	50,04	0,38	1,27	6,30	1	2012	none
Virosi	H	N58.0259°; E27.2551°	66,1	0,34	1,26	5,45	7	1995–2012	none
Partsi Saarjärv	H	N57.9978°; E27.1662°	64,8	0,34	1,25	5,30	6	1995–2012	none
Heisri Mustjärv	H	N58.0249°; E26.8312°	33,28	0,43	1,29	8,20	n/a	n/a	n/a
Kuulma	H	N57.9569°; E27.1613°	47,1	0,41	1,30	4,50	n/a	n/a	n/a
Loosalu	H	N58.9361°; E25.0824°	17,41	0,44	1,28	4,70	14	multiple	none
Matsimäe Pühajärv	H	N59.0611°; E25.5135°	41,63	0,39	1,22	n/a	n/a	n/a	n/a
Meelva	H	N58.1407°; E27.3852°	47,77	0,38	1,28	5,60	7	1994–1995	*Anisus vortex, Planorbis corneus*
Paidra	CW	N57.9110°; E27.1910°	10,23	0,68	1,39	6,70	n/a	n/a	n/a
Hino	CW	N57.5766°; E27.2298°	13,91	0,84	1,56	8,65	2	2001	*Lymnaea stagnalis*
Verijärv	CW	N57.8106°; E27.0470°	16,78	0,73	1,53	8,50	1	2002	*Lymnaea stagnalis, Ancylus fluviatilis, Valvata piscinalis, Bithynia tentaculata*

[1]H – humic-water lake; CW – clear-water lake

[2]Snail occurrence data was obtained from Estonian Environmental Monitoring database (https://www.keskkonnaagentuur.ee)

[3]none – no gastropod species observed, n/a – data non-available

Lake	Type[1]	Geographic coordinates	Water chemistry				Gastropod species occurence[2]		
			Dissolved organic carbon (DOC, mg/l)	Freshness index	Fluorescence index	pH	Number of sampling visits	Sampling year	Gastropod species[3]
Saadjärv	CW	N58.5535°; E26.6059°	11,24	0,75	1,51	8,75	14	multiple	*Lymnaea stagnalis, Bithynia tentaculata, Physa fontinalis, Radix balthica, Myxas glutinosa, Valvata piscinalis, Valvata pulchella, Valvata depressa, Gyraulus albus, Anisus vortex*
Uiakatsi	CW	N57.9532°; E26.6365°	6,684	0,72	1,46	8,35	2	2007–2012	*Lymnea stagnalis, Hippeutis complanatus*
Piigandi	CW	N58.0176°; E26.7913°	8,337	0,73	1,48	7,00	1	2012	none
[1]H – humic-water lake; CW – clear-water lake									
[2]Snail occurrence data was obtained from Estonian Environmental Monitoring database (https://www.keskkonnaagentuur.ee)									
[3]none – no gastropod species observed, n/a – data non-available									

RNA expression and unmapped read analysis

Total RNA was extracted from the whole eye tissues collected in 2016, and libraries were sequenced with Illumina HiSeq 3000. Reads that passed quality control were mapped onto the reference genome of *Perca fluviatilis* [38] using hisat2 2.1.0 [39] (Supplementary appendix). Differential expression analysis between the two groups of lakes (humic vs. clear-water) was performed using the DEseq2 package1.22.2 [40] in R 3.3.4 (R Core Team, 2018). All genes with an adjusted *p*-value ≤ 0.05 [41] were considered as significantly differentially expressed between populations from two groups of lakes. Human orthologue gene symbols were searched for using complete gene names in NCBI. Gene Ontology (GO)-enrichment analysis of differentially expressed genes against all orthologous gene symbols as a background was performed using Gorilla [42]. The GO terms with a false discovery rate (FDR) ≤ 0.05 were considered as significant.

Unmapped reads from each sample were further analysed to detect the occurrence of parasite reads among the whole-eye RNA-seq data. Briefly, NCBI's blastn 2.6.0 [43] was applied to align the non-redundant sets of unmapped reads to the sequences in a non-redundant nucleotide database. To reveal the presence of the eye fluke parasites' sequences (of the Digenea class Trematoda, and of the *Diplostomidae* family) among the unmapped reads, the taxonomic analysis of blastn outputs was processed in Megan Community Edition 6.8.18 [44].

PCR-based confirmation of Diplostomidae in perch eye

DNA was extracted from the whole eye using a standard salt extraction method [45], and PCR-based screening was performed in 212 perch eye samples (Table S1) using diplostomid-specific primers that amplified a fragment of the cytochrome c oxidase

subunit I (*COI*) gene [31]. Primers were modified to include linkers for Illumina-compatible adapters at their 5' ends [46, 47]. Both eyes were screened in 172 individuals, while only the left eye was screened in the remaining 40 individuals. PCR products were visualised on a 1.5% agarose gel, and the presence of a ~ 500 bp amplification product was recorded as evidence of *Diplostomidae* infection in a given eye (Table S1).

Metabarcoding of theDiplostomidae community in perch eye

We used whole eyes as the starting material for the analysis; that is, the *Diplostomidae* were not individually extracted from the eye, but rather sequenced together as a naturally pooled sample [29]. Libraries were prepared from SPRI-bead-purified PCR products of 142 *Diplostomidae* positive samples identified with the PCR described above (Table S1) by attaching Illumina adapters and unique individual indexes following the PCR protocol described in [47] with minor modifications (see Supplementary appendix). Samples were pooled and sequenced using an Illumina MiSeq instrument (Illumina Inc., San Diego, California, USA) at the Turku Centre for Biotechnology (Turku, Finland). The paired-end raw reads were demultiplexed (Table S2) and merged using PEAR 0.9.6 [48]. For robust downstream analysis, we followedaconservative approach –only the samples containing ≥ 1000 sequences [49] were retained (115 of 142; Table S2).

Taxonomic classification was performed with Kraken 2.0.6-beta [50]. In addition, to validate the Kaken results with a probabilistic approach the sequences were classified by applying a naïve Bayesian classifier using RPD11.5 [51] following [52]. For both classifiers, a custom database was generated using the available Platyhelminthes *COI* gene sequences from NCBI GenBank (https://www.ncbi.nlm.nih.gov/; Supplementary appendix). As both taxonomic classifiers showed consistent results, the further analyses are based only on the Kraken classification. To avoid biases related to unequal number of reads per sample [53], the presence of a particular parasite genus/species in a sample was considered as highly supported if ≥ 5% of the sequences were assigned to that parasite genus/species per eye sample.

Next, we rigorously filtered the sequences to further minimize technical artefacts that could lead to overestimation of haplotype diversity [25, 54]. As the majority of parasite sequences belonged to *Tylodelphys clavata* (mean = 83.6%; median = 94.0%; Fig. 1; Table S2), we further characterized the intraspecific variation of this species. All of the sequences assigned to *T. clavata* were extracted and clustered with cd-hit 4.7 [55, 56] using 100% similarity to remove redundancy and exclude unique sequences, as the latter could appear due to technical PCR or sequencing errors. For the subsequent analyses, we used only representative sequences of the clusters with more than 2.5% of the total number of sequences assigned to *T.clavata* per sample. In addition, the haplotypes that were observed only in a single sample were excluded, as they may represent sequencing artefacts [25, 54]. However, this procedure might potentially eliminate rare haplotypes from the subsequent analysis. The final dataset contained 348 *T. clavata* sequences from 113 eye samples (79 individuals). For comparative purposes we added seven partial sequences of *T. clavata COI* found in GenBank (accession numbers: KR271473.1, KR271475.1, KR271480.1, KT751175.1, KT768015.1, KT961707.1, KY271544.1). All sequences were aligned using Muscle 3.8.31 [57], and the NCBI sequences were trimmed to the same size of the *COI* fragments generated during NGS sequencing, using BioEdit 7.2.5 [58]. To visualize the relationships among haplotypes, a TCS haplotype network [59] was generated using PopART1.7 (http://popart.otago.ac.nz).

Results

Initial insights from eye transcriptomes

Altogether, 94% of the reads from the fourteen RNA-seq libraries generated from whole-eye tissue were mapped to the reference perch genome (Table S3). In total, 265 perch genes were found to be differentially expressed ($p_{adj} \leq 0.05$) between fish originating from humic and clear-water lakes (Fig. 1; Table S4). Gene Ontology (GO) analysis indicated that the differentially expressed genes were enriched for 69 GO process terms (GOrilla, FDR ≤ 0.05), with the top three terms (FDR < $1.14*10^{-5}$), consisting of immune system process (GO:0002376, n = 50), adaptive immune response process (GO:0002250, n = 15) and immune response process (GO:0006955, n = 27; Table S5).

Evaluation of unmapped whole-eye RNA-seq reads revealed that the samples from four out of six clear-water lakes contained sequences that originated from parasitic flatworms of the Strigeidida order or/and *Diplostomoidea* superfamily, but no reads were detected in any of the eight humic water lakes (Fig. 1).

PCR-based validation

The ~ 500 bp *Diplostomidae COI* gene fragment was successfully amplified in 95 of the 212 individuals additionally sampled in 2017 (142 of 384 eye samples; TableS1). All of the collected samples from the eight humic lakes were free of *Diplostomidae* parasites, whereas perch from four of the five clear-water lakes were infected. Infection prevalence was very high in three clear-water lakes (prevalence 96–100%, Fig. 1, Table S1).

Targeted metabarcodingof eye parasites

The majority of the PCR-positive eye samples produced a large number of sequences belonging to *Diplostomidae* parasites (mean number of reads = 53,171; median = 39,651). In total, 99.3% of the sequences were assigned to the *Diplostomoidea* superfamily. The majority of sequences (mean = 83.6%) were assigned to *Tylodelphys clavata* (Table S2) while a small number of sequences were assigned to three species from the genus *Diplostomum* (*D. Baeri* complex sp. 2 SAL-2014 (n_{eyes}=7), *D. spathaceum* (n_{eyes}=4) and *D. pseudospathaceum* (n_{eyes}=7); Table S2).

Altogether, 34 distinct *T. clavata* haplotypes were identified in the 113 analysed eye samples collected from 79 perch; four of those haplotypes were identical to the published GenBank sequences. The most common haplotype was found in 107 samples, while the other haplotypes were observed in 2 to 16 samples (Fig. 2). Majority of the eyes contained 1 to 5 haplotypes. Most of the haplotypes formed genetically close star-like network, whereas two smaller haplotype groups were more distant from the former (Fig. 2).There was no evidence of strong genetic structuring, as common haplotypes were present in all four lakes.

Discussion

The extent to which extrinsic environmental conditions shape host-pathogen coevolution and contribute to the emergence of locally adapted populations are still poorly understood. Here, we demonstrate how integrated use of complementary NGS approaches can provide novel insights on such complex associations [2, 15, 18, 60]. By analysing both host-specific and unmapped whole-eye RNA-seq reads, we discovered that perch individuals from humic and clear-water lakes differ in immune system related gene expression, and that this difference could be explained by contrasting *Diplostomidae* parasite pressure between the two habitats (Fig. 1). We subsequently developed a targeted metabarcoding approach to further investigate the molecular diversity of this parasite group. We found that *T. clavata* is the dominant eye parasite in perch, with high prevalence and haplotype diversity in the four clear-water lakes (Fig. 2). While high prevalence and abundance of *T. clavata* in perch has been observed earlier [26], our work provides strong support for the hypothesis that the humic environment is likely unfavourable for *Diplostomidae* eye parasites to successfully complete their life cycle. Moreover, we show for the first time that in addition to the head-kidney, which is the main lymphoid organ involved in piscine immune defence [61], the presence of eye parasites also alters the expression patterns of a number of immune system genes measured from the whole eye.

Differential expression of immune genes

The adaptive importance of gene functions can be studied by analysing gene expression differences in an ecological context [62]. Among the genes that were differentially expressed between eyes of perch from clear-water and humic lakes, those with immune system-related functions were strongly overrepresented. Specifically, of the 174 genes that were up-regulated in clear-water lake perch, 48 (27.5%,Table S5) had immune-related functions; of the 91 that were down-regulated in clear-water lake perch, 9 (9.9%, Table S5) had immune-related functions (Fig. 1). Differentially expressed genes included interferons, interleukins, and other protein (e.g. interferon regulatory factor 1, interferon induced proteins, interleukin-8 like protein, MHC class II beta subunit, T cell antigens) that are involved in immune cell activation and antigen presentation.

In wild populations, immune system genes are often found to be at the very centre of evolutionary change [63–65]. Nevertheless, the expression of immune-related genes in the perch eye was initially unexpected, as traditionally the eye has been thought to bean “immunopriviliged organ [66–69]. However, accumulating evidence has started to paint a more complex picture of ocular immunity by, for instance, showing that leucocytes can selectively penetrate the retina-blood barrier [70], and that immune system related genes are expressed in various eye microhabitats [71, 72].

One interesting differentially expressed gene found in our study is catalase (CAT; EC1.11.1.6), which is a principal enzyme in antioxidant pathway that functions by converting reactive H_2O_2 to H_2O and O_2. CAT showed a marked down-regulation in clear-water lakes (Table S5). CAT enzymatic activity has been studied in various compartments of the eye in humans and model organisms [73, 74], and reduced CAT activity was linked to decreased parasitosis [75]. However, because here we have analysed gene expression of the whole eye rather than that of specific eye structures and tissues, and without blood expression data for contrast, we cannot determine the extent to which the observed expression differences are driven by the processes in blood versus internal eye structures. Nevertheless, our results indicate that *T. clavata* is most likely influencing immune gene expression patterns of the host. Most of the current – and limited – information we have on eye immunity comes from mammalian models; we know very little about immune processes in the eye of other taxa [68, 69, 76, 77]. More studies targeting multiple eye tissues [78] are therefore clearly needed to evaluate the "immunopriviliged" status of fish eyes in response to eye parasites.

Humic lakes as eye parasite-free environments

To explain the excess of differentially expressed immune-related genes (Fig. 1) between humic and clear-water perch populations, we hypothesized that observed differences in transcript abundances may be driven by eye parasites. In order to test the potential link between humic substances and occurrence of *Diplostomidae* parasites, we scanned the proportion of RNA-seq reads that were not mapped to the perch genome. For individuals originating from humic lakes, none of the unmapped RNA-seq reads were assigned to the *Diplostomoidea* (Fig. 1). This initial result was later confirmed with PCR-based screening of additional samples collected the following year when a very high prevalence of *Diplostomoidea* parasites was observed in four out of six clear-water lakes (Fig. 1). This result is consistent with previous studies in perch and other fish species, which showed the absence of some parasite taxa in potentially challenging habitats [34, 79–81]. *Diplostomidae* parasites have a complex life cycle with three hosts and free-living stages, making this group particularly sensitive to biotic and abiotic elements of their environment. Because both clear-water and humic lake pairs are in very close geographic proximity (see Fig. S1 in Supplementary appendix), the difference in parasite prevalence cannot be explained by the lack of dispersal opportunities for the parasite [79]. The most obvious difference between lakes is their colour, which is tightly linked to water chemistry, particularly DOC and pH (Pearsons's r = -0.64; P = 0.003). Monitoring data of gastropods' diversity indicated their absence in mos tof the studied humic lakes (Table 1). In clear-water lakes, however, at least one species of gastropod was recorded that included *Lymnaea* sp. or *Radix* sp., which are both considered as first intermediate hosts for *Diplostomoidea* and *T. clavata.* Moreover, high density of underwater vegetation in clear-water lakes likely supports high density of gastropods, while humic lakes are typically very poor in aquatic vegetation. Taken together, this suggests that interactive effects driven by the humic content on *Diplostomoidea* parasite free-living stage and the availability of first intermediate gastropod host [34, 82] most likely create a 'life-cycle bottleneck' for this group host [79].

Cryptic diversity in T. clavata

DNA analysis of naturally pooled fish eye parasites has previously been used in combination with pyrosequencing [29]. However, the early attempts to harness the power of NGS for intra- and interspecific analysis were severely hampered by very short read length (e.g. only 22 bp were sequenced in [29]. In the current study, we developed targeted metabarcoding of a longer (~ 500 bp) diplostomid-specific *COI* fragment for whole-eye parasite community analysis. While currently there is no consensus about *Diplostomidae* species delineation [32], our conservative approach of eliminating singletons and rare reads allowed us to assign most of the *COI* fragments to *T. clavata* (Fig. 1).

We observed high *T. clavata* haplotype diversity among our studied lakes, as well as a lack of genetic structuring (Fig. 2), consistent with previous studies [32, 83]. Together, this suggests that *T. clavata* forms a large well-connected population system, as is expected for parasites with highly mobile definitive hosts such as piscivorous birds. The high haplotype diversity in *T. clavata* observed here also suggests that earlier Sanger sequencing efforts have likely managed to capture only a fraction of the genetic diversity within this species. It is likely that this finding also holds for other *Diplostomoidae* species – current molecular studies of fish eye flukes are typically based on analysis of less than a hundred individually sampled parasites (but see [32]), yet a single fish eye may harbour hundreds of parasites (e.g., [26]). Thus, it was not surprising that the developed *Diplostomoidae* metabarcoding approach revealed, for the first time, an extensive intra-specific diversity in *T. clavata.* Our study also showed that the majority of perch were infected by several *T. clavata* haplotypes (Fig. 2).The latter result would indicate continual infection by differ, ent haplotypes that co-exist in the same lakes – a result also observed for other eye flukes [84, 85].

Conclusions

Taken together, this study demonstrates how components of the abiotic environment drastically shape common parasite communities and host immune response, highlighting the significance of analysing results of host-parasite studies in an ecological context. In addition, our study illustrates the utility of integrating RNA-seq and targeted metabarcoding approaches in host-parasite community studies. The striking intraspecific diversity of *T.clavata* recovered from our targeted metabarcoding approach suggests that NGS of naturally pooled samples represents an efficient and powerful strategy for shedding light on cryptic

Abbreviations

CAT- catalase

DOC-dissolved organic carbon

COI- cytochrome c oxidase subunit1

NGS- next generation sequencing

Declarations

Ethics Approval And Consent To Participate

Not applicable. The handling of fish during sampling adhered to the regulations of the Estonian Animal Protection Act. Fishing permits were issued by the Estonian Ministry of the Environment (permits no. 54/2016 and 37/2017).

Consent For Publication

All authors consent to publication

Competing interests

None

Availability of data and material

Transcriptome reads of *P. fluviatilis* eye are available in the NCBI SRA (SRR10441590 - SRR10441602 and SRR7091762) as a part of BioProjectsPRJNA589499 and PRJNA450919, respectively. Short Illumina linked-reads of *Diplostomidae* mtDNA cytochrome oxidase I fragment are available in the NCBI SRA (SRR10490070 - SRR10490211) as a part of BioProjectPRJNA590324.

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Authors’ contributions

AV conceived the study; SK, VK and AV conducted fieldwork; AP, MS, TK performed laboratory analyses; MO, FA, EJV analyzed the data; KN wrote the first draft of the MS; all coauthors approved the final version of the manuscript.

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Figures

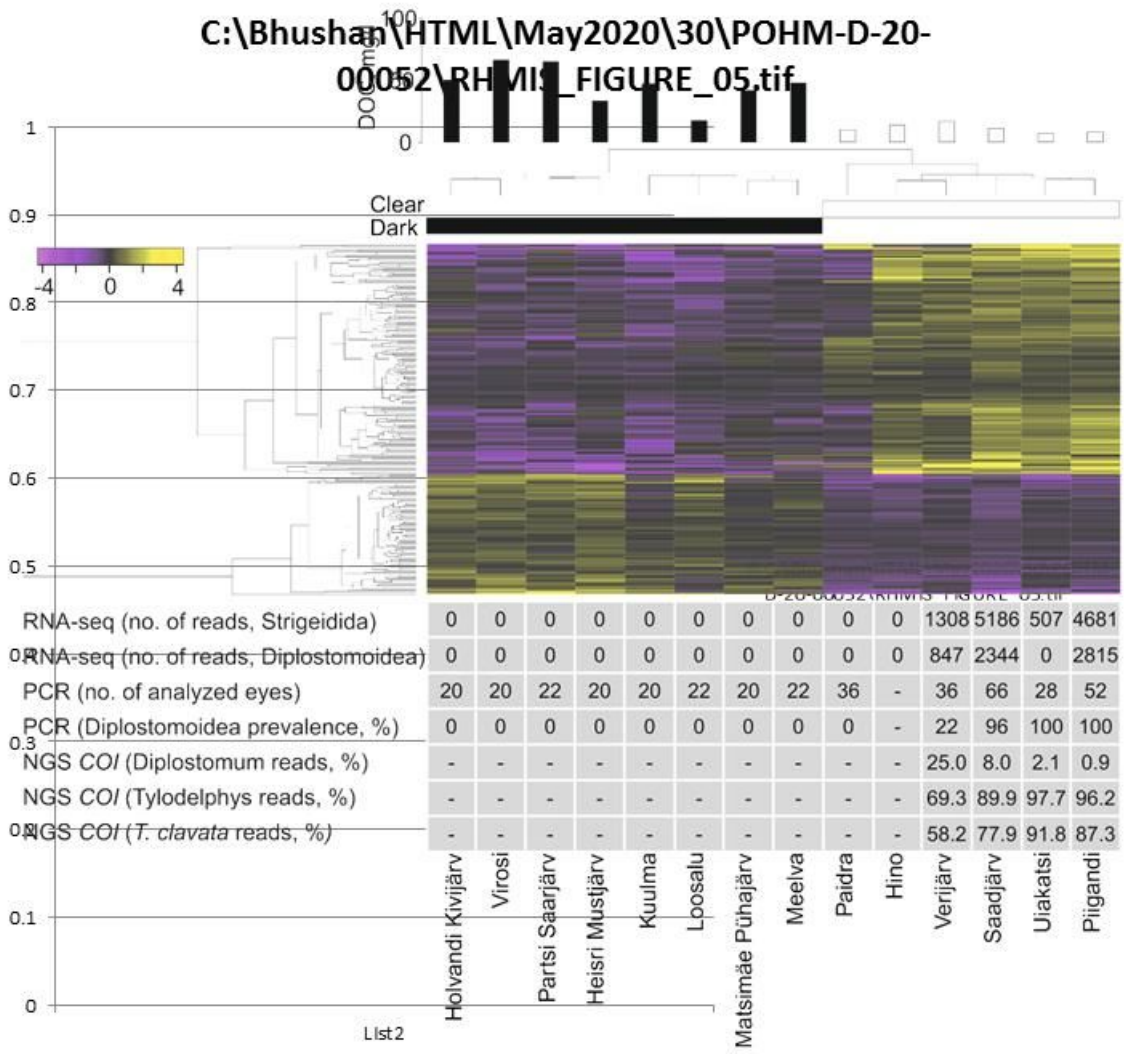

Figure 1

Heatmap showing differentially expressed genes (DESeq2, n=265,padj ≤ 0.05) between individuals (n=14) from humic and clear-water lakes. Yellow and violet colour correspond to an increased and decreased transcript abundance, respectively, in the clear-water lakes. The bar plot above the figure illustrates dissolved organic carbon (DOC) concentration (mg/l) in each studied lake. The table indicates the number of reads that were assigned to the order Strigeidida and the superfamily Diplostomoidea; the results of PCR

amplification of Diplostomid-specific COI gene in humic and clear-water lakes; and the proportion of Diplostomid-specific COI reads assigned to the genus Diplostomum and Tylodelphys and to the species Tylodelphys clavata in four clear-water lakes.

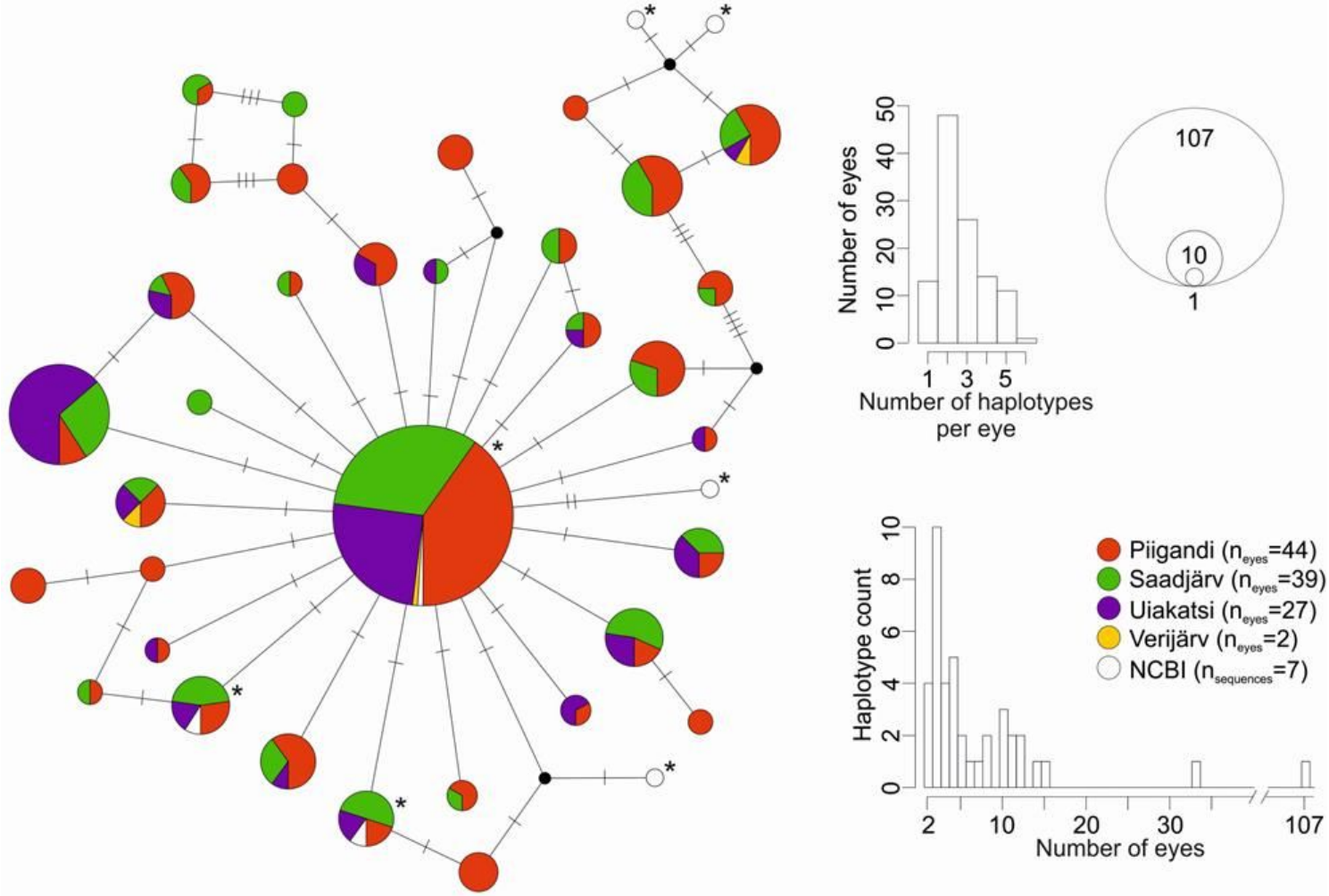

Figure 2

Haplotype network of COI sequences for Tylodelphys clavata. Circle size is proportional to the frequency of each haplotype. Perch populations from different lakes are represented by different colours. The haplotypes that include NCBI sequences are highlighted with a star; neyes refers to the number of eye samples; nsequences refers to the number of sequences from the NCBI database. The insert histograms illustratethe number of haplotypes observed per eye and the frequency of haplotypes.

Supplementary Files

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