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Unique genetic structure of the human tapeworm *Dibothriocephalus latus* from the Alpine lakes region – a successful adaptation?

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Research Article

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Abstract

Dibothriocephalus latus is the most frequent causative agent of fish-borne zoonosis (diphyllobothriosis) in Europe, where it is currently circulating mainly in the Alpine lakes region (ALR) and Russia. Three mitochondrial genes (*cox*1, *cob* and *nad*3) and 6 microsatellite loci were analysed to determine how is the recently detected triploidy/parthenogenesis in tapeworms from ALR displayed at the DNA level. A geographically distant population from the Krasnoyarsk Reservoir in Russia (RU-KR) was analysed as a comparative population. One or 2 alleles of each microsatellite locus was detected in plerocercoids from RU-KR, corresponding to the microsatellite pattern of a diploid organism. In contrast, 1–3 alleles were observed in tapeworms from ALR, in accordance with their triploidy. The high diversity of mitochondrial haplotypes in *D. latus* from RU-KR implied an original and relatively stable population, but the identical structure of mitochondrial genes of tapeworms from ALR was probably a consequence of a bottleneck typical of introduced populations. These results indicated that the diploid/sexually reproducing population from RU-KR was ancestral, located within the centre of the distribution of the species, and the triploid/parthenogenetically reproducing subalpine population was at the margin of the distribution. The current study revealed the allelic structure of the microsatellite loci in the triploid tapeworm for the first time.

Introduction

The broad fish tapeworm *Dibothriocephalus latus* (Cestoda; Diphyllobothriidea) is the most frequent causative agent of diphyllobothriosis, a fish-borne zoonosis, in Europe. Copepods and freshwater fish are the first and second intermediate hosts, respectively. The European perch *Perca fluviatilis* (Percidae), Northern pike *Esox lucius* (Esocidae), burbot *Lota lota* (Lotidae) and ruffe *Gymnocephalus cernua* (Percidae) are the most suitable and frequent fish hosts of *D. latus* (for a review, see Králová-Hromadová *et al.*, 2021). Fish-eating mammals (mainly Canidae and Felidae) may serve as definitive hosts of *D. latus* (Bouvier *et al.*, 1963; Zottler *et al.*, 2019), but humans play the most important role in the circulation of *D. latus* in the environment (von Bonsdorff, 1977). The life cycle of *D. latus* is maintained when humans consume raw or insufficiently cooked fish products harbouring larval stages, plerocercoids.

The tapeworm is distributed in Eurasia and North and South America (for a review, see Králová-Hromadová *et al.*, 2021). Some studies support the theory of the introduction of *D. latus* to North America by immigrants from endemic European regions (von Bonsdorff, 1977), but others have suggested that *D. latus* was already present in North America prior to European immigration (Jenkins *et al.*, 2013). The tapeworm was also introduced to some Patagonian lakes in South America (for a review, see Torres and Yera, 2018; Kuchta *et al.*, 2019).

Several foci of diphyllobothriosis with different dynamics of occurrence have developed in Europe. The level of infection decreased substantially or was eliminated after massive health campaigns and effective treatment in Fennoscandia, the Baltic region, and the Danube Delta in the last decades of the 20th century. In contrast, the ongoing circulation of *D. latus* in the natural environment and in humans has been documented in the Alpine lakes region (ALR) (for a review, see Králová-Hromadová *et al.*, 2021) and throughout Russia (von Bonsdorff, 1977; Dugarov and Pronin, 2017; Chugunova *et al.*, 2020).

Diphyllobothriosis has persisted in ALR despite great efforts to eliminate the further spread of the disease, mainly due to eating habits and the proliferation of restaurants serving raw fish, insufficient disinfection of wastewater and the many yachters and fishermen using the lakes

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(Dupouy-Camet and Peduzzi, 2004; Jackson *et al.*, 2007). Since 2000, diphyllobothriosis has been diagnosed in human patients from Switzerland, Italy and France and *D. latus* has also been frequently detected in fish from various subalpine lakes (Wicht *et al.*, 2010*a*, 2010*b*; Dupouy-Camet *et al.*, 2015; Menconi *et al.* 2020, 2021).

The ongoing circulation of *D. latus* in the natural environment of subalpine lakes has offered biological material for molecular and genetic studies of the parasite (Nicoulaud *et al.*, 2005; Yera *et al.*, 2006, 2008; Wicht *et al.*, 2007, 2010*a*, 2010*b*; Bazsalovicsová *et al.*, 2018). A recent in-depth karyological analysis of 2 *D. latus* specimens from European perch from Iseo lake (northern Italy) found a very atypical chromosomal structure (Orosová *et al.*, 2021). Both tapeworms contained majority of the cells with the triploid number of chromosomes ($3n = 27$, 46.7%), but 5.9% of cells displayed diploid ($2n = 18$) chromosomal set. A high level of aneuploidy, represented by 20, 22, 24 and 26 chromosomes, was also detected in 3.7, 7.0, 12.9 and 23.9% of the cells, respectively. Fluorescent *in situ* hybridization using a probe for the small ribosomal subunit demonstrated the presence of 3 clusters of hybridization signals on chromosome triple no. 7, thus confirming the triploidy of the specimens. The course of meiosis was aberrant and indicated an absence of fully developed spermatic cells and mature functional spermatozoa. Parthenogenetic reproduction was concluded to be the most probable method of reproduction of triploid *D. latus* from Iseo lake (Orosová *et al.*, 2021).

The unique chromosomal structure of *D. latus* raised the question of how intraindividual chromosomal polymorphism is displayed at the DNA level and how is parthenogenesis linked to the genetic structure of the species. To answer these questions, 3 mitochondrial genes (mtDNA) were first used to analyse the structure and overall level of intraspecific polymorphism of *D. latus* populations from the ALR and a selected locality in Russia. Microsatellites (short tandem repeats, STRs) have been used for obtaining a more detailed assessment of the genetic structure of populations relative to mtDNA. Six microsatellite loci, previously designed for *D. latus* by microsatellite library screening (Bazsalovicsová *et al.*, 2018), were analysed to determine the number of allelic variants at each locus and their possible association with the variable number of chromosomes and method of reproduction.

Materials and methods

Study areas

Plerocercoids of *D. latus* were isolated from fish intermediate hosts from 6 lakes in ALR: (1) Iseo lake, Italy (IT-IS), (2) Como lake, Italy (IT-CO), (3) Maggiore lake, Italy (IT-MA), (4) Lake Geneva, Switzerland (CH-GE), (5) Neuchâtel lake, Switzerland (CH-NE) and (6) Biel lake, Switzerland (CH-BI) (Fig. 1; Table 1). Maggiore lake is on the border between Italy and Switzerland; our samples originated from the Italian side of the lake. Similarly, Lake Geneva lies between Switzerland and France; plerocercoids were obtained from the Swiss part of the lake.

Iseo lake was our first choice for collecting samples because intraindividual chromosomal polymorphism has recently been detected in *D. latus* from this lake (Orosová *et al.*, 2021). The other 5 subalpine lakes were chosen as study areas due to their similar ecological conditions. The Italian lakes Iseo, Como and Maggiore were originally oligotrophic but underwent severe cultural eutrophication caused by urban, industrial and/or agricultural pollution (Manca *et al.*, 2007; Menconi *et al.*, 2021). Lakes Geneva, Biel and Neuchâtel in Switzerland suffered from eutrophication but later became mainly mesotrophic (Lang, 1989; Anneville *et al.*, 2007). Lakes Iseo, Como, Maggiore and Geneva represent isolated aquatic biotopes with no connection *via* common tributaries or outlets; Neuchâtel and Biel lakes are connected by the Thielle channel (Fig. 1). The high prevalence

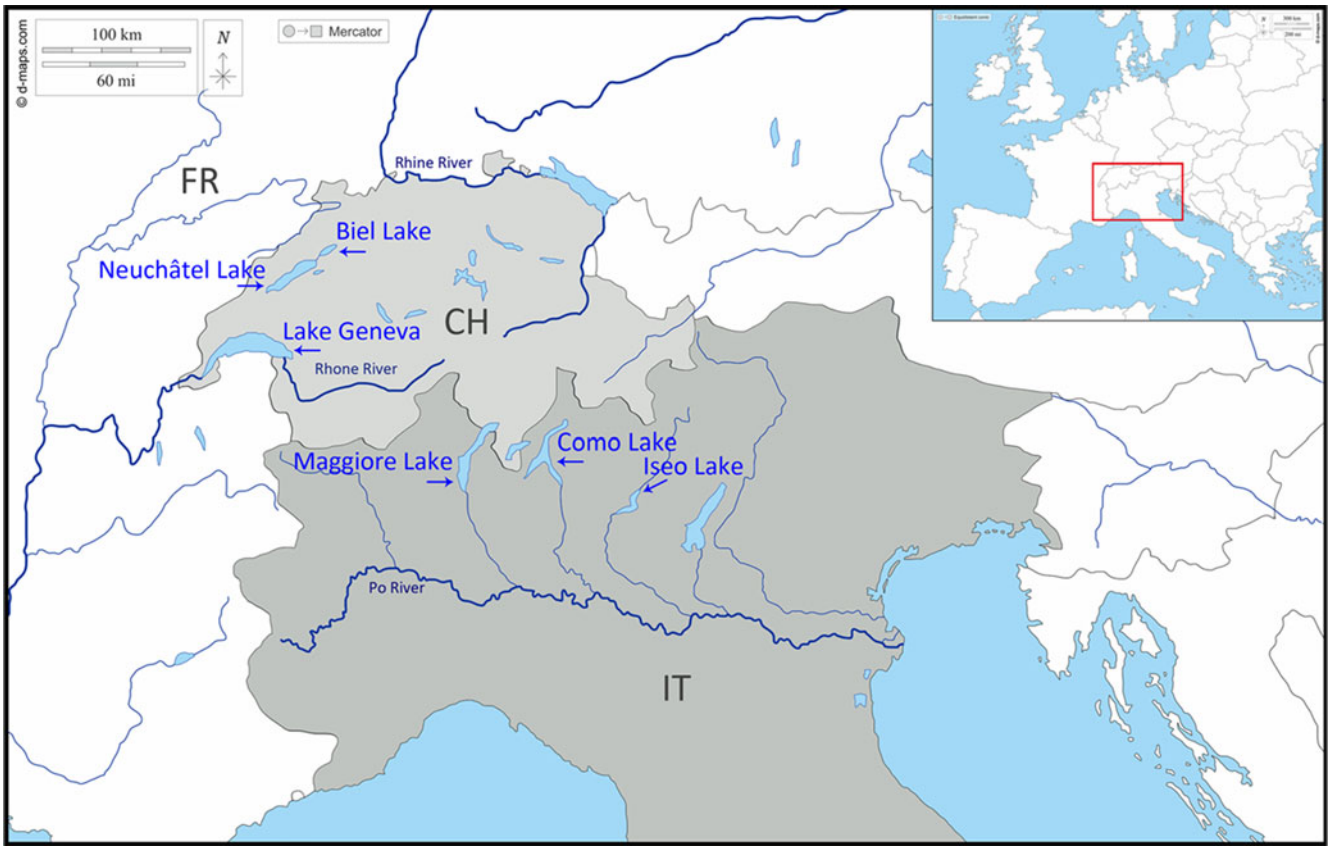

Fig. 1. Hydrological map of the study area of *Dibothriocephalus latus* from the ALR. CH, Switzerland; FR, France; IT, Italy.

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Table 1. Details of the *Dibothriocephalus latus* populations from ALR and RU-KR

Country	Locality	Host	Sample code	No. of samples
Italy (IT)	Iseo lake (IS)	*Perca fluviatilis*, *Esox lucius*	IT-IS	43 P
Italy	Como lake (CO)	*P. fluviatilis*, *E. lucius*, *Lota lota*	IT-CO	47 P
Italy	Maggiore lake (MA)	*P. fluviatilis*	IT-MA	8 P
Switzerland (CH)	Lake Geneva (GE)	*P. fluviatilis*	CH-GE	11 P
Switzerland	Biel lake (BI)	*P. fluviatilis*	CH-BI	3 P
Switzerland	Neuchâtel lake (NE)	*P. fluviatilis*	CH-NE	1 P
Switzerland	Various localities	*Homo sapiens*, human (H)	CH-H	14 A
Switzerland	Canton Grisons	*Vulpes vulpes*, red fox (F)	CH-F	1 A
Russia (RU)	Krasnoyarsk Reservoir (KR)	*P. fluviatilis*, *E. lucius*, *L. lota*	RU-KR	18 P
			Subtotal	113 plerocercoids (P) from ALR
				15 adults (A) from ALR
				18 plerocercoids (P) from RU-KR
			Total	146 samples

of *D. latus* has been confirmed in European perch, Northern pike and burbot in all 6 subalpine lakes (for a review, see Králová-Hromadová *et al.*, 2021).

Dibothriocephalus latus specimens from fish from a distant locality, the Krasnoyarsk Reservoir in Russia (RU-KR) (southern Siberia, Asian part of Russia), were analysed as a comparative population (Fig. 2A; Table 1). This reservoir is one of the largest artificial reservoirs in the world. The construction of its dam began in 1956, and subsequent filling with water from Yenisey River ended in 1970. The reservoir is >388 km long and 15 km wide with an average depth of 36.7 m (Vishegorodtsev *et al.*, 2005). Several major rivers flow into the reservoir and form bays. The section of the Yenisey River downstream from the city of Krasnoyarsk never freezes in winter as the dam is located at the Krasnoyarsk hydroelectric power station, even though the river is in Siberia. Fish, mainly European perch and Northern pike, have an important role in human nutrition in this region. Diphyllobothriosis remains an important problem due to the frequent consumption of fish and ranks third in the statistics of helminthiases (11%) in the Krasnoyarsk territory (Chugunova *et al.*, 2020).

Parasitic material

A total of 146 samples of *D. latus* (113 plerocercoids and 15 adult tapeworms from ALR and 18 plerocercoids from RU-KR) was analysed (Table 1). Details for each specimen are provided in Supplementary material Table 1. Plerocercoids ($n = 131$) were obtained mainly from the musculature and/or abdominal cavity and internal organs of *P. fluviatilis*, *E. lucius* and *L. lota* caught by professional fishermen in the 6 subalpine lakes (Fig. 1) and RU-KR (Fig. 2A) in 2017 and 2018. Fish were kept on ice and dissected immediately without previous freezing. Complete helminthological dissection of each fish included examination of the abdominal cavity and internal organs and detailed inspection of thin slices (approximately 5 mm) of musculature. Plerocercoids were rinsed in a saline solution and preserved in 96% ethanol for analysis.

Pieces of strobilae of adult tapeworms ($n = 15$) were collected by Dr Barbara Wicht from Switzerland, deposited in the helminthological collection of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic, and kindly provided by Professor Tomáš Scholz and Dr Roman Kuchta. Samples from human patients ($n = 14$) originated from different localities in the Swiss cantons of Geneva, Vaud and Valais (shore of lake Geneva) and from the canton of St. Gallen (the northeastern part of Switzerland) near lake Constance. A single adult tapeworm isolated from a red fox (*Vulpes vulpes*) from the canton of the Grisons was also included in the analysis (Table 1; Supplementary material Table 1).

Isolation of DNA

Genomic DNA was isolated either from entire plerocercoids or from 20 mg of tissue from adult tapeworms using the QIAamp® DNA mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. The DNA was diluted in deionized water and stored at −20°C for molecular analysis.

Molecular identification by multiplex polymerase chain reaction

A differential multiplex polymerase chain reaction (PCR) test based on the mitochondrial cytochrome *c* oxidase subunit I gene (*cox*1 mtDNA) was designed by Wicht *et al.* (2010*b*) for discriminating between the most common diphyllobothriid species infecting humans: *D. latus*, *Dibothriocephalus dendriticus*, *Dibothriocephalus nihonkaiensis* and *Adenocephalus pacificus*. This test was applied for the species-specific identification of all specimens. Five primers were used in each multiplex PCR. One reverse primer (MulRevCom) was common for all diphyllobothriid species, and 4 forward primers were species-specific: MulLat3 for *D. latus*, MulDen4 for *D. dendriticus*, MulPac2 for *A. pacificus* and MulNih5 for *D. nihonkaiensis*. Details of the primers are provided in Table 2 (see section I). The PCR amplification was performed in a total volume of 20 μL containing 10–20 ng of genomic DNA, 10 pmol of each of the 5 primers and 1× Master Mix (Thermo Fisher Scientific Inc., Waltham, USA). The PCR amplification conditions were 5 min at 95°C as an initial denaturation step, then 40 cycles of 30 s at 95°C, 1 min at 60°C, 1 min at 72°C and a final polymerization step for 10 min at 72°C. The PCR products were visualized on a 1.5% agarose gel.

Selection and PCR amplification of mitochondrial genes

Three mitochondrial genes were analysed: (1) partial *cox*1 (426 bp; primers MulLat3 and MulRevCom) obtained during the identification of species by multiplex PCR, (2) complete

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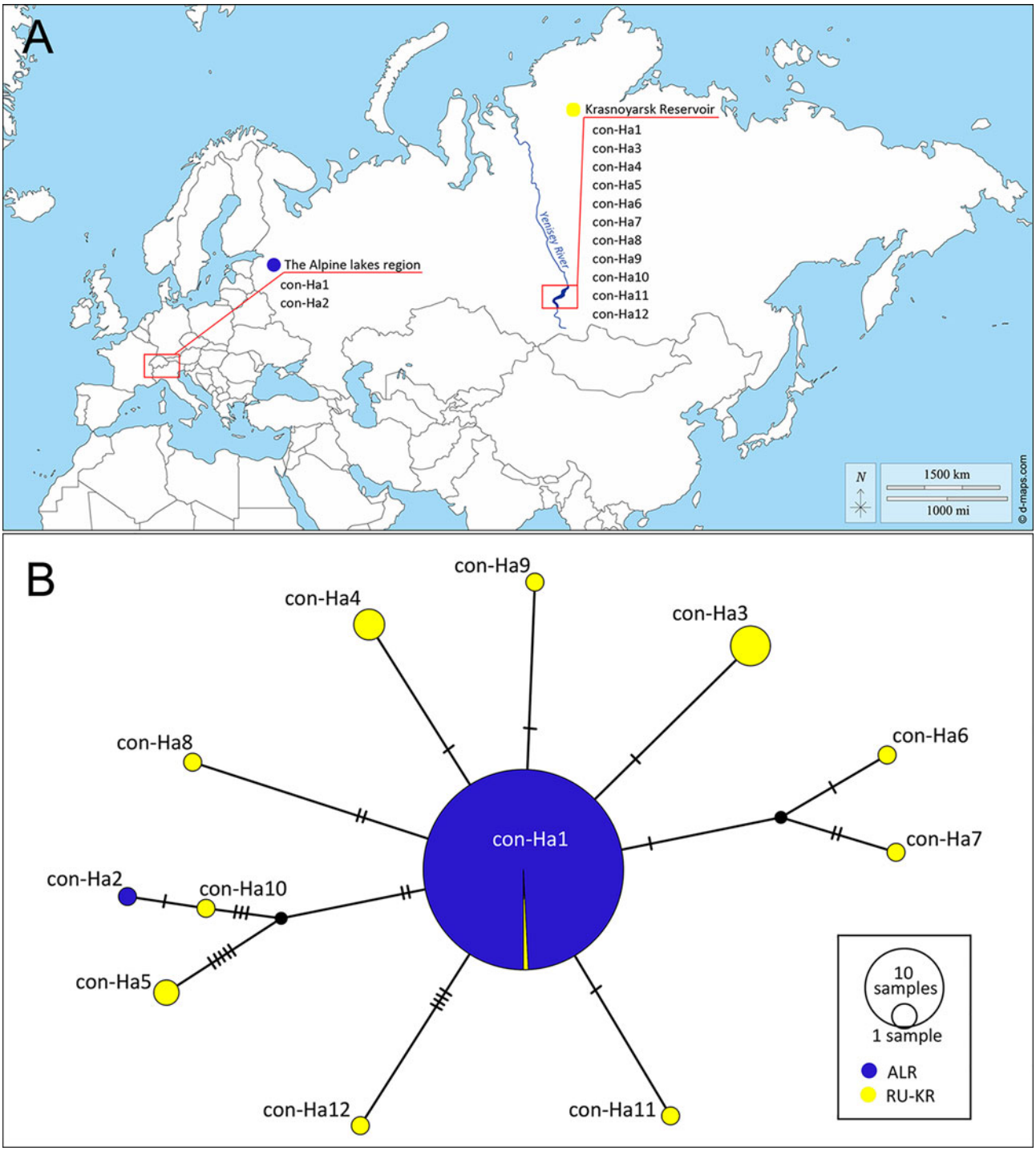

Fig. 2 (A) Map of the ALR and the RU-KR, with detected concatenated mitochondrial haplotypes (con-Ha). The map was obtained from d-maps.com. (B) Haplotype network of *D. latus* from the ALR and RU-KR, based on con-Ha of the mitochondrial *cox*1, *cob* and *nad*3 genes. The sizes of the haplotypes are proportional to the number of samples. Each hatch mark represents a mutation, and black dots represent intermediate missing or unsampled haplotypes.

cytochrome *b* (*cob*, 1107 bp; primers F2Dnihcob and R2Dnihcob) and (3) complete nicotinamide dehydrogenase subunit 3 (*nad*3, 357 bp; primers DnND3Fo and DnND3Re). The sequences of the primers used for the PCR amplification and sequencing of the mitochondrial genes are presented in Table 2 (see section II); the scheme of the amplified regions and locations of the primers is provided in Supplementary material Fig. 1. The PCR amplification of partial *cox*1 was as described above. The amplification of the *cob* and *nad*3 genes was carried out using the following conditions: 5 min at 95°C as an initial denaturation step, then 35 cycles of 1 min at 94°C, 30 sec at 56°C (*cob*) or 1 min at 50°C (*nad*3), 1 min at 72°C and a final polymerization step for 4 min (*cob*) or 10 min (*nad*3) at 72°C.

Purification and sequencing of the mitochondrial genes

The PCR products were visualized on a 1.5% agarose gel and purified using ExoProStar™ 1-Step (Illustra™, GE Healthcare, Little Chalfont, UK). The products were sequenced using a 3500 Genetic Analyser (Thermo Fisher Scientific) and the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific). Contiguous sequences were assembled and inspected

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Table 2. Summary and details of the primers

Purpose of application	Mitochondrial gene Microsatellite locus	Primer name	Primer sequence (5′–3′)	Ref.
I. Identification by multiplex PCR	*cox*1	F: MulLat3	GGGGTGTTACGGGTATTATACTC	1
		F: MulDen4	GTGTTTTTCATTTGATGATGACCAGTC	1
		F: MulPac2	ACATGTGTGTAGTAACCTTGGC	1
		F: MulNih5	CTTTGTTGTCTGGCCTTCCT	1
		R: MulRevCom	ATGATAAGGGAYAGGRGCYCA	1
II. Determination of mitochondrial	*cox*1	F: MulLat3	GGGGTGTTACGGGTATTATACTC	2
haplotypes		R: MulRevCom	ATGATAAGGGAYAGGRGCYCA	2
	cob	F: F2Dnihcob	GTTTTACTGATAGGTTATTTAAAC	2
		R: R2Dnihcob	CAATTTAAAAAACGAGTTAAAGAT	2
	*nad*3	F: DnND3Fo	CGTTAAGATGAATGAG	3
		R: DnND3Re	GACCTTACATCACTTAGTG	3
III. Determination of allelic variants of	DL_6	F: DL_6	TTAGGAGAGGAATCATGCGG	4
microsatellite loci		R: DL_6	TAGGGAACGGACTTCACTCG	4
	DL_8	F: DL_8	TCGGAAGTAATTCTTGACGCT	4
		R: DL_8	CGTGGAGTACATAGACCGGC	4
	DL_10	F: DL_10	GGGCTATTCGCCTACTACTGC	4
		R: DL_10	TGCCCTGTGCATTCATACTC	4
	DL_36	F: DL_36	TTCTGACACTGTCTCCGACG	4
		R: DL_36	TCTCACAAATTCCCAACATTCA	4
	DL_104	F: DL_104	GCTGCCTTTTGAGGTTGAAG	4
		R: DL_104	AATGCCGCAAGTTTTCTCAC	4
	DL_112	F: DL_112	AACCCACCTTTTCGCATACA	4
		R: DL_112	AGGACAGTTCGGCACATAGG	4

Ref., reference; 1, Wicht *et al.* (2010*b*); 2, Wicht *et al.* (2010*a*); 3, Yera *et al.* (2006); 4, Bazsalovicsová *et al.* (2018).

for errors using Geneious software (version 10.0.5, Biomatters, Auckland, New Zealand). The *cox*1, *cob* and *nad*3 datasets were concatenated using SeaView 4.2 (Gouy *et al.*, 2010).

Statistical analysis of the sequences of the mitochondrial genes

Genealogical information was visualized by a haplotype network using concatenated mitochondrial data in PopArt (Leigh and Bryant, 2015) with the TCS 1.21 algorithm (Clement *et al.*, 2000). Haplotype diversity (Hd), nucleotide diversity (Pi) and neutrality test statistics (Tajima's *D*, Fu and Li's F^* and Ramos-Onsins and Rozas' R_2) were calculated for the concatenated data using DNASP 6 (Rozas *et al.*, 2017). The statistical parameters were calculated independently for (1) the entire dataset, (2) the ALR population and (3) the RU-KR population. The significance of all tests was obtained using 10 000 coalescent simulations.

PCR amplification and fragment analysis of microsatellite loci

Six microsatellite loci, DL_6/$(agg)_n$, DL_8/$(tcc)_n$, DL_10/$(cta)_n$, DL_36/$(ctt)_n$, DL_104/$(tag)_n$ and DL_112/$(ttc)_n$, were previously identified for *D. latus* by screening a microsatellite library (Bazsalovicsová *et al.*, 2018). Primers for their PCR amplification are summarized in Table 2 (see section III). The PCR amplification for the microsatellite assays was performed in a total volume of 20 μL containing 10–20 ng of genomic DNA, 5 pmol of each primer (1 was fluorescently labelled), and 1× HOT FIREPol® Blend Master Mix with 7.5 mM $MgCl_2$ (Solis Biodyne, Tartu, Estonia). The conditions of the PCRs were: 15 min at 95°C, followed by 26 cycles of 15 s at 95°C, 90 s at 60°C, 90 s at 72°C and a final step for 10 min at 72°C.

For the fragment analysis, 1 μL of the amplified PCR product was mixed with 8.5 μL of HiDi formamide and 0.5 μL of GeneScan-LIZ 500 Size Standard (Thermo Fisher Scientific). The mixture was then denatured for 5 min at 95°C and capillary electrophoresis on 3500 Genetic Analyser (Thermo Fisher Scientific) was performed. GeneMapper v.3.7 (Applied Biosystems) was used for genotyping the samples.

Statistical analysis of the microsatellite allelic variants

Genodive 3.0 (Meirmans, 2020) was used for the calculation of basic statistical parameters of genetic diversity (number of alleles, effective number of alleles, observed heterozygosity and heterozygosity within a population), Nei's *D* genetic distance (Nei, 1972) and genotype assignment within populations. A principal coordinates analysis (PCoA) was used for visualizing the pattern of genetic relationships of *D. latus* individuals and populations using GenAlex 6.6 (Peakall and Smouse, 2012). Data for PCoA were exported from Genodive 3.0 and analysed independently in 2 dataset formats. The first format was based on an analysis that included all alleles detected in each individual, including 3 alleles of the triploid individuals, and the analysis of the second format was based on 2 alleles, which were randomly downsampled in the triploid individuals. They were assigned to unique multilocus

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Table 3. Molecular variability and tests of neutrality for the 3 mtDNA genes of *D. latus*

Population	*N*	Hn	Hd	Pi	Fu and Li's *F**	Tajima's *D*	Ramos-Onsins and Rozas' R_2
Entire dataset	146	12	0.230798	0.00033	−3.31232**	−2.46005***	0.019505*
ALR	128	2	0.015625	0.00005	−4.43106***	−1.9792***	0.0880
RU-KR	18	11	0.908497	0.00218	−1.57926	−1.62181	0.072602**

ALR, Alpine lakes region; RU-KR, Krasnoyarsk reservoir in Russia; *N*, number of samples; Hn, number of haplotypes; Hd, haplotype diversity; Pi, nucleotide diversity.
*$P < 0.05$; **$P < 0.02$; ***$P < 0.001$.

genotypes (genets) per population using the Meirmans and Van Tienderen's (2004) algorithm, which tested the probability of finding the observed genotype assignment under random mating. A threshold distance of 3 was chosen based on the 'valley' between the first and second peaks in a histogram of pairwise distances. Samples from CH-BI, CH-NE and CH-F were not included in the analyses of Nei's *D* genetic distance and genotype diversity due to the small number of samples. Statistical support for the pairwise Nei's *D* values was obtained by 10 000 permutations of the data.

Results

Structure and number of mitochondrial haplotypes

Partial *cox*1 (426 bp), complete *cob* (1107 bp) and complete *nad*3 (357 bp) were sequenced for 146 *D. latus* specimens from all localities; their accession numbers are provided in Supplementary material Table 1. We detected totals of 3 *cox*1 (CO1-Ha), 11 *cob* (COB-Ha) and 3 *nad*3 (ND3-Ha) haplotypes. Of the 3 polymorphic sites (all transitions) detected in *cox*1, two were responsible for a change in amino acid sequence. Twenty-two substitutions (transitions/transversions ratio of 21/1) were identified for the *cob* gene, 9 of which changed the amino acid sequence. The analysis of the *nad*3 sequences identified 2 transitions, one of which caused a change in amino acid. Concatenation of the data resulted in 12 haplotypes (con-Ha) (Supplementary material Table 2). The concatenated dataset contained 25 variable characters, 13 of which were parsimoniously informative.

All plerocercoids from fish from lakes Iseo, Como, Maggiore, Geneva, Biel and Neuchâtel, 1 plerocercoid from fish from RU-KR (RU-KR/6) and adult tapeworms from all humans except for 1 and from the fox from Switzerland possessed identical sequence structures of the *cox*1, *cob* and *nad*3 genes, resulting in a common concatenated con-Ha1 haplotype (total of 128 individuals) (Supplementary material Table 1; Fig. 2A). One adult tapeworm isolated from a human patient from Switzerland (CH-H/12) possessed a specific COB-Ha2, which resulted in a unique con-Ha2.

Intrapopulation polymorphism was substantially higher in the population of *D. latus* from RU-KR. Three *cox*1 haplotypes (CO1-Ha1–Ha3), 10 *cob* haplotypes (COB-Ha1, COB-Ha3–Ha11) and 3 *nad*3 haplotypes (ND3-Ha1–Ha3) were detected in 18 *D. latus* individuals. They resulted in 11 concatenated haplotypes, particularly con-Ha1 (detected only in RU-KR/6) specific also for tapeworms from ALR, and con-Ha3–Ha12 (Supplementary material Tables 1 and 2) unique to the population from RU-KR (Fig. 2A).

Haplotype network

The haplotype network based on the concatenated dataset of the *cox*1, *cob* and *nad*3 sequences showed differences between the ALR and RU-KR populations (Fig. 2B). The analysis found a star-like pattern, with 1 dominant central haplotype (con-Ha1) shared by the majority of the *D. latus* samples ($n = 128$; for details see above). In contrast to ALR, notable genetic diversity in the *D. latus* population from RU-KR was demonstrated by a higher number of concatenated haplotypes. Of 11 haplotypes, 6 unique haplotypes from RU-KR were separated from the central haplotype by 1 or 2 mutations (con-Ha3, 4, 6, 8, 9 and 11). Haplotype con-Ha10 from RU-KR was placed on the mutation pathway with a specific haplotype detected in 1 adult tapeworm from a human infection (con-Ha2; CH-H/12) from Switzerland. Three additional concatenated haplotypes (con-Ha5, 7 and 12) from RU-KR differed from the central haplotype by 3–7 mutations.

Characterization of the populations of *D. latus* from ALR and RU-KR performed using DNASP produced similar results. The ALR population had fewer haplotypes and lower haplotype (Hd) and nucleotide (Pi) diversities compared to the RU-KR population (Table 3).

The neutrality tests, Tajima's *D* and Fu and Li's *F**, which were applied to indicate past demographic events or other processes, found significant negative values in the ALR population and the entire dataset. Ramos-Onsins and Rozas' R_2 test value was significantly low for the entire dataset. Negative values of the neutrality tests, indicating an excess of rare nucleotide-site variants under the neutral model of evolution, are usually interpreted as a sign of population expansion after a bottleneck or a selective sweep. The lower degree of mitochondrial polymorphism, however, may be a result of either purifying or adaptive natural selection. Mitochondrial DNA acts as a single locus due to the lack of recombination, so selection anywhere in mtDNA would exactly mimic a demographic bottleneck/expansion (Bazin *et al.*, 2006). However, the results of statistical tests of neutrality can provide only hypothetical explanations of the past events responsible for the genetic structure of the population.

Tajima's *D* and Fu and Li's *F** tests identified negative but non-significant values in the RU-KR population, whereas the value of Ramos-Onsins and Rozas' R_2 test was significantly low (Table 3). These results suggested that the RU-KR population may also have experienced a sudden expansion in its recent evolutionary history.

Variations in allelic numbers of microsatellite loci

A standard number of 1 (homozygous) or 2 (heterozygous) alleles per microsatellite locus was detected in all plerocercoids from fish from RU-KR and in 2 *D. latus* adults (CH-H/1, CH-H/12) obtained from the human patients from Switzerland (Supplementary material Table 1). This type of microsatellite pattern was assigned to allelic type A1-2 and is typical for a diploid organism. Higher percentages (65–90%) of heterozygous forms were observed in all loci except for one (DL_6) (Table 4).

Fragment analysis of *D. latus* from fish from ALR, from the fox, and from the majority of the humans from Switzerland identified 1, 2 or 3 peaks corresponding to 1, 2 or 3 alleles, respectively. This allelic type of microsatellite pattern was assigned to A1-3 and corresponded to a triploid organism. The majority of loci (DL_10, DL_36, DL_104 and DL_112) had 1 to 3 alleles, with a notably lower percentage (0.79–8.73%) of variants of 1 allele (homozygous) (Table 4). Locus DL_6 completely lacked the 1-allele variant, whereas heterozygous variants of 3 alleles were

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Table 4. Numbers of alleles (A) present in sampled individuals for each microsatellite locus with the percentages (%) and number of individuals (no. of ind.) with 1 allele (1A), 2 alleles (2A) and 3 alleles (3A)

Microsatellite locus	Numbers of A per individual	% and (no. of ind.) with 1A	% and (no. of ind.) with 2A	% and (no. of ind.) with 3A
Allelic type A1-2 (20 individuals)				
DL_6	1 or 2	65% (13)	35% (7)	–
DL_8	1 or 2	35% (7)	65% (13)	–
DL_10	1 or 2	10% (2)	90% (18)	–
DL_36	1 or 2	20% (4)	80% (16)	–
DL_104	1 or 2	15% (3)	85% (17)	–
DL_112	1 or 2	10% (2)	90% (18)	–
Allelic type A1-3 (126 individuals)				
DL_6	2 or 3	–	3.96% (5)	96.04% (121)
DL_8	1 or 2	0.79% (1)	99.21% (125)	–
DL_10	1 or 2 or 3	8.73% (11)	69.04% (87)	22.23% (28)
DL_36	1 or 2 or 3	0.79% (1)	1.59% (2)	97.62% (123)
DL_104	1 or 2 or 3	2.38% (3)	76.98% (97)	20.63% (26)
DL_112	1 or 2 or 3	7.94% (10)	80.95% (102)	11.11% (14)

Table 5. Descriptive statistics for the 6 microsatellite loci of *D. latus*

Population	Num	Eff_num	Ho	Hs
IT-IS	4.167	2.581	0.784	0.605
IT-CO	4.000	2.867	0.772	0.634
IT-MA	3.333	2.843	0.729	0.663
CH-GE	2.667	2.593	0.869	0.608
CH-H	4.167	2.686	0.770	0.622
RU-KR	8.333	4.814	0.778	0.783

Num, number of alleles observed; Eff_num, effective number of alleles; Ho, observed heterozygosity; Hs, heterozygosity within populations.
See Table 1 for the population codes.

present in most of the specimens (96.04%). Three alleles were not observed only at the locus DL_8, which had 2 alleles in 99.21% (125 of 126 tapeworms).

Basic statistics of allelic variants of microsatellite loci and genetic diversity

The level of polymorphism was evaluated across the 6 microsatellite loci in 141 individuals from ALR (IT-IS, IT-CO, IT-MA, CH-GE and CH-H) and RU-KR (Table 5). Populations that contained fewer than 4 samples (CH-BI, CH-NE and CH-F) were excluded from the descriptive statistics, although a correction for the bias from sampling a limited number of individuals per population was included in some tests.

The total number of observed (Num) and the effective number of alleles (Eff_num) differed between the populations from ALR and RU-KR (Table 5). The number of alleles per population was highest in *D. latus* from RU-KR (8.333), and fewer alleles were generally detected in ALR, with the fewest in larvae from lake Geneva (CH-GE, 2.667). Similarly, Eff_num was highest in *D. latus* from RU-KR (4.814) and lower in the ALR populations (2.581–2.867).

Observed heterozygosity (0.729–0.869) was similar in the entire dataset, indicating more heterozygotes in both populations. The heterozygosity within populations (Hs, gene diversity), however, was highest in RU-KR (0.783) (Table 5). The results indicated similar Hs values and close genetic relationships amongst *D. latus* from ALR, but the population from RU-KR was genetically diverse.

Genetic relatedness/dissimilarity and genotype diversity amongst populations

Genetic distance amongst the populations was determined using Nei's *D* matrix of genetic distances, which included a bias correction for small sample sizes. The populations from ALR were genetically very similar. The RU-KR population was genetically the most distant based on the means of Nei's *D* (mean distance = 0.17, Table 6), consistent with the geographic distance between the RU-KR and ALR populations.

Genotype assignment analysis identified 51 genets in the entire dataset. In the ALR population, 123 individuals comprised of only 36 genets, substantially less than expected under random mating. In contrast, the RU-KR population had equal numbers of observed and expected genets (Table 7).

PCoA of microsatellites

The results of the PCoA were in agreement with the descriptive statistics but identified a more detailed pattern of relationships amongst the populations. Slightly different results were obtained with the 2 formats of input data applied for the PCoA.

The results of the first dataset, which accepted all alleles detected in each individual, including the 3 alleles present in triploids in the input data, identified 2 clusters of *D. latus* individuals collected from all populations. The first cluster was formed by tapeworms from RU-KR and 2 specimens from the human infections from Switzerland (CH-H/1 and CH-H/12) (Fig. 3A). These data imply that the 2 patients from Switzerland probably acquired diphyllobothriosis abroad, although *D. latus*

Table 6. Pairwise population matrix of Nei's *D* genetic distance for the populations

	IT-IS	IT-CO	IT-MA	CH-GE	CH-H	RU-KR	Mean[a]
IT-IS	0						0.08
IT-CO	0.069533	0					0.01
IT-MA	0.062447	0.10313	0				0.10
CH-GE	0.063253	0.085135	0.123431	0			0.10
CH-H	0.03255	0.065513	0.061303	0.043805	0		0.07
RU-KR	0.190452	0.178098	0.141137	0.172813	0.156858	0	0.17

[a]Mean distance of a population was determined by dividing the sum of all pairwise distances of a particular population by 6, which is the number of populations paired with the concerned population.
All pairwise values were statistically significant at $P < 0.001$.
See Table 1 for the population codes.

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Table 7. Number of observed (Num_obs) and expected (Num_exp) genotypes assigned to specific populations

Population	Num_obs	Num_exp	*P*
Entire dataset	51.0	139.8	0.000
IT-IS	10.0	42.7	0.000
IT-CO	15.0	46.9	0.000
IT-MA	5.0	8.0	0.000
CH-GE	2.0	11.0	0.000
CH-H	4.0	13.7	0.000
RU-KR	18.0	18.0	1.000

P, low value rejects the null hypothesis that expected and observed values are equal. See Table 1 for the population codes.

was circulating in the aquatic environment of the Swiss lakes. The second cluster was formed by *D. latus* from the Swiss and Italian lakes (IT-IS, IT-MA, IT-CO, CH-GE, CH-BI and CH-NE) and by the majority of adult tapeworms obtained from the humans (CH-H) and fox from Switzerland (CH-F). The tapeworms from ALR did not form separate clusters; in fact, several individuals from Italy overlapped with plerocercoids and the adults from Switzerland. Note that many individuals from ALR are not visible in Fig. 3A as separate points due to the presence of identical multilocus genotypes across all loci.

The PCoA based on the populations (Fig. 3B) supported the distinct position of the genetically diverse population from RU-KR. In contrast, the populations from ALR clustered differently than the individuals (Fig. 3A). Populations of *D. latus* from 3 lakes in Switzerland (CH-NE, CH-GE and CH-BI) formed a separate cluster, and adult tapeworms isolated from the humans and fox from Switzerland (CH-H and CH-F) were genetically related to the Italian samples.

The second dataset, based on 2 alleles randomly downsampled from the 3 alleles in the triploid specimens, provided slightly different results. At the larger scale, the PCoA pattern for individuals supported the distant geographic origin of *D. latus* from RU-KR and indicated a genetic structure similar to the samples from ALR (Fig. 3C and D). Again, 2 samples from the human infections from Switzerland had affinity to the individuals from Russia (Fig. 3C). The downsampled data at a more detailed scale, however, provided less-informative results on the clustering of individuals and populations (Fig. 3C and D). *Dibothriocephalus latus* from different subalpine lakes and hosts overlapped and clustered in adjoining assemblages.

Discussion

A polyploid (triploid/aneuploid) population of *D. latus* from ALR was analysed and its population genetics was compared with a geographically distant population from Siberia in Russia using mitochondrial and microsatellite DNA. Both mtDNA and microsatellite data differed greatly between the allopatric populations and were associated with the differences in ploidy, mode of reproduction, the historical colonization of the Alpine region and the geographic distance. The data and factors contributing to the reconstructed population genetic patterns are discussed below.

Microsatellite allelic patterns and chromosomal ploidy

Microsatellites in tapeworms have been predominantly applied in studies of diploids, such as the caryophyllidean *Caryophyllaeus*

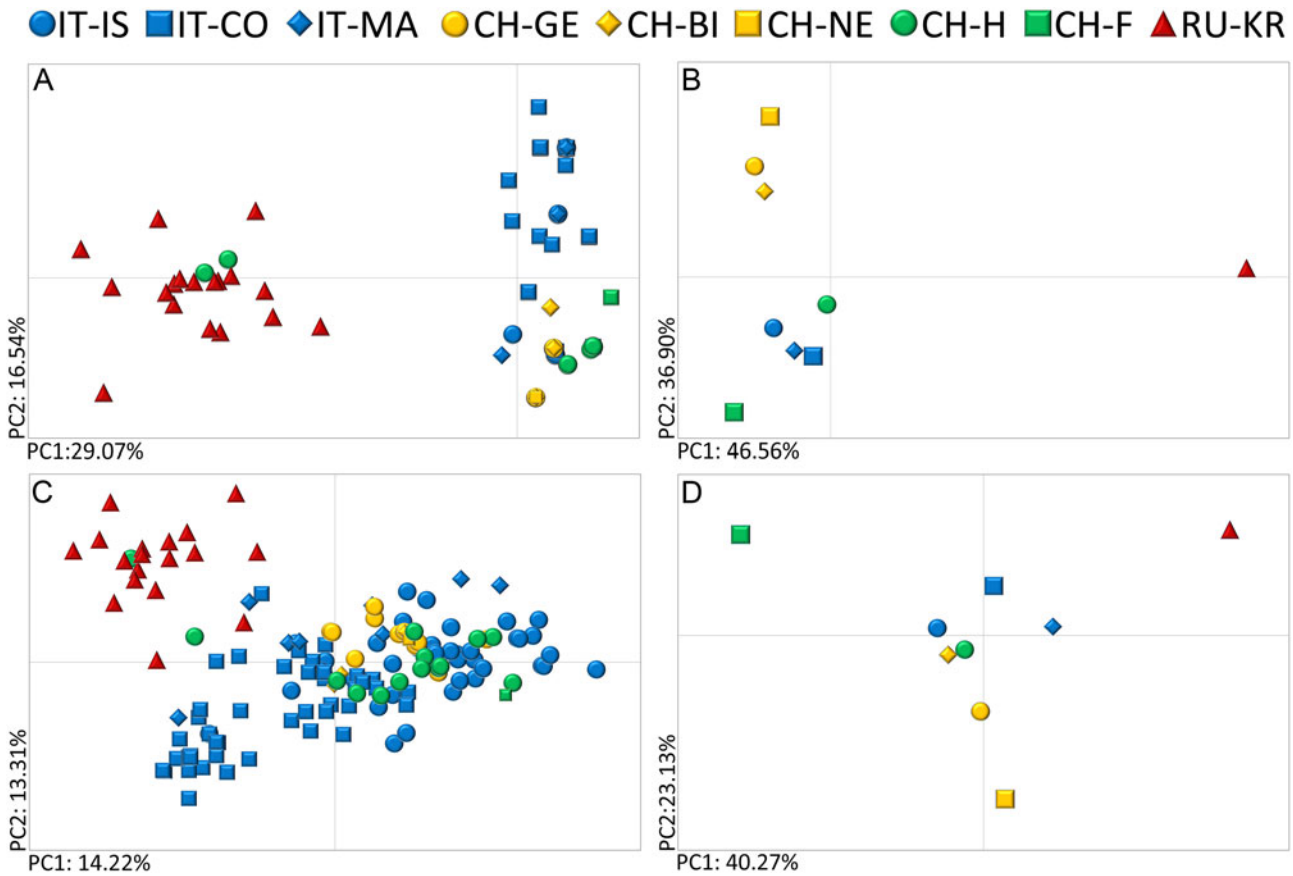

Fig. 3. Population genetic structure of *D. latus* individuals (A, C) and populations (B, D) from the ALR and the RU-KR, derived from the PCoA based on the triploid dataset (A, B) and a randomly downsampled dataset of diploids (C, D). See Table 1 for the population codes.

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laticeps ($2n = 20$; Bombarová and Špakulová, 2015; Králová-Hromadová *et al.*, 2015; Bazsalovicsová *et al.*, 2020) and the diphyllobothriideans *Ligula intestinalis* ($2n = 18$; Petkevičiūtė and Kuperman, 1992; Štefka *et al.*, 2007, 2009) and *D. dendriticus* ($2n = 18$; Wikgren and Gustafsson, 1965; Bazsalovicsová *et al.*, 2020). Microsatellites have rarely been used in genetic studies of polyploid organisms. In platyhelminths, STR markers were applied to study the triploid liver flukes of the genus *Fasciola* (Trematoda; Fasciolidae) from Japan, in which 3 alleles were present in 1 of 12 STR loci in the majority of the flukes (Ohari *et al.*, 2021*a*). Identical genetic types of all STR loci determined in different generations of the species suggested that aspermic triploid *Fasciola* reproduce clonally by parthenogenesis, and possibly gynogenesis (Ohari *et al.*, 2021*b*).

Our study is the first to use STR markers in triploid tapeworms. The results of the microsatellite analysis of the ALR population were in accordance with the previously detected triploidy of *D. latus* from Iseo lake (Orosová *et al.*, 2021); 1 to 2 alleles were observed in 5 of 6 loci analysed in individuals from ALR. Three alleles detected at a particular locus represented a fully heterozygous form and were recognized as 3 distinct peaks in a fragment analysis. The third allele missing in the variant of 2 alleles (2A), or 2 alleles missing in the variant of 1 allele (A1), can be explained by the presence of homozygous forms of allele/alleles within the particular locus, which could not be detected as an individual peak during the fragment analysis.

In contrast to ALR, the standard pattern of 1 and 2 alleles (allelic type A1-2) was detected in all 6 loci in *D. latus* from fish from RU-KR. The tapeworms from RU-KR have not yet been studied cytogenetically, but a diploid set of chromosomes ($2n = 18$) would be anticipated. Studies of the basic karyological characteristics and chromosomal architecture of *D. latus* from RU-KR are needed to verify this assumption.

The allelic type A1-2 was also found in 2 adult *D. latus* specimens obtained from humans from Switzerland. Detailed medical and travel history and knowledge of the eating habits of patients are necessary for identifying the origin and exact source of infections. Such information was not available to us, but the genetic structure of the tapeworms from the 2 patients from Switzerland implies an allochthonous (imported) infection. One tapeworm with allelic type A1-2 was characterized by the most frequent con-Ha1, typical for ALR, and 1 sample from RU-KR. The second tapeworm with A1-2 possessed a unique con-Ha2, which was placed on the same mutation pathway with the haplotypes specific for RU-KR. These 2 patients were probably not infected by the consumption of raw fish from the local aquatic resources of subalpine lakes but acquired diphyllobothriosis abroad.

All other patients from Switzerland were infected with tapeworms whose genetic structure (allelic type A1-3 and haplotype con-Ha1) was identical to the plerocercoids isolated from fish from the subalpine lakes. This type of infection was evidently local (autochthonous).

Triploidy and parthenogenesis

Chromosome numbers and levels of ploidy in platyhelminths can vary considerably within species and even within individuals (Grey and Mackiewicz, 1980; Petkevičiūtė, 1996; Pongratz *et al.*, 2003). Hermaphroditic platyhelminths have versatile mechanisms of reproduction; populations with high chromosome numbers tend to reproduce asexually, whereas those possessing low and even ploidy levels (di-, tetra- or hexaploid) reproduce sexually (Lorch *et al.*, 2016).

Polyploidy is frequently associated with parthenogenesis (Otto and Whitton, 2000), which can be apomictic (ameiotic), automictic (meiotic) and generative (haploid) (Whitfield and Evans, 1983). Polyploidy combined with parthenogenesis offers advantages for plasticity and the evolution of broad ecological tolerance in species (Lorch *et al.*, 2016).

Until recently, the cytogenetic studies on *D. latus* were rather scarce. The first data on unusual features of spermatogenesis were documented in the adult tapeworms from Russia and Finland (von Bonsdorff and Telkkä, 1965; Levron *et al.*, 2006). The latest study on chromosomes and meiotic division of *D. latus* from the ALR was carried out by Orosová *et al.* (2021) who found some stages of spermatocyte division in the middle part of the strobila. The early stages of developing spermatozoa were also found but fully developed spermatozoa were missing. The nuclear divisions during oogenesis were exclusively mitotic and meiotic figures were observed only very sporadically. Spermatogenesis was abnormal and meiotic figures occurred only very sporadically in the part of strobila which often contained proglottids full of eggs. No mature functional spermatozoa were detected in *D. latus* from Iseo lake, so parthenogenesis was likely the only possible method of reproduction (Orosová *et al.*, 2021).

The currently identified allelic structure of the STR loci corresponded to the specific chromosomal structure, abnormal meiosis and asexual reproduction of *D. latus* from Iseo lake. First, a low number of observed and effective alleles was detected in tapeworms from all subalpine lakes. Next, the variant of 3 alleles of a particular locus was constant in the vast majority of individuals. In particular, locus DL_6 was displayed by multilocus genetic type 106/109/112 in 96.04% (121 samples) of the individuals from ALR, and no other combination of 3 alleles was observed. Identical genetic types of the STR loci were detected in all loci and the majority of individuals, indicating that the aspermic triploid *D. latus* from ALR reproduce asexually.

Triploid parthenogens (ALR) vs diploid sexuals (RU-KR)

Parthenogens have better colonizing capacities and wider geographic distributions because they can colonize areas where sexuals have difficulties establishing a population (Lorch *et al.*, 2016). Ancestral diploid/sexual populations dominate in stable habitats and are in the centre of the distribution of the species, while parthenogens are at the margins of the distributions (Pongratz *et al.*, 2003).

The latest results on the chromosomal architecture of *D. latus* from ALR (Orosová *et al.*, 2021) and the allelic structure of the STR loci (current results) provided the first and preliminary indication that the diploid/sexually reproducing population from RU-KR was ancestral, located within the centre of the distribution of the species, but the triploid/parthenogenetically reproducing subalpine population seems to be at the margin of the distribution. However, we have to be careful when interpreting the results, since the number of plerocercoids from RU-KR was rather low ($n = 18$) in comparison with number of plerocercoids from ALR ($n = 113$). Nevertheless, the outputs based on the microsatellite data were also well supported by the results on the mtDNA of *D. latus* from RU-KR (11 haplotypes/18 plerocercoids), in strong contrast to the mitochondrial structure of the tapeworms from ALR (1 haplotype/113 plerocercoids).

The mitochondrial diversity displayed by the 11 haplotypes in 18 larvae from RU-KR implied an original and relatively stable population, with some previous changes in the past in population size. In contrast, the identical structure of the mitochondrial haplotypes of all *D. latus* specimens from ALR indicated relatively 'recent' population expansion, supported by the tests of neutrality. Such high homogeneity is a consequence of a bottleneck effect typical for the recent (possibly post-glacial) introduction of the tapeworm to the area, followed by an expansion in range with limited gene flow. The high homogeneity may also be due to a

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reduction of mitochondrial polymorphism by natural selection and an increased effect of drift in the population of parthenogens.

The 'recent' introduction of the Alpine population of *D. latus* has to be perceived in a broader historical context. The long co-existence of broad fish tapeworm with humans in western Europe was evidenced by palaeo-parasitological analyses of coprolites, cesspit material and soil sediments, which showed its presence in Europe at least since the Bronze age (Gonçalves *et al.*, 2003; Flammer *et al.*, 2018; Ledger *et al.*, 2019). In Switzerland, the palaeo-parasitological study documented the presence of diphyllobothriosis caused by *D. latus* in the lakeside of Lake Chalain since about 3000 BC (Dommelier *et al.*, 1998). The first recognizable description of *D. latus* in the ALR was reported more than 430 years ago by the 2 Swiss physicians (Thaddeus Dunus in Lucarno and Gaspard Wolphius in Zürich) who published data on human diphyllobothriosis at least since 1592 (Grove, 1990).

In Eurasia, the last glacial period resulted in the modification of river systems and creation of millions of lakes as the glacier retreated (Pongratz *et al.*, 2003). Fish parasites moved along with their fish hosts from Siberia to Europe during interglacial period (11 700 years ago until now) (Rumyantsev, 1984). Introduction of *D. latus* from Siberia to Europe could be linked with the postglacial expansion of European perch, in which specific colonization routes depended on the retreated glacier (Nesbø *et al.*, 1999). As the Alps presented solid ice fields and montane glaciers (Spötl *et al.*, 2021), present-day populations of *D. latus* in this region must be descendants which persisted in the refugia. The dispersal history and introductions of *D. latus* to the distant regions was sustained by large-scale migrations of humans throughout history (Katkar, 2011; Haak *et al.*, 2015).

The detection of identical haplotypes in all plerocercoids from ALR suggests the existence of only 1 population, even though the Italian lakes and lake Geneva (Neuchâtel and Biel lakes are connected by the Thielle channel) represent isolated biotopes with no aquatic connections. Humans, however, represent the most important hosts of *D. latus* and have contributed substantially to the spread and maintenance of diphyllobothriosis in the subalpine environment, providing an efficient means of gene flow between the populations.

Factors maintaining the unique genetic structure of the ALR population

The specific molecular and genetic structure of *D. latus* from ALR has likely been maintained by several biological and environmental factors. In particular, the number of adult tapeworms in the intestine of a definitive host is the crucial biological factor influencing the reproduction of the tapeworm. *Dibothriocephalus latus* in ALR has usually been found as a single plerocercoid in the musculature of the most common second intermediate fish host, the European perch (Gustinelli *et al.*, 2016; Radačovská *et al.*, 2019*a*). Humans acquire diphyllobothriosis by consuming raw or insufficiently cooked fish products, including marinated or smoked fish-harbouring plerocercoids. Infection is thus attributable to particular geographic regions with local dietary habits and traditions of consuming raw fish.

ALR is specific in this aspect, because the consumption of raw fish is more occasional than regular in this part of Europe. The increased risk of diphyllobothriosis for consumers in the subalpine territory is mainly due to the popularity of local food specialities, such as perch carpaccio, and extreme food choices (e.g. raw-food diet) (Wicht, 2008). Humans consequently usually acquire diphyllobothriosis only sporadically by the consumption of infected perch fillets, which mainly harbour only 1 plerocercoid in the musculature, developing into a single adult in the definitive host.

A different situation has been documented in Russia and Estonia, historically amongst the oldest foci of diphyllobothriosis in Europe. Patients harbouring up to 11 tapeworms (Kondrateva, 1961; Rossolovskaya, 1968), or even as many as 106 *D. latus* specimens (Grant, 1930), were diagnosed in these countries. A high intensity of infection can be explained by the historic eating habits of consuming raw fillets of fish and/or pike caviar on a regular basis. Pike is a paratenic host of *D. latus* that may harbour numerous plerocercoids in the musculature, but also on internal organs, including ovaries (for a review, see Králová-Hromadová *et al.*, 2021). Consumption of infected pike caviar may thus lead to multiple *D. latus* infections, which is a good prerequisite of sexual reproduction of tapeworms. Indeed, the statistical analysis of STR loci with 1 or 2 alleles implied a sexually reproducing panmictic population in RU-KR. Sexual reproduction was also supported by the analysis of genotype diversity with a random segregation of alleles.

Ecological stress may be another factor contributing to the specific genetic composition of the ALR population. The probability of errors during meiosis and mitosis can increase under severe environmental conditions (Lorch *et al.*, 2016). The subalpine lakes belong to anthropized water systems affected by intensive industrial, agricultural and urban activities (Lang, 1989; Anneville *et al.*, 2007; Manca *et al.*, 2007; Menconi *et al.*, 2021). It could contribute to spontaneous polyploidization and aneuploidy and to changes in reproductive strategy, which are common in harsh environments and bottlenecked populations (Lorch *et al.*, 2016).

Restricted distribution in Europe vs ancestral range

ALR and RU-KR represent only a fragment of the Eurasian distribution of *D. latus*. The logical questions would be, what is the genetic make-up of other populations of the tapeworm and where is the boundary between 'ancestral diploids' and 'recent triploids'? These questions, no matter how interesting they are, are very difficult to answer, because the broad fish tapeworm represents a very specific human parasite in its past and present occurrence.

Several foci of diphyllobothriosis have developed in Europe: (1) Fennoscandia, (2) the Baltic region, (3) ALR, (4) the Danube river region and (5) Russia (for a review, see Králová-Hromadová *et al.*, 2021). Large-scale monitoring, massive health campaigns, implementation of preventive measures and effective treatment, however, led to a substantial decrease in the numbers of infections in Fennoscandia, the Baltic region and the Danube river region in the last decades of the 20th century. Consequently, collecting *D. latus* from its historically important endemic regions is rather difficult. Indeed, all our attempts to enlarge the *D. latus* dataset for the current study were unsuccessful. We have genotyped adult diphyllobothriid tapeworms from a grey wolf (*Canis lupus*) and a Eurasian lynx (*Lynx lynx*) from Latvia (Bagrade *et al.*, 2021) and from lynx from Finland (Čisovská Bazsalovicsová *et al.*, 2022). All tapeworms from both regions, however, were another diphyllobothriid, *Spirometra erinaceieuropaei*, the causative agent of sparganosis. The broad fish tapeworm was described from the further Baltic country, Poland, where eggs of *D. latus* were morphologically detected from European otter (*Lutra lutra*), wolf and lynx from Białowieża Primeval forest (Górski *et al.*, 2006, 2010; Szczęsna *et al.*, 2008) and *D. latus* plerocercoids were found in European perch from Pomeranian Bay in the southwestern part of the Baltic Sea (Bielat *et al.*, 2015). *Dibothriocephalus latus*, however, was not found during our ichthyoparasitological

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examinations of perch and pike from Pomeranian Bay and Białowieża Primeval forest (Radačovská *et al.*, 2019*c*). The eggs detected in otter, lynx and wolf (Górski *et al.*, 2006, 2010; Szczęsna *et al.*, 2008) likely belonged to the recently intensively studied *S. erinaceieuropaei* in northeastern Poland (Kondzior *et al.*, 2020). Finally, the examination of 700 European perch from the Slovak part of the Danube river also did not reveal any *D. latus* (Radačovská *et al.*, 2019*b*).

The only comparable molecular and genetic data on *D. latus* from localities other than ALR and RU-KR are from Finland. The number of chromosomes (15, 16, 17, 18, 20, 24 and 28) in somatic cells, with 18 chromosomes the most frequent, varied considerably in *D. latus* from Finland (Wikgren and Gustafsson, 1965). These authors concluded that *D. latus* was a diploid ($2n = 18$) species with a relatively large variation in the number of chromosomes. We analysed STR loci and mitochondrial haplotypes in 2 adult *D. latus* obtained from patients from Finland without any details on travel history and the origin of the samples (data not shown). The allelic type A1-3, specific for *D. latus* from ALR, was observed in both tapeworms. One specimen from Finland was in addition characterized by the mitochondrial concatenated haplotype con-Ha1, thus having the identical genetic structure with the tapeworms from ALR. The patient either acquired diphyllobothriosis in the Alpine region, or the infection was local indicating common genetic structure of populations from Finland and the Alpine lakes. The second tapeworm possessed unique con-Ha13, which was either specific for Finland, or introduced from abroad. The allelic type A1-3 of the tapeworms from Finland provided very preliminary evidence that triploidy and the specific genetic structure of *D. latus* may be a feature characteristic for more European populations of the parasite.

Many European populations of *D. latus* are either extinct or extremely difficult to sample, so the continuous geographic lineage of *D. latus* cannot be examined. The only promising territory for further collection and a detailed investigation of *D. latus* is Russia, where the tapeworms have been found in several European and Asian regions (Rumyantsev, 2007; Dugarov and Pronin, 2017; Chugunova *et al.*, 2020).

The unique genetic structure of *D. latus* from the subalpine territory indicated a successful adaptation of the parasite introduced to novel and extreme habitats, which followed a bottleneck induced by colonization. More explicit assessments of genetic diversity, metapopulation dynamics, demographic changes and migratory routes of *D. latus* require additional genetic data based on a larger sample set. Further studies would probably identify a broader genetic diversity of *D. latus* and would hopefully specify the areas of distribution and boundaries between 'ancestral diploids' and 'recent triploids'.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182022000634

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Author contributions. A. R., E. Č. B. and I. K.-H. conceived the study, performed molecular analyses and wrote the manuscript. A. R., E. Č. B., I. K.-H., A. G. and J. K. C. collected the parasitic material. K. Š. and G. M. performed fragment analysis and sequencing. J. Š., A. R. and E. Č. B. performed statistical analyses. All authors discussed the results, revised the manuscript and approved its final version.

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Ethical standards. There is no ethical issue involved in this study.

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