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Different hosts in different lakes: prevalence and population genetic structure of plerocercoids of *Ligula intestinalis* (Cestoda) in Czech water bodies

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Abstract: *Ligula intestinalis* (Linnaeus, 1758) is a tapeworm parasite with a worldwide distribution that uses a wide variety of fish species as its second intermediate host. In the present study, we investigated the prevalence and population genetic structure of plerocercoids of *L. intestinalis* in five common cyprinoid species, roach *Rutilus rutilus* (Linnaeus), freshwater bream *Abramis brama* (Linnaeus), white bream *Blicca bjoerkna* (Linnaeus), bleak *Alburnus alburnus* (Linnaeus), and rudd *Scardinius erythrophthalmus* (Linnaeus), collected in six water bodies of the Czech Republic (Milada, Most, Medard, Jordán, Římov and Lipno). Of the six study sites, the highest frequency of parasitism was recorded in Lake Medard (15%). The overall prevalence rate among the species was as follows: roach > rudd ≥ freshwater bream > bleak > white bream. Two mitochondrial genes (*cyt*b and *COI*) were used to compare the population genetic structure of parasite populations using selected samples from the five fish species. The results of the phylogenetic analysis indicated that all populations of *L. intestinalis* were placed in Clade A, previously identified as the most common in Europe. At a finer scale, haplotype network and PCoA analyses indicated the possible emergence of host specificity of several mtDNA haplotypes to the freshwater bream. Moreover, pairwise Fixation indices (F_{ST}) revealed a significant genetic structure between the parasite population in freshwater bream and other host species. Parasite populations in roach not only showed the highest rate of prevalence but also depicted a maximum number of shared haplotypes with populations from bleak and rudd. Our results suggest that recent ecological differentiation might have influenced tapeworm populations at a fine evolutionary scale. Thus, the differences in prevalence between fish host species in different lakes might be influenced not only by the parasite's ecology, but also by its genetic diversity.

Keywords: tapeworm, Czech Republic, host specificity, freshwater, fish parasite

This article contains supporting information (Tables S1–S3, Figs. 1–4) online at http://folia.paru.cas.cz/suppl/2022-69-018.pdf

Host specificity is a crucial factor shaping the distribution and diversity patterns of parasite species. Understanding host specificity is essential in order to characterise the diversity and study the biogeography of parasites (Štefka et al. 2009, Martinů et al. 2018, Bernard et al. 2019, Wells and Clark 2019). Given that the global species richness of parasites is higher than the diversity of non-parasitic species (Windsor 1998) host specificity is of utmost importance for the studies of co-evolution (Blatrix and Herbers 2003), but also co-extinction, i.e. the extinction of host species resulting in the extinction of their associated parasite species (Stork and Lyal 1993). Moreover, host specificity can act as an isolation barrier restricting gene flow among parasite populations (Schirrmann and Leuchtmann 2015). Accordingly, the emergence of host specificity can be associated with the earliest stages of speciation in parasites (Meinilä et al. 2004).

Host specificity may be either associated with the ability of a parasite species to attach and adapt to a given host species or with the limitations of a parasite species in finding a host species (i.e., adaptive *vs* non-adaptive factors) (Nosil 2015). Consequently, estimation of the source of adaptive evolutionary change in parasites requires population genetic studies of host specificity (Huyse et al. 2005). On the contrary, phylogenetic analyses can uncover morphologically indistinguishable but genetically distinct species, i.e., cryptic species, differing in their biology

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Table 1. Sampling locations in the Czech Republic

Reservoirs	Area	Maximum depth	Locality
Lipno	48.70 km^2	25 m	48.7000N, 14.0666E
Římov	2.10 km^2	43 m	48.8341N, 14.4814E
Jordán	0.51 km^2	12.5 m	49.4213N, 14.6650E
Most	3.11 km^2	71 m	50.5396N, 13.6460E
Medard	4.93 km^2	50 m	50.1794N, 12.5958E
Milada	2.50 km^2	22 m	50.6536N, 13.9444E

(Nazarizadeh et al. 2022). Complexes of cryptic species or lineages have been revealed multiple times in helminths (e.g., Blouin 2002, Bouzid et al. 2008a, Ristau et al. 2013). Lastly, studying patterns of population genetic structures in parasitic helminths can shed light on the phylogeographic history of parasites and their host species, and more importantly, identify the ecological drivers of speciation patterns in parasites (Cole and Viney 2019).

The diphyllobothriidean cestode *Ligula intestinalis* (Linnaeus, 1758) and its related (cryptic) species have become a favourite object of phylogenetic and population genetic studies (e.g., Bouzid et al. 2008b, Štefka et al. 2009) due to their wide host spectrum and a geographical distribution covering the Holarctic (Dubinina 1980), Paleotropical (Britton et al. 2009) and Australasian realms (Morgan 2003, Chapman et al. 2006, Lagrue et al. 2018). *Ligula intestinalis* is an endoparasite demonstrating a life cycle with two intermediate hosts: a diaptomid or cyclopoid copepod as the first host and a planktivorous fish as the second intermediate host. Cyprinoid fishes in natural and artificial reservoirs are frequently exposed to infection with *L. intestinalis* (Arme and Owen 1968, Kennedy 1974). In addition to its common cyprinoid fish hosts, *L. intestinalis* and other species of the genus also infect other fish taxa, including species of the families Salmonidae, Galaxiidae and Catostomidae (Dubinina 1980, Bean and Winfield 1991, Baruš and Prokeš 1995, Groves and Shields 2001). This tapeworm spends a maximum of five days in a fish-eating bird as the final host (Dubinina 1980), during which it reaches sexual maturity and begins to produce eggs that are released into the water.

The fish stage (plerocercoid) plays a significant role in the behaviour, fecundity and health of the fish which may consequently lead to considerable aquaculture damage (Sweeting 1977, Wyatt and Kennedy 1989, Carter et al. 2005). Moreover, the parasite can significantly affect fish growth, stress resistance and susceptibility to predators (Longshaw et al. 2010, Iwanowicz 2011). Mortality of fish infected with *L. intestinalis* may occur either directly from their inability to survive through winter (Wyatt and Kennedy 1989) or indirectly through increased predation risk by birds and by other fish (Van Dobben 1952, Holmes and Bethel 1972, Sweeting 1977, Palm et al. 2018). Therefore, the prevalence of *L. intestinalis* in its fish hosts has been monitored by multiple studies (Dence 1958, Ergonul and Altindag 2005, Ivankov et al. 2020). Furthermore, variation in the rate of prevalence between different host species can indicate the emergence of host specificity and thus it is an important tool in ecological parasitology (Britton et al. 2009).

Most population genetic studies on *L. intestinalis* have concentrated on analysing evolutionary lineages at a large scale (global or continental). For instance, using mtDNA sequences Bouzid et al. (2008b) revealed the existence of two evolutionary lineages of *Ligula* (Clades A and B) in Europe and the Mediterranean, which was later confirmed by a microsatellite study (Štefka et al. 2009). Clade A samples were specific to fishes of the Leuciscide subfamilies Leuciscinae and Alburninae, whereas the Clade B was specific to other, less abundant, cyprinoid species, such as gudgeon *Gobio gobio* (Linnaeus), bitterlings (*Rhodeus* sp.) and barbels (*Barbus* sp.). Despite their genetic differentiation, both lineages shared the definitive hosts, great crested grebe *Podiceps cristatus* (Linnaeus) and goosander *Mergus merganser* (Linnaeus). Therefore, differences in fish-host specificity were proved for the Euro-Mediterranean clades.

These results confirmed findings of an earlier population genetic study performed at a local scale in Lough Neagh (Northern Ireland) (Olson et al. 2002), in which *Ligula* parasites from roach *Rutilus rutilus* (Linnaeus) and gudgeon were found to represent distinct strains, considering their effect on the gonadal development of their hosts. Furthermore, *Ligula* from minnow *Phoxinus phoxinus* (Linnaeus) from Wales was found genetically similar to *Ligula* from roach in Lough Neagh. Olson et al. (2002) proposed that the introduction of roach and a subsequent increase in final host populations (*P. cristatus*) was likely the cause for the presence of separate strains in Lough Neagh.

Here, we aimed to monitor the occurrence and genetic structure of plerocercoids of *L. intestinalis* among the five most abundant cyprinoid species in the Czech Republic. We attempted to (a) compare the prevalence of plerocercoids of *L. intestinalis* in six freshwater lakes and reservoirs in the Czech Republic, and (b) compare the phylogenetic relationship of *Ligula* populations from the five host species to specimens analysed in previous genetic studies. Using a subsample of the collected specimens, we also aimed to (c) test for parasite genetic structure associated with host specificity at a smaller spatial scale than in the majority of previous studies.

MATERIALS AND METHODS

Description of the study area

There are almost 24,000 fishponds and reservoirs on the territory of the Czech Republic, with a total area of 52,000 hectares (Ministry of Agriculture of the Czech Republic 2019). In the present study, we focused on six artificial lakes and reservoirs covering the western half of the Czech Republic (Bohemia), including Milada and Most lakes in the north, Medard lake in the west, and the Lipno, Římov and Jordán reservoirs in the south (Fig. 1, Table 1).

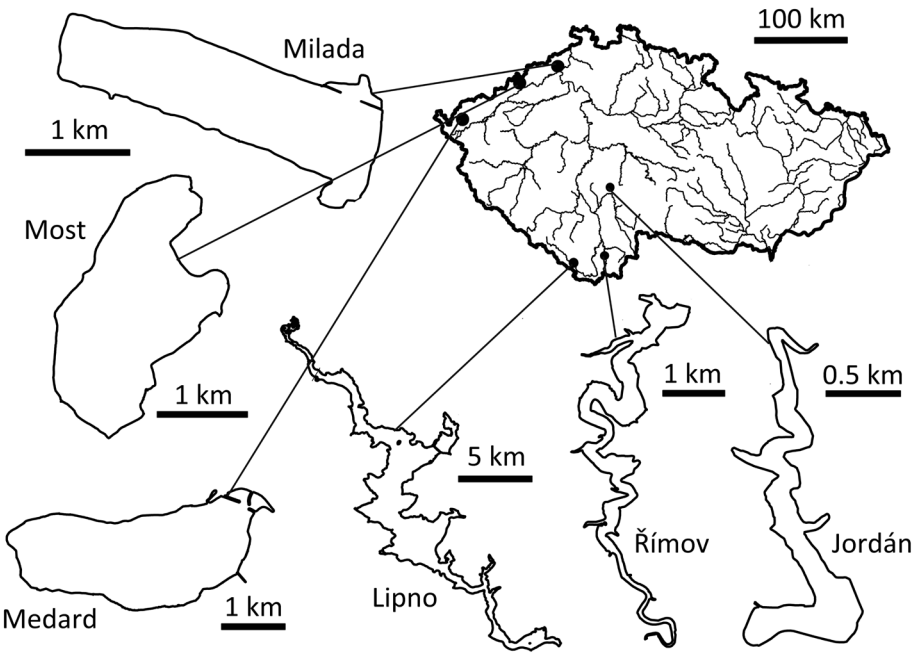

Fig. 1. Map of the six lakes and reservoirs studied.

Sampling

Permits for sampling were issued by the state enterprise Povodí Vltavy for the Lipno and Římov reservoirs, by the municipality Tábor for the Jordán Reservoir, by the state enterprise Palivový kombinát Ústí for the Milada and Most lakes, and by the joint company Sokolovská uhelná for the Medard Lake. Between July and September 2020, a total of 6,630 fish belonging to five species of the family Leuciscidae were collected: freshwater bream, *Blicca bjoerkna* (Linnaeus), white bream, *Abramis brama* (Linnaeus), bleak, *Alburnus alburnus* (Linnaeus), roach, *Rutilus rutilus* (Linnaeus), and rudd, *Scardinius erythrophthalmus* Linnaeus. They were captured using benthic and pelagic CEN multi-mesh gillnets with stretched mesh sizes ranging from 5 to 55 mm (knot-to-knot), supplemented by benthic and pelagic large-mesh gillnets with stretched mesh sizes ranging from 70 to 130 mm (knot-to-knot) (Blabolil et al. 2017). To capture the fish, the gillnets were set overnight. The fish catch was standardised as the number of fish per area of installed gillnets (individuals per 1,000 m^2).

After extraction from the nets, the fish individuals were immediately killed and their body cavities were examined for the presence of plerocercoids of *Ligula intestinalis*. In particular, the anterior part of the body has a rounded shape, and no external segmentation of the strobila is apparent. The genital complexes extend in an irregular longitudinal row in the strobila of plerocercoids and the ovaries, testes, genital ducts and vitelline follicles are located in the transverse segments (Bykhovskaya-Pavlovskaya 1964). The plerocercoids were fixed in 96% ethanol and stored at freezing temperatures.

Estimating the rate of prevalence

The prevalence was calculated as the percentage of infected fish individuals. The significance of the differences in prevalence in different host species and reservoirs was tested with the non-parametric Kruskal-Wallis H test and pairwise Wilcox test (Zar 1999). In addition, fish abundance was estimated for the five host species based on catch from the multi-mesh gillnet method (Appelberg et al. 1995). Then, a linear regression test was carried out between the fish abundances (average between pelagic and benthic standardised abundance) and the prevalence of infection to test any possible connection between the abundance and rate of infection. All statistical analyses were performed using the stats package in R version 4.0.1 software (R Core Team 2021).

DNA isolation, amplification and PCR

In total, 30 *Ligula* specimens covering the five host species from Lipno, Medard, Římov and Most were selected to verify their assumed mtDNA clade membership (Clade A; Bouzid et al. 2008b) and to reveal possible internal population structure. DNA extraction was performed using the DNeasy Blood and Tissue kit (Qiagen). Then, DNA was eluted into 60 µl of the AE buffer. Two fragments of the mtDNA containing 914 bp of the mitochondrial cytochrome b gene (*Cyt*b) and 396 bp of the mitochondrial cytochrome oxidase subunit I gene (*COI*) loci were amplified using four pairs of primers (F2Dnihcob: 5′-GTT TTA CTG ATA GGT TAT TTA AAC-3′, R2Dnihcob_mod: 5′- CAG TTT AAA AAT CGA GTT AAA GAT-3′) (Wicht et al. 2010) and COIA2: 5′-CAT ATG TTT TGA TTT TTT GG-3′ and COIB2: 5′- AKA ACA TAA TGA AAA TGA GC-3′ (Li et al. 2000, Bouzid et al. 2008b, Štefka et al. 2009). PCRs were carried out in a 12 µl volume using 1 µl of extracted DNA, 10 pM of each primer, 6.25 µl 2× concentrated PPP Master Mix (Top-Bio, Vestec, Czech Republic). The amplification protocol consisted of one denaturation step at 94 °C for 15 min, then 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 50 °C for 45 seconds and an extension step at 72 °C for 45 seconds, followed by the last elongation step at 72 °C for 10 min. PCR products were enzymatically cleaned up with VWR ExoCleanUp FAST PCR reagent (VWR, USA) following manufacturer's protocol. Purified PCR products were sequenced using the PCR primers in a commercial laboratory (SeqMe, Dobříš, Czech Republic).

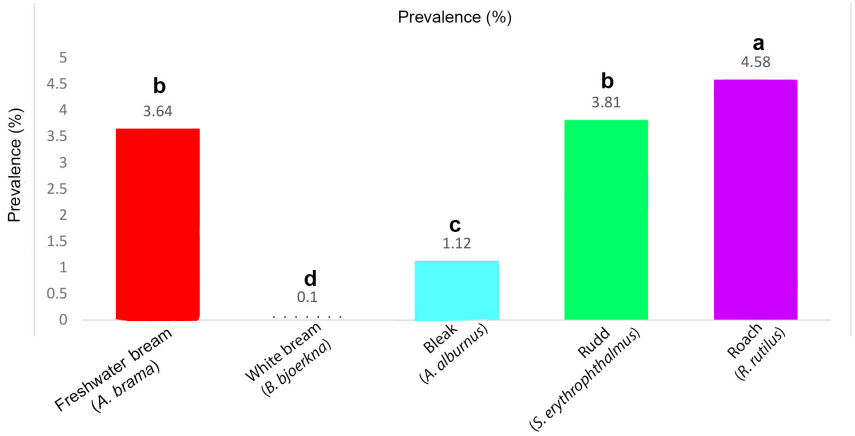

Fig. 2. Prevalence of plerocercoids of *Ligula intestinalis* (Linnaeus, 1758) in five cyprinid species across all localities. Shared letters denote non-significant results, while different letters denote significant differences (Kruskal-Wallis test, $p \leq 0.05$), all localities (values above columns).

Sequence analysis

The Geneious Prime software (https://www.geneious.com; version 20.1.1) was applied to edit the raw sequences. A multiple sequence alignment was generated using ClustalW in MEGA version 5 (Tamura et al. 2011). Then, all sequences were translated into protein-coding sequences based on the invertebrate mitochondrial genetic code to examine any possible stop codons. A substitution saturation test was carried out with the plotting of transversions and transitions against genetic distance and estimating the index of substitution saturation (Iss) and its critical value (Iss.c) in DAMBE Version 6.0.4 (Xia and Xie 2001, Xia and Lemey 2009). DnaSP version 6.0 (Librado and Rozas 2009) was used to estimate nucleotide diversity, haplotype diversity and sequence polymorphisms. Genetic distances among the parasite populations were determined using the uncorrected pairwise genetic distances with 1,000 bootstraps.

Phylogenetic relationships

We generated a concatenated dataset of two fragments of mtDNA (*Cyt*b and *COI*) comprising 1,310 bp in length for the 30 selected samples of *L. intestinalis* and aligned it with 82 sequences from two previous studies (Bouzid et al. 2008b, 2013; Table S1). Bayesian Inference (BI) and Maximum Likelihood (ML) approaches were carried out to reconstruct the phylogenetic relationships of the newly sampled plerocercoids. Using PartitionFinder version 2.1.1 (Lanfear et al. 2017), we identified the appropriate substitution nucleotide model for genes and codons based on the Bayesian Information Criterion (BIC). Bayesian phylogenetic analyses were performed via MrBayes version 3.2.2 (Ronquist and Huelsenbeck 2003) using the selected model of sequence evolution (Table S2). We ran one cold and three heated chains for 40 million generations, with trees being sampled every 1,000 generations. We discarded the first 25% of the runs as burn-in. Then, by combining the post-burn-in trees, a 50 percent majority rule consensus tree was constructed. Bayesian posterior probabilities (PP) were computed to estimate support of the Bayesian tree. Using the best-fitting model, a maximum likelihood analysis was applied to construct an ML tree in IQTREE version 2.1.2, (Minh et al. 2020). Branch support of the ML tree was computed using 1,000 rapid bootstrap replicates (Hoang et al. 2018). *Dibothriocephalus latus* (Linnaeus, 1758), *D. nihonkaiensis* (Yamane, Kamo, Bylund et Wikgren, 1986) and *Diphyllobothrium stemmacephalum* Cobbold, 1858 (GenBank accession number AB269325, AB268585 and AP017648, respectively) were used as outgroups based on data provided by Waeschenbach et al. (2017).

Population genetic structure

Population genetic relationships among populations of *L. intestinalis* in the Czech water bodies were reconstructed using the concatenated mtDNA dataset by a maximum parsimony algorithm in PopArt (Leigh and Bryant 2015) and by the Principal Coordinates Analysis (PCoA) in adegenet R package (Jombart 2008) in R 4.0.1 software. Moreover, to compare the newly studied populations with the Clade A haplotypes from the previous study (Bouzid et al. 2008b, Štefka et al. 2009) a haplotype network containing a total of 80 sequences (801 bp in length) was constructed. Pairwise F-statistics for the newly sampled Czech populations were executed with 10,000 permutations in Arlequin version 3.5 and parasite populations were categorised into five groups based on their hosts.

RESULTS

Fish abundance and rate of infection

Roach was the most abundant fish species in Milada (208 individuals/1,000 m^2), Most (90 individuals/1,000 m^2) and Jordán (310 individuals/1,000 m^2) water bodies. Bleak was numerically the most dominant species in Lipno (236 individuals/1,000 m^2) and Římov (148 individuals/1,000 m^2) reservoirs. In Lake Medard, rudd had the highest fish abundance (26 individuals/1,000 m^2; Table S3). Analysis of prevalence for five cyprinoid species, calculated cumulatively for all six water bodies, is presented in Fig. 2. Prevalence differed significantly among all species pairs ($p \leq 0.05$), except for freshwater bream and rudd. For example, roach (4.6%) and white bream (0.1%) had the highest and lowest significant prevalence, respectively. The prevalence for rudd (3.8%) was significantly higher than the prevalence for bleak (1.1%) or white bream (0.1%).

Table 2. Prevalence of infection of five cyprinoid fishes with *Ligula intestinalis* (Linnaeus, 1758) in six reservoirs (N = total number samples, n = total number of infected fish, P = prevalence in %). Numbers followed by a letter in column P mark the level of significance in the prevalence rate of roach between pairs of different reservoirs in a Kruskal-Wallis test. Shared letters [a–c] denote non-significant results, while different letters denote significant differences ($p \leq 0.05$).

	Medard			Jordán			Milada			Most			Lipno			Římov		
	N	n	P	N	n	P	N	n	P	N	n	P	N	n	P	N	n	P
Freshwater bream	6	0	**0**	39	0	**0**	0	0	**0**	0	0	**0**	60	4	**6.66**	169	6	**3.55**
White bream	8	0	**0**	1617	2	**0.12**	0	0	**0**	0	0	**0**	236	0	**0**	45	0	**0**
Bleak	18	0	**0**	224	0	**0**	0	0	**0**	0	0	**0**	1001	4	**0.39**	589	18	**3.05**
Rudd	37	2	**5.4**	64	0	**0**	33	0	**0**	128	8	**6.25**	0	0	**0**	0	0	**0**
Roach	60	17	**28.3** [a]	176	0	**0**	412	22	**5.39** [b]	483	52	**10.7** [c]	636	0	**0**	434	10	**2.30** [b]
Total	129	19	**14.72**	2120	2	**0.094**	455	22	**4.49**	611	60	**9.81**	1933	8	**0.41**	1237	34	**2.74**

Table 3. Genetic characteristics of the concatenated dataset (1310 bp) estimated for samples of *Ligula intestinalis* (Linnaeus, 1758) from the five host species (samples comprising more than three individuals) including number of individuals (N), nucleotide diversity (Pi), number of haplotypes (h), haplotype diversity (Hd), (K) the total number of mutations.

Parasite populations	N	h	hd	pi	k
Freshwater bream	5	5	1.00	0.0021	7
White bream	2	2	-	-	-
Bleak	4	1	0.00	0.0000	0
Rudd	4	4	1.00	0.0016	4
Roach	15	11	0.93	0.0035	25

The prevalence recorded for each species in each freshwater reservoir is given in Table 2. According to our observations, roach was either the most abundant or the second most abundant cyprinoid species in lakes Medard, Milada and Most (gillnet catches; Table S3), where it also had the highest prevalence (28.3%, 5.3% and 10.7%, respectively). In contrast, freshwater bream in most lakes and reservoirs (except Římov) had the lowest abundance of all species studied. However, in two reservoirs (Lipno and Římov) the prevalence of *Ligula intestinalis* was higher in freshwater bream than in other host species (Table 2, Fig. S1). Overall, Medard and Jordán demonstrated the highest and lowest prevalence of *L. intestinalis*, respectively. Roach was the only species sampled frequently enough to allow comparison of prevalence among reservoirs. Its prevalence rate was significantly higher in the Medard than in Římov, Milada and Most ($p \leq 0.05$; Table 2). No correlation was found between fish abundance and prevalence of infection with *L. intestinalis* ($p > 0.05$; Fig. S2).

Sequences analyses

Our concatenated dataset (*Cyt*b and *COI*) containing 30 sequences comprised 21 haplotypes, showing 1,274 invariable sites, 22 parsimony informative sites, and 14 singleton sites, out of the 1,310 bp aligned positions. Neither stop codons nor insertions/deletions were observed in the datasets. Table 3 demonstrates genetic characteristics for the parasite populations comprising more than three sampled individuals. Minimum and maximum nucleotide diversities were calculated to be 0.000 and 0.00348 (the samples from roach and bleak, respectively). Also, haplotype diversities ranged between 0.0 and 1 in bleak and rudd, and freshwater bream, respectively (Table 3).

Phylogenetic relationships

The substitution saturation analysis showed that the Iss was significantly smaller than Iss.c ($p < 0.005$), suggesting the suitability of the concatenated dataset for phylogenetic analyses. The pattern of transitions and transversions plotted against the genetic distance also confirmed that the dataset retains sufficient phylogenetic signals (Fig. S3).

Both phylogenetic trees (ML and BI) recovered identical topologies. Consequently, only the BI tree is demonstrated (Fig. 3). The phylogenetic analysis disclosed six distinct lineages within the monophyletic *L. intestinalis* complex which was consistent with the previous studies (Bouzid et al. 2008b, Štefka et al. 2009). Our phylogenetic results depicted that all samples of plerocercoids from the five host species in the Czech water bodies were placed within Clade A as expected.

Population genetic structure

The pattern of population structure revealed by haplotype network analysis showed haplotype sharing among the parasite samples in rudd, bleak and roach. In contrast, samples from the freshwater bream were separated from the other haplotypes by three mutational steps (Fig. 4). Similarly, the haplotype network also containing samples from the previous studies, based on the 405 bp of *Cyt*b and 396 bp *COI* (801 bp), revealed no shared haplotypes between *L. intestinalis* samples from the freshwater bream and other host species whereas common haplotypes were discovered between rudd and roach hosts (Fig S4). However, despite the different status of the bream related haplotypes, they did not create a single cluster in this larger sample containing other European populations. The PCoA analysis of the current samples revealed a similar clustering pattern, including the assignment of three parasite populations (rudd, bleak and roach) to one cluster and clear separation of the freshwater bream population from the rest. The first and the second axes in the PCoA explained 43.9% and 16.3% of the total variations, respectively (Fig. 5).

The maximum and minimum values of pairwise Fixation indices (F_{ST}) were estimated to be 0.71 and 0.02, respectively (Table 4). In accordance with the haplotype network and PCoA results, the parasites from freshwater bream demonstrated a significant ($P < 0.05$) genetic differentiation in comparison to other samples. Similarly, the highest and the lowest genetic distances (0.06% and 0.01%) were calculated between the freshwater bream and the roach, bleak and rudd, respectively.

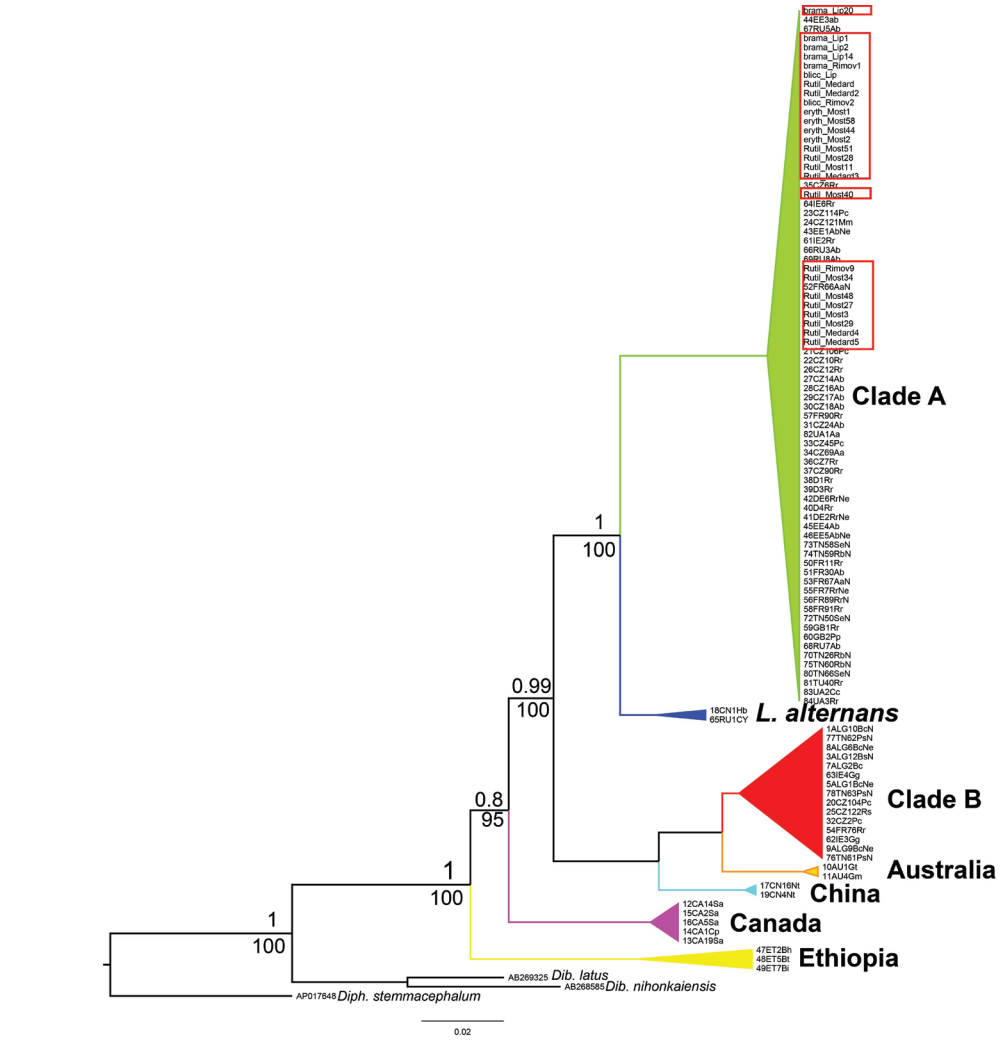

Fig. 3. Bayesian phylogenetic tree reconstructed for the *Ligula intestinalis* (Linnaeus, 1758) species complex using a concatenated mtDNA dataset (*Cyt*b and *COI*, 1,310 bp). For each node, nodal supports indicate Bayesian Inferrence (BI, top) and Maximum Likelihood (ML, base) support values. Newly obtained sequences from the Czech Republic are highlighted by red rectangles.

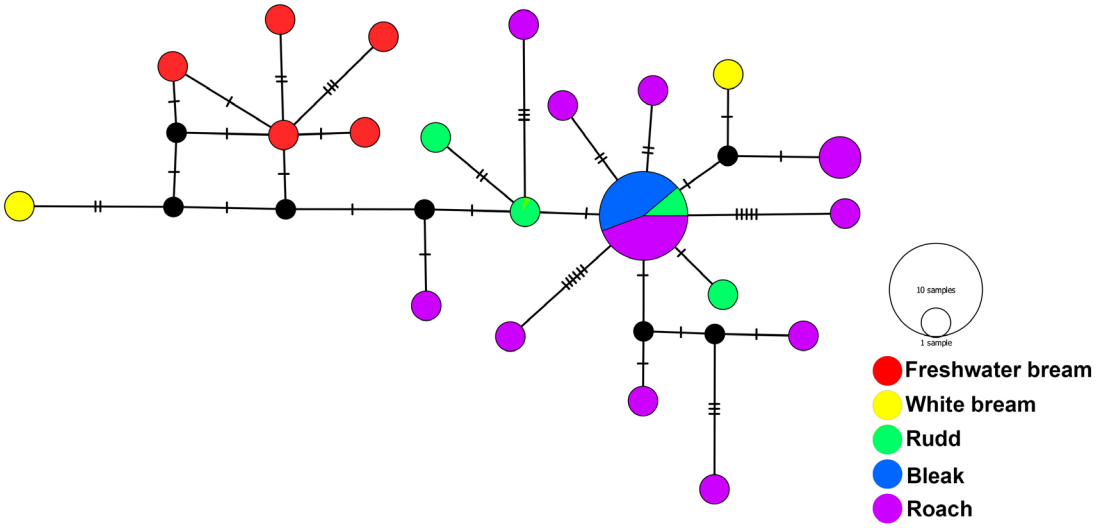

Fig. 4. Haplotype network of 30 samples of plerocercoids of *Ligula intestinalis* (Linnaeus, 1758) selected to represent hosts from the Czech Republic. The network was generated using a concatenated mtDNA dataset (1,310 bp). Individual haplotypes were coloured based on their respective fish hosts (using the same colours as in Fig. 1). The size of the circles is proportional to haplotype frequencies. Putative unsampled haplotypes are shown by black circles and dash symbols indicate mutational steps.

Table 4. Pairwise values of Fst (below diagonal) and genetic distance (above diagonal) for samples of *Ligula intestinalis* (Linnaeus, 1758) from the five host species. P-values lower than 0.05 are flagged with an asterisk (*).

	N	Freshwater bream	White bream	Bleak	Rudd	Roach
Freshwater bream	5	0	0.05%	0.04%	0.04%	0.06%
White bream	2	0.31*	0	0.04%	0.05%	0.03%
Bleak	4	0.55*	0.14	0	0.01%	0.02%
Rudd	6	0.45*	0.10	0.02	0	0.02%
Roach	15	0.71*	0.38	0.13	0.10	0

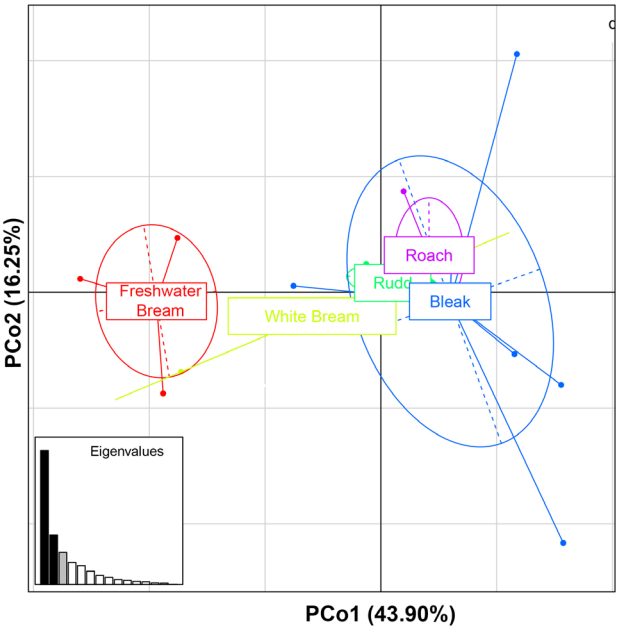

Fig. 5. Principle coordinate analysis (PCoA) based on Euclidean genetic distance for the parasite samples from the five host species, freshwater bream *Abramis brama* (Linnaeus), white bream *Blicca bjoerkna* (Linnaeus), bleak *Alburnus alburnus* (Linnaeus), rudd *Scardinius erythrophthalmus* (Linnaeus) and roach *Rutilus rutilus* (Linnaeus).

DISCUSSION

In the present study, a total number of 6,630 fish of five cyprinoid species from three lakes and three reservoirs (Medard, Milada, Most, Jordán, Římov and Lipno) in the Czech Republic were captured and examined for the presence of plerocercoids of *Ligula intestinalis*. While the presence of *L. intestinalis* in Lipno has already been demonstrated in a population genetic study (Štefka et al. 2009), the presence of the parasite in Medard, Milada, Most, Jordán and Římov has not been previously reported. Each of the host species studied (freshwater bream, white bream, bleak, rudd and roach) was infected in at least one of the lakes. Then, we used population genetic analysis on a selected set of samples to analyse the level of population structure and test for possible occurrence of host specificity in the plerocercoids. Obtained results suggest an emergence of a bream-specific genetic cluster. Below we discuss the genetic results with regard to the obtained rates of prevalence in the Czech water bodies.

Prevalence of *Ligula intestinalis* in different fish and lakes

We attempted to monitor the infection rate of *L. intestinalis* within one summer season (July and September 2020). The results showed that the total prevalence (across all fish species) is less than 15% in all the reservoirs studied in the Czech Republic, whereas roach in Medard had the highest prevalence (28%) when host species were considered separately. Earlier studies show a large variation in prevalence both in the Czech Republic and elsewhere. In a study conducted in the Nové Mlýny reservoirs (South Moravia, Czech Republic), the infection rate of *L. intestinalis* in white bream ranged from 1.5% in April-May to 11.5% in November-December (Baruš and Prokeš 1995). In contrast, in a study of *L. intestinalis* in freshwater bream from the Aras Dam (Iran) the prevalence was 45%, with a significantly higher infection rate in autumn compared to winter (Nezafat et al. 2008). In France, the highest value of prevalence was observed at the end of the summer and in the autumn (Brown et al. 2001). In Zimbabwe, the highest seasonal infection rate (prevalence 21–46%) was reported in the cooler months of July to September (Barson and Marshall 2003). In addition to the season and temperature, which are generally considered to be major factors affecting the speed of development of this parasite, *L. intestinalis* may exhibit also epizootic cycles over multi-seasonal periods, during which it rapidly increases its numbers in the lake resulting in increased fish mortality (Kennedy et al. 2001).

Our single season data did not pick up excessively high prevalence rates suggesting an epizootic peak, although the rate for roach in Medard was relatively high with possible ecological impact. The pronounced infection rate in roach compared to the other four species appears to be consistent with previous reports from the United Kingdom, where infection rates were higher in roach than in gudgeon, rudd, freshwater bream, minnow and dace *Leuciscus leuciscus* (Linnaeus) (Arme and Owen 1968, Kennedy and Burrough 1981). Further research has shown that although *L. intestinalis* prevalence fluctuates in irregular cycles, epizootics of the parasite are associated with rapid increases in roach populations (Kennedy et al. 2001).

However, our research showed that the component of a hidden population structure needs to be considered when interpreting the ecological impact of prevalence rates. For example, roach in Lipno was free of *L. intestinalis* despite the large sample size, while the prevalence in freshwater bream was considerable (6.7%). At the first sight, this result could imply that different phases of the *L. intestinalis* infection cycle are present in the lake, switching between different hosts. However, even though our sequencing analysis confirmed that all Czech samples belong to the same mtDNA Clade A (as in Bouzid et al. 2008b, Bouzid et al. 2013) we also found emerging genetic differentiation between freshwater bream plerocercoids and the rest of population of *L. intestinalis* from the samples. Thus, the ecology of roach and bream is probably highly independent of each other.

For *L. intestinalis* populations to thrive, they must succeed in all three stages of their life cycle: a copepod as the first host, a zooplanktivorous fish as the second intermediate host, and a bird as the final host, in which they reach sexual maturity. The adult worm spends only a maximum of five days in its final host (Kennedy et al. 2001), but the migratory birds play an important role in the life cycle of *L. intestinalis*, as they are the main means of dispersal (Štefka et al. 2009). Among birds in the Czech Republic, the presence of adult *L. intestinalis* was detected in the great crested grebe and great cormorant *Phalacrocorax carbo* (Linnaeus) in southern Moravia (Ryšavý and Sitko 1995, Levron et al. 2009, Moravec and Scholz 2016).

During our fish surveys, we spotted six potential final hosts – great cormorants (*P. carbo*), grebes *Podiceps auritus* (Linnaeus), *Podiceps cristatus*, *P. nigricollis* Brehm, and ducks (*Mergus merganser*, *Aythya ferina* (Linnaeus) at the visited reservoirs and lakes. However, several other species common to the area, such as herons and gulls, are thought to frequent the studied localities. Although the role of some of these species in the introduction and spread of the parasite between lakes seems to be of minor importance because their abundance is low or they rarely forage on fish, cormorants, grebes and herons are widespread and highly mobile species, thus allowing dispersal of *L. intestinalis*. Nevertheless, whilst dispersal rates seem sufficient to maintain gene flow across relatively large distances (Štefka et al. 2009), it is questionable whether they are sufficient to modulate differences in plerocercoid prevalence between the same hosts in different lakes. Whereas prevalence in some fish species was low in most lakes and cannot be directly contrasted, other species (roach and possibly freshwater bream, Table 2) showed marked differences. Although several ecological factors (e.g., fish abundance, primary host density, etc.) contribute to creating lake-specific patterns, we suggest that the propagule size and frequency of introduction of *L. intestinalis* by definitive hosts should also be considered. Our data do not allow for testing all possible factors, but we did not find a link between the fish host abundance and prevalence rate across the studied lakes (Fig. S2).

Phylogeny and population genetic structure of *L. intestinalis*

It has been previously acknowledged that the evolution of *Ligula* tapeworms is defined by the interplay between geography and host specificity at a global scale (Bouzid et al. 2008b). Interestingly, at a local scale, epidemiological differences between freshwater bream and roach infecting plerocercoids are known to exist (Loot et al. 2001), but neither a microsatellite study (Štefka et al. 2009), nor the most densely sampled mtDNA study of the Clade A to date (Bouzid et al. 2008b, Štefka et al. 2009) recovered any genetic differences between the freshwater bream and roach (plus bleak and rudd) samples. Somewhat unexpectedly, the analysis of population genetic structure at a fine scale in our study revealed a high degree of host specificity of several haplotypes of *L. intestinalis* to freshwater bream. Even though our sample size is small, the pattern becomes obvious also in a larger sample, when the new data are analysed with all available Clade A sequences (Fig. S4). Plerocercoids from breams create several clusters, or even if scattered into individual haplotypes they are not shared with other hosts. Such a pattern might indicate an ongoing process of lineage sorting. The results of both the PCoA analysis and the pairwise F_{ST} of the current samples also showed that *L. intestinalis* collected from the freshwater bream diverged from the rest of the samples, while the samples in roach, rudd and bleak demonstrated shared haplotypes and low F_{ST} differentiation. White bream samples were less frequently collected and whilst their haplotypes were not shared with any other species, they also did not create a single cluster.

Our results might indicate an emerging barrier between ecologically differentiated (host-specific) parasite populations. However, our data are too limited, both in the number of sequenced samples and the volume of genetic information, to conclude if the parasite populations have been influenced by an ecological speciation process and whether any reproductive barriers evolved between the host-specific clusters. Nevertheless, our finding represents a promising candidate for testing a hypothesis of speciation due to divergent natural selection in different hosts, as defined by Nosil (2012). Even though ecological speciation is understood as one of the primary mechanisms of sympatric speciation, the process can play a role in all geographical settings, including parapatric and allopatric layouts (Rundle and Nosil 2005, Nosil 2012).

Preliminary population genomic data, a RADseq analysis of plerocercoids performed by Kočová (2018), also suggest a differentiation between bream and the rest of the samples within Clade A. Other processes, such as a historical separation of populations of *L. intestinalis* from different hosts during the quaternary glaciation period followed by admixture, may have contributed to creating the current pattern of population structure. Thus, it is evident that additional epidemiological, genomic and transcriptome data are needed for populations of *L. intestinalis* sampled across different hosts before a firm conclusion could be reached on the ecological speciation routes of this parasite.

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