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Mitochondrial genetic diversity and phylogenetic relationships of *Echinococcus multilocularis* in Europe ☆

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☆ Note: Nucleotide sequence data reported in this paper are available in GenBank™ under the accession numbers **OR058958 — OR058984** and **OR060928 — OR060942**.

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ABSTRACT

The cestode *Echinococcus multilocularis* is the causative agent of alveolar echinococcosis, a fatal zoonotic parasitic disease of the northern hemisphere. Red foxes are the main reservoir hosts and, likely, the main drivers of the geographic spread of the disease in Europe. Knowledge of genetic relationships among *E. multilocularis* isolates at a European scale is key to understanding the dispersal characteristics of *E. multilocularis*. Hence, the present study aimed to describe the genetic diversity of *E. multilocularis* isolates obtained from different host species in 19 European countries. Based on the analysis of complete nucleotide sequences of the *cob*, *atp6*, *nad2*, *nad1* and *cox1* mitochondrial genes (4,968 bp), 43 haplotypes were inferred. Four haplotypes represented 62.56 % of the examined isolates (142/227), and one of these four haplotypes was found in each country investigated, except Svalbard, Norway. While the haplotypes from Svalbard were markedly different from all the others, mainland Europe appeared to be dominated by two main clusters, represented by most western, central and eastern European countries, and the Baltic countries and northeastern Poland, respectively. Moreover, one Asian-like haplotype was identified in Latvia and northeastern Poland. To better elucidate the presence of Asian genetic variants of *E. multilocularis* in Europe, and to obtain a more comprehensive Europe-wide coverage, further studies, including samples from endemic regions not investigated in the present study, especially some eastern European countries, are needed. Further, the present work proposes historical causes that may have contributed to shaping the current genetic variability of *E. multilocularis* in Europe.

1. Introduction

Echinococcus multilocularis is a cyclophyllid tapeworm (Taeniidae, Cestoda) distributed in the northern hemisphere and causing alveolar echinococcosis (AE) in humans and other mammals. Humans are dead-end hosts and develop AE after the ingestion of viable parasite eggs. Human AE is a malignant, chronically evolving condition caused by the progressive and infiltrative development of *E. multilocularis* parasitic metacestode vesicles into a tumour-like intrahepatic parasitic mass with possible metastases (spread and proliferation of secondary lesions) within extra-hepatic organs.

An average of 141 newly diagnosed human AE cases has been reported annually between 2016 and 2020 by the European Centre for Disease Prevention and Control (ECDC) (ECDC, 2022). This number is likely to be an underestimation, since AE is often misdiagnosed and underreported by national health systems. Moreover, the ECDC reports lack representative data from countries such as Croatia, the Czech Republic, Hungary, and Slovenia, where the presence of human AE is well documented (Logar et al., 2007; Kolářová et al., 2015; Dušek et al., 2020; Dezsényi et al., 2021).

The adult stage of *E. multilocularis* dwells in the small intestine of the definitive hosts (mainly red foxes), which contaminate the environment by spreading infective eggs with their faeces. Among several wild carnivores that are competent definitive hosts of *E. multilocularis* (raccoon dog, golden jackal, wolf, Arctic fox) (Kapel et al., 2006; Fuglei et al., 2008; Lalošević et al., 2016; Deplazes et al., 2017), the red fox is acknowledged as the main reservoir in temperate Europe (Oksanen et al., 2016). Due to its large geographic distribution, adaptability to various food sources and the seasonal dispersal patterns ranging up to 300 km (Oksanen et al., 2016; Zecchin et al., 2019), the red fox may sustain, in many endemic areas, the life cycle of the parasite. Diversely, in the Arctic regions of Europe, the Arctic fox is the definitive host for *E. multilocularis* (Henttonen et al., 2001; Fuglei et al., 2008). In Europe, several Arvicolinae species sustain the sylvatic cycle of *E. multilocularis* (Oksanen et al., 2016; Romig et al., 2017). Regarding the infection in the definitive host, *E. multilocularis* in Europe is found from the High Arctic Svalbard archipelago (Norway) to southern Croatia (Henttonen et al., 2001; Beck et al., 2018). Since the 2000 s, the highest prevalence rates of infections in red foxes in Europe have been found in the Netherlands (59 % in the Limburg area, Maas et al., 2014), in Poland (50 % in the Warmińsko-Mazurskie province, Karamon et al., 2014), southern Belgium (33.1 %, Losson et al., 2003), Germany, Slovakia and the Baltic countries (from 24.5 % to 58 %, pooled prevalence rates, Oksanen et al., 2016) and Switzerland (41.4 % in the Zurich area, Otero-Abad et al., 2017). It should be noted that a few European countries, namely Finland, mainland Norway, Malta, the United Kingdom and Ireland, have no reports of *E. multilocularis* in red foxes and have surveillance programs in place to document continued freedom from disease (EFSA, 2022).

Switzerland, Austria, southern Germany and eastern France constituted a historical endemic core area of *E. multilocularis* in Europe, where human AE has been reported since the 19th century (Kern et al., 2003; Knapp, 2015; Eckert and Thompson, 2017). Later, several other central, eastern and Baltic European countries recorded cases of human AE, even if the historical endemic area still contributes to most of the registered cases (Marcinkute et al., 2015; Vuitton et al., 2015; Eckert and Thompson, 2017; ECDC, 2022). In Switzerland, an increase of the incidence of human AE cases was observed from the early 2000 s, after decades of stable endemicity (Schweiger et al., 2007). Similar increasing trends also were reported in countries such as Poland and Lithuania (Nahorski et al., 2013; Šarkūnas et al., 2019; ECDC, 2022). Whether this trend represents a true emergence or is caused by an increased awareness of the disease is difficult to determine (Vuitton et al., 2015). Some attention is now focused on newly endemic countries in the Balkan peninsula such Croatia and Serbia, where positive animal (Lalošević et al., 2016; Beck et al., 2018) and human cases (Dušek et al., 2020; M. Miljević, personal communication) have been reported, and Bosnia-Herzegovina, where positive animal (but no human cases yet) have been detected (Omeragić et al., 2022).

Compared with the *Echinococcus granulosus sensu lato* species complex, *E. multilocularis* is much less genetically diverse, having a 10 times lower nucleotide diversity (Haag et al., 1997) which, therefore, hampers intraspecific differentiation. The identification

of *E. multilocularis* polymorphic markers for typing has been a long-term goal. While nuclear gene markers have not provided satisfactory results (Nakao et al., 2009, 2010), approaches based on mitochondrial genes and autosomal microsatellite markers have been implemented for population studies of *E. multilocularis*.

Based on the polymorphisms of three protein-coding mitochondrial genes (cytochrome b, NADH dehydrogenase subunit 2 and cytochrome c oxidase subunit 1), Nakao and colleagues (2009) analysed 76 isolates from Europe, Asia, and North America. They identified four different geographic clades: Asian, Mongolian, European and North American (Nakao et al., 2009). This typing scheme, using one or three mitochondrial genes, has been adopted also in several other European and international studies (e.g. Gesy and Jenkins, 2015; Karamon, 2017; Šnábel et al., 2019; Alvarez Rojas 2020; Jarošová et al., 2020; Herzig et al., 2021), while other mitochondrial markers (ATP synthase membrane subunit 6, NADH dehydrogenase subunit 1 and 5 and 12S ribosomal RNA gene) tested in Asia and North America (Yang et al., 2005; Gesy et al., 2014; Avcioglu et al., 2016; Li et al., 2018; Heidari et al., 2019) were less studied in the European context.

Based on the microsatellite marker EmsB, a mainland-island model was proposed to explain the genetic flow of *E. multilocularis* between historical European endemic areas and the more recently colonized areas. Similarly, Šnábel and colleagues (2019) investigated 25 isolates from eight European countries with one nuclear and four mitochondrial gene fragments, and confirmed the mainland-island model based on EmsB (Šnábel et al., 2019). Country-level studies using EmsB data or mitochondrial genes have been conducted in Denmark and Sweden (Knapp et al., 2019), Estonia (Laurimaa et al., 2015), France (Umhang et al., 2014), Germany (Herzig et al., 2021), Italy (Casulli et al., 2009), Luxembourg (Martini et al., 2022), Poland (Karamon et al., 2017; Umhang et al., 2017), Russia (Konyaev et al., 2013), Slovakia (Jarošová et al., 2020) and Switzerland (Knapp et al., 2021).

No known additional studies based on mitochondrial markers have yet been attempted to explore the genetic diversity of *E. multilocularis* populations at a wider European scale. In this context, this study aims to fill a gap in knowledge, providing a comprehensive epidemiological picture of the genetic diversity of *E. multilocularis* specimens collected from a high number of European countries, by comparative molecular analysis using five mitochondrial markers. Further, historical causes that may have contributed to shaping the current genetic variability of *E. multilocularis* in Europe are discussed.

2. Materials and methods

2.1. Sampling

From 2019 to 2022, parasitic material (biological tissue or genomic DNA) of different stages (worm, egg or metacestode) of *E. multilocularis* was sent to the European Union Reference Laboratory for Parasites (EURLP, Rome, Italy) from universities, research or health institutes of 18 European countries (Austria, Belgium, Croatia, Denmark, Estonia, France, Germany, Hungary, Italy, Latvia, Lithuania, Luxembourg, Svalbard (Norway), Poland, Serbia, Slovakia, Slovenia and Switzerland). Samples from the Czech Republic and part of the samples from Switzerland were already stored at the EURLP prior to 2019, for a total of 19 European countries included in this study. The parasitic material was collected in the context of national surveys, medical collections, research projects or surveillance programmes, and preserved in ethanol or frozen at −80 °C. The epidemiological unit for this study, hereafter referred to as an isolate, was represented by the individual specimen sampled from a single host. Countries were regarded as populations in the comparative genetic analyses.

2.2. DNA extraction, PCR conditions and Sanger sequencing

Parasite tissues were stored in 70 % or 95 % ethanol before molecular analyses. Prior to DNA extraction, ethanol was removed by centrifugation and the tissue was left to air-dry for 30 min. Genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. A negative control (nuclease-free water) was included in each working session. DNA was stored at −20 °C until use. A multiplex PCR (Trachsel et al., 2007) was used to confirm the identification of the specimens as *E. multilocularis*. In the case of amplification failure, a second DNA extraction was performed after overnight incubation of the tissue sample in PBS to completely remove residual ethanol. Multiplex PCR-positive samples were subsequently amplified at the following five full-length mitochondrial genes: cytochrome b (*cob*, 1,068 bp), ATP synthase membrane subunit 6 (*atp6*, 516 bp), NADH dehydrogenase subunit 2 (*nad2*, 882 bp), NADH dehydrogenase subunit 1 (*nad1*, 894 bp) and cytochrome c oxidase subunit 1 (*cox1*, 1,608 bp) (Table 1).

Fragments were amplified using the following cycling conditions: 95° C/15 min for initial denaturation; 35 cycles of 94° C/30 s, 55° C/30 s, 72° C/1 min; and 72° C/5 min final extension. For each PCR, 2 µl of DNA were added to 28 µl of the reaction mixture containing 15 µl of 2 × PCR Master Mix HotStart (Qiagen GmbH, Hilden, Germany), 15 pmol of each primer and nuclease-free water. In each PCR assay, Internal Reference Material of *E. multilocularis* DNA, as a positive control, and nuclease-free water as a negative control, were included. PCR products were visualized by capillary gel electrophoresis (Qiaxcel, Qiagen GmbH, Hilden, Germany). Purification and Sanger sequencing was performed by GENEWIZ (Leipzig, Germany), using the same primer pairs as the initial PCR. When early termination of the sequencing reaction occurred, an internal forward primer was used for a new sequencing reaction to ensure complete coverage on both strands (Table 1). This was often the case with the *cox1* gene, where the presence of a poly-T region (position 207 → 218) caused a frequent polymerase drop-off.

Forward and reverse sequences were aligned with CLC Main Workbench 20.0.4 to generate a single consensus; mismatches were corrected manually. Multiple alignments of single gene markers and of the concatenated five markers (4,958 bp) were generated. Additionally, to allow comparison with haplotypes from Nakao et al. (2009), a *cob* + *nad2* + *cox1* multiple alignment (3,558 bp) was constructed. All the alignments are accessible at Mendeley Data, https://doi.org/10.17632/vpv7gfpmdj.1.

Sequence data were deposited in GenBank (Accession numbers: **OR060928 - OR060942**; **OR058958 - OR058984**) (Supplementary Table S3).

2.3. Data analysis

The alignments were analyzed using DnaSP v6 software (Rozas et al., 2017) to identify the different haplotypes. Haplotype networks were graphically represented with the software PopART using the TCS inference method (https://popart.otago.ac.nz). Diversity indices such as numbers of segregating sites (S), parsimony-informative sites, singletons, haplotype number (h), haplotype diversity (hd), nucleotide diversity (π), and the neutrality indices of Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997), were calculated with DnaSP v6 for each population and for the whole dataset. To measure the relative abundance of haplotypes in each population, the Simpson's index of diversity was computed (Simpson, 1949).

Table 1
PCR Primer pairs and sequencing primers used in this study.

Primer name	Sequence (5′-3′)	PCR fragment (bp)	Gene	Reference
1.*cob*_for	GTTTAAACTGGTAGATTGTGGTTC	654	*cob*	Nakao et al., 2009
1.*cob*_rev	ACGGATTACTGTTACCACTC			This article
2.*cob*_for	TGATAGTTTGCCGTTGGTTG	845	*cob*	This article
2.*cob*_rev	CTCCACAGTAGAAATCACCATCA			Nakao et al., 2009
*atp6*_for	AAGGTGATTAGTTGTCCGT	808	*atp6*	Li et al., 2018
*atp6*_rev	TGCTAACCTACACAACTCC			Li et al., 2018
*nad2*_for	GCGTTGATTCATTGATACATTGT	1031	*nad2*	Nakao et al., 2009
*nad2*_rev	TAGTAAAGCTCAAACCGAGTTCT			Nakao et al., 2009
*nad1*_for	GAGTTTGCGTCTCGATGATAGG	1126	*nad1*	Li et al., 2018
*nad1*_rev	TCCCCA AAACCCACATTCTAC			Li et al., 2018
1.*cox*1_for	CTGGTTGGTCATCTTATGGA	894	*cox1*	This article
1.*cox*1_rev	ATGATGACCCCAAACACTAC			This article
2.*cox*1_for	ATTTTGATGCGTTTGGGTTT	894	*cox1*	This article
2.*cox*1_rev	TTCACAGCTATACAACCAGG			This article
*nad*1_seqfor	AGGTTATTCTCAGTTTCGTAAGGG	sequencing primer		This article
*nad*2_seqfor	TATCGTCTGTGCTATTAGTCTCTG	sequencing primer		This article
1.*cox*1_seqfor	TTGATGCCTATATTAATTGGCG	sequencing primer		This article

Nucleotide substitutions were categorized into non-synonymous and synonymous. Reference sequences used to reconstruct the haplotype networks were retrieved from GenBank (Supplementary Table S1). For the *cob* and the *nad1* genes, only gene fragments (not full length) of 663 bp and 588 bp, respectively, were used for the comparison, to allow inclusion of the maximum number of reference sequences available. The pairwise genetic distance among the populations was estimated by calculating the fixation index (Fst) with Arlequin 3.5.2 (Excoffier and Lischer, 2010).

3. Results

3.1. Analyzed samples

A total of 386 samples was collected from 19 countries (16 European Union (EU) member states and three non-member states). At least one sample was available from each European country where *E. multilocularis* is considered to be endemic, with the exception of Bosnia and Herzegovina, Belarus, Liechtenstein, the Netherlands, Romania, Russia, Sweden, Turkey and Ukraine (Table 2). In the case of Norway, samples were collected in the Svalbard archipelago that is the only Norwegian territory where *E. multilocularis* is found, while the mainland country currently remains *E. multilocularis*-free. *Echinococcus multilocularis* DNA, worms or eggs were collected from the following definitive hosts: Arctic fox (*Vulpes lagopus*), golden jackal (*Canis aureus*), raccoon dog (*Nyctereutes procyonoides*) red fox (*Vulpes vulpes*) and wolf (*Canis lupus*). *Echinococcus multilocularis* DNA and metacestodes were collected from the following intermediate or accidental hosts: common vole (*Microtus arvalis*), crab-eating macaque (*Macaca fascicularis*), domestic dog (*Canis lupus familiaris*), domestic pig (*Sus scrofa domesticus*), Eurasian beaver (*Castor fiber*), human (*Homo sapiens*), Japanese macaque (*Macaca fuscata*), muskrat (*Ondatra zibethicus*), ring-tailed lemur (*Lemur catta*), wild boar (*Sus scrofa*), western gorilla (*Gorilla gorilla*) and unspecified mouse species (Table 2; Supplementary Table S2).

3.2. Mitochondrial gene sequences

In total, 227 out of 386 collected *E. multilocularis* isolates were fully sequenced for all five genes and a final concatenated alignment of 4,968 bp was produced. The number of sequences obtained for individual genes was 256 for *cob*, 265 for *atp6*, 241 for *nad2*, 249 for *nad1* and 245 for *cox1*. The final *cob* + *nad2* + *cox1* concatenated alignment (*sensu* Nakao) included 227 isolates as well. Table 2 summarizes the isolates included in the final alignment (4,968 bp) per country and host species.

Table 2
Country of origin, animal hosts and number of isolates of *Echinococcus multilocularis* included in the final alignment of the mitochondrial sequences.

Country	Host species	*n* isolates
Austria	7 humans, 4 red foxes, 1 macaque	12
Belgium	5 red foxes	5
Croatia	14 red foxes, 1 human	15
Czech Republic	15 red foxes	15
Denmark	3 red foxes, 1 raccoon dog	4
Estonia	2 red foxes, 1 beaver	3
France	15 red foxes, 1 mouse	16
Germany	8 red foxes, 2 humans, 1 common vole, 1 mouse	12
Hungary	5 red foxes	5
Italy	5 red foxes	5
Latvia	25 red foxes, 3 raccoon dogs, 1 wolf	29
Lithuania	4 red foxes	4
Luxembourg	3 muskrats	3
Svalbard (Norway)	3 Arctic foxes	3
Poland	16 red foxes	16
Serbia	1 red fox	1
Slovakia	15 red foxes, 6 humans	21
Slovenia	13 red foxes, 1 jackal	14
Switzerland	13 dogs, 11 domestic pigs, 7 beavers, 3 humans, 3 lemurs, 3 macaques, 2 gorillas, 1 mouse, 1 wild boar	44
Total	-	227

3.3. Identification and characteristics of the mitochondrial haplotypes

Of 227 isolates, 73 polymorphic sites were identified, 56 of which were parsimony-informative while 17 were singletons (Supplementary Table S4). The highest frequency of substitutions was observed for *cob* (1.69 %, 18/1,068), followed by *atp6* (1.55 %, 8/516), *nad2* (1.47 %, 13/882), *cox1* (1.43 %, 23/1,608) and *nad1* (1.23 %, 11/894). In total, 43 haplotypes were identified. The number of haplotypes decreased to 28 if singletons were not considered. The π was 0.00081 and hd was 0.897, respectively. Results of neutrality tests scored negative (Tajima's D = -2.02902 and Fu's Fs = −22.136). Table 3 summarizes diversity and neutrality indices for each population. Tajima's D values were negative for all populations, with the exception of Austria, Denmark, Italy and Poland, indicating an excess of low-frequency polymorphisms, but none of these was statistically significant.

Table 3
Haplotype analysis performed by DnaSP v.6 software.

Country	*n*	h	hd	π	Tajima's D	Fu's Fs	Haplotype frequency	1-*D*
Austria	12	5	0.788	0.00052	0.46078	0.404	H1(5), H2(2), H3(3), H8(1), H17(1)	0.79
Belgium	5	2	0.400	0.00008	−0.81650	0.090	H2(4), H18(1)	0.40
Croatia	15	7	0.848	0.00036	−1.03115	−2.264	H2(1), H3(5), H8(3), H11(3), H15(1), H19(1), H20(1)	0.85
Czech Rep.	15	11	0.9333	0.00052	−0.90293	−6.551	H2(2), H3(4), H5(1), H8(1), H12(1), H21(1), H22(1), H23(1), H24(1), H25(1), H26(1)	0.93
Denmark	4	2	0.667	0.00013	1.63299	0.540	H2(2), H3(2)	0.67
Estonia	3	2	0.667	0.00013	na	na	H2(1), H4(2)	0.67
France	16	5	0.533	0.00019	−1,19959	−1.535	H2(2), H3(1), H6(11), H13(1), H27(1)	0.53
Germany	12	5	0.818	0.00030	−0.41412	−0.952	H1(1), H2(4), H3(3), H5(3), H12(1)	0.82
Hungary	5	2	0.400	0.00008	−0.81650	0.090	H1(4), H9(1)	0.40
Italy	5	3	0.800	0.00020	0.24314	−0.475	H2(2), H3(2), H12(1)	0.80
Latvia	29	7	0.596	0.00065	−1,69366	1.034	H1(1), H2(5), H4(18), H14(2), H28(1), H29(1), H30(1)	0.60
Lithuania	4	1	0.0000	0.00000	na [a]	na [a]	H4(4)	0.00
Luxembourg	3	3	1	0.00027	na	na	H2(1), H3(1), H31(1)	1.00
Svalbard (Norway)	3	2	0.667	0.00027	na	na	H32(1), H33(2)	0.67
Poland	16	7	0.858	0.00164	0.52191	2.607	H2(2), H4(5), H10(3), H14(3), H34(1), H35(1), H36(1)	0.86
Serbia	1	1	-	-	na	na	H4(1)	na
Slovakia	21	7	0.719	0.00045	−0.00683	−0.787	H1(11), H2(2), H3(2), H10(2), H16(2), H37(1), H38(1)	0.72
Slovenia	14	4	0.396	0.00023	−0.32983	−0.149	H1(11), H2(1), H3(1), H13(1)	0.40
Switzerland	44	13	0.866	0.00050	−0.07871	−3.887	H1(10), H2(3), H3(11), H5(3), H7(6), H9(4), H13(1), H15(1), H39(1), H40(1), H41(1), H42(1), H43(1)	0.87

n, number of isolates analysed; h, number of haplotypes detected; hd, haplotype diversity; π, nucleotide diversity; 1-*D*, complement of the Simpson's Diversity Index; na, test was not applied because the sample size was less than 4.
[a] Index was not computed because no polymorphism was found.

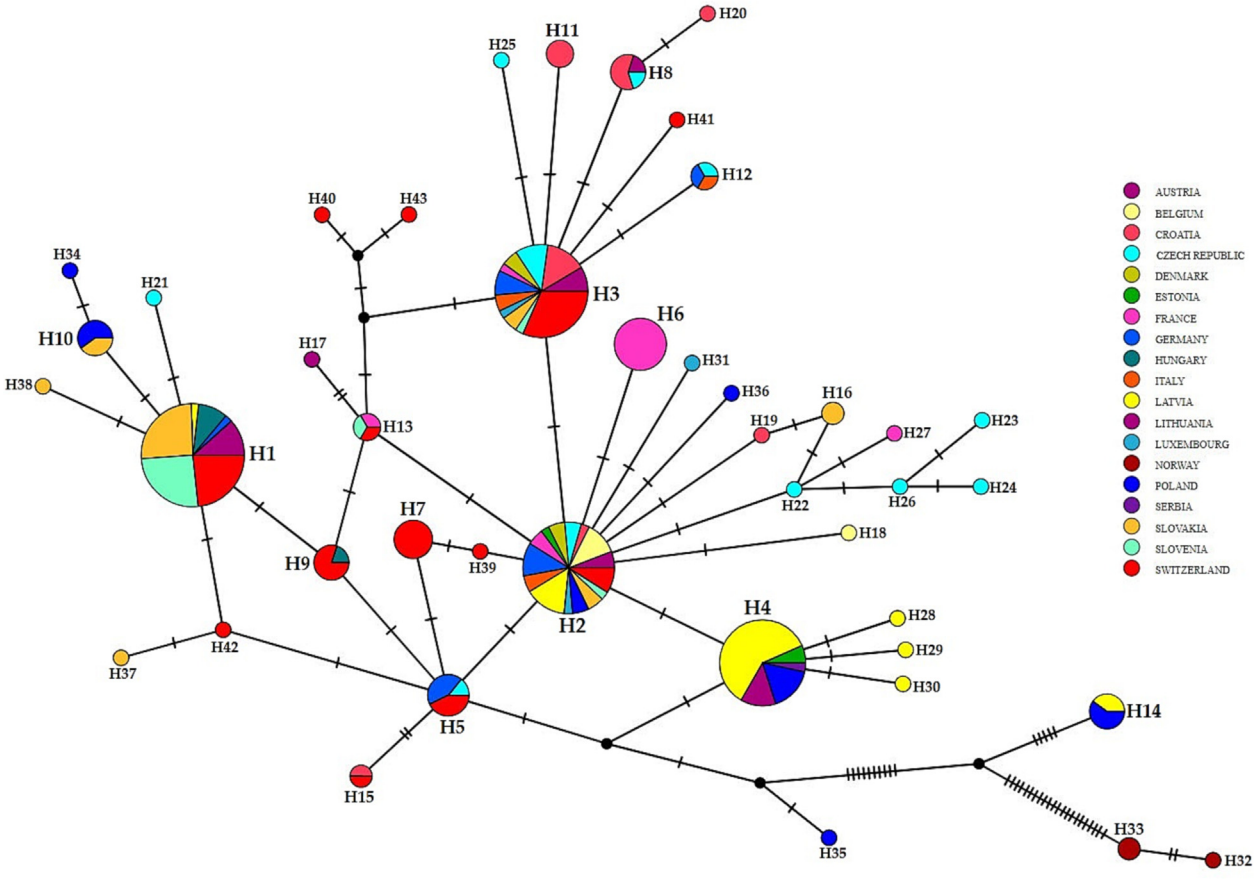


Fig. 1. Haplotype network. TCS network representing the 43 *Echinococcus multilocularis cob + atp6 + nad2 + nad1 + cox1* haplotypes (4,968 bp) detected in this study, obtained from 227 isolates. Each haplotype is represented by a circle, with the area of the circle proportional to its frequency. Hatches depict single mutational steps and small black dots display hypothetical haplotypes. A colour version of this figure is available on-line.

Forty-four of the 73 (60.27 %) nucleotide substitutions were in the third codon position, and 43 of these were synonymous (Supplementary Table S4). Relationships between haplotypes are illustrated in Fig. 1. Twelve haplotypes were shared by two or more populations and 17 haplotypes were shared by at least two isolates. Thirty-one haplotypes were population-specific, 26 of which were identified in a single isolate. The four most common haplotypes (namely H1-H4) accounted for 142 of 227 isolates

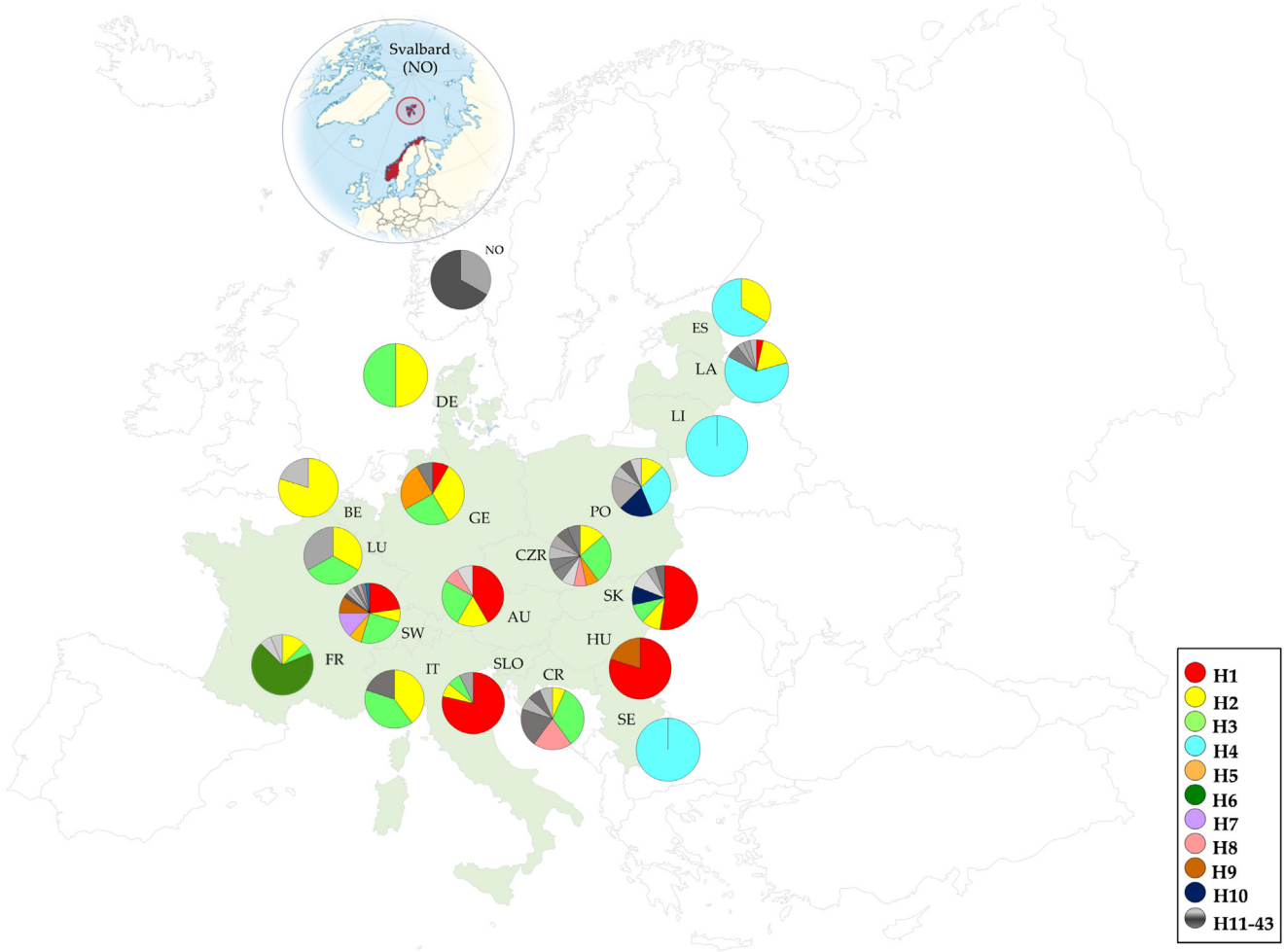


Fig. 2. Geographical haplotype distribution. Map of Europe showing country-level haplotype distribution of *Echinococcus multilocularis* used in this study. Location codes: AU, Austria; BE, Belgium; CR, Croatia; CZR, Czech Republic; DE, Denmark; ES, Estonia; FR, France; GE, Germany; HU, Hungary; IT, Italy; LA, Latvia; LI, Lithuania; LU, Luxembourg; NO, Svalbard (Norway); PO, Poland; SE, Serbia; SK, Slovakia; SLO, Slovenia; SW, Switzerland. A colour version of this figure is available on-line.

(62.56 %) and, with the exception of Svalbard (Norway), at least one of these four haplotypes was present in all the 19 populations investigated (Fig. 2) (Supplementary Table S2). The Simpson's Index of Diversity was minimum for Lithuania (0 %) and maximum for Luxembourg (100 %); for only those populations where number of isolates were ≥ 5, the index ranges from 40 % (Belgium, Hungary, and Slovenia) to 93 % (Czech Republic).

In the case of France, H6 was the predominant haplotype (11/16). This haplotype was defined by the presence of an 'A' in position 222 of the *nad2* gene (1,806 in the final alignment); observing the chromatograms, this position showed two overlapping peaks (A and G) of almost equal intensity, on both forward and reverse strands; the flanking sequences were clearly defined with no further overlapping. This polymorphism could be attributed to heteroplasmy, i.e. the presence of more than one mitochondrial genome in the same cell or individual. Heteroplasmy has never been described in *E. multilocularis*, however it was reported for *E. granulosus* (Bowles et al., 1995), other cestodes (Kinkar et al., 2020; Tuli et al., 2022), and is known to occur quite frequently in eukaryotes (Parakatselaki and Ladoukakis, 2021).

3.4. Characteristics of the haplotype networks

In general, most haplotypes were part of a network where each haplotype was separated from the closest by one or two mutation steps (Fig. 1). Geographical clustering by country was not detected. One haplotype (H14), representing two and three isolates from Latvia and Poland, respectively, set apart from the main group, and the two haplotypes from Svalbard (Norway) (H32-H33) clustered even more distantly (Fig. 1).

When the alignment *sensu* Nakao (*cob* + *nad2* + *cox1*) was analysed, the number of variable positions decreased to 54 and that of haplotypes to 36 (hd: 0.888). Fig. 3 illustrates the relationships among haplotypes *sensu* Nakao from the present study (n = 36) and those described by Nakao and colleagues (n = 18) (Nakao et al., 2009). As expected, most haplotypes from this study were identical (e.g., H1-H3) or closely related to the European haplotypes of Nakao. Nevertheless, haplotype H14 was located at the edge of the Asian cluster, while the Svalbard haplotypes (H32-H33) were even more distant and placed between the North American N1 and N2 haplotypes (Fig. 3).

In order to extend the comparison, haplotype networks were computed for individual markers using reference mitochondrial sequences retrieved from GenBank (Supplementary Table S1 and Supplementary Figs. S1–S5). In each of the five networks, one or two main European clusters, including European reference sequences and most isolates from the present study, were observed. Into these European clusters, sequences from Canada and the USA also grouped eventually (not seen in *atp6* and *nad1* due to a lack of reference sequences). Some sequences from Latvia

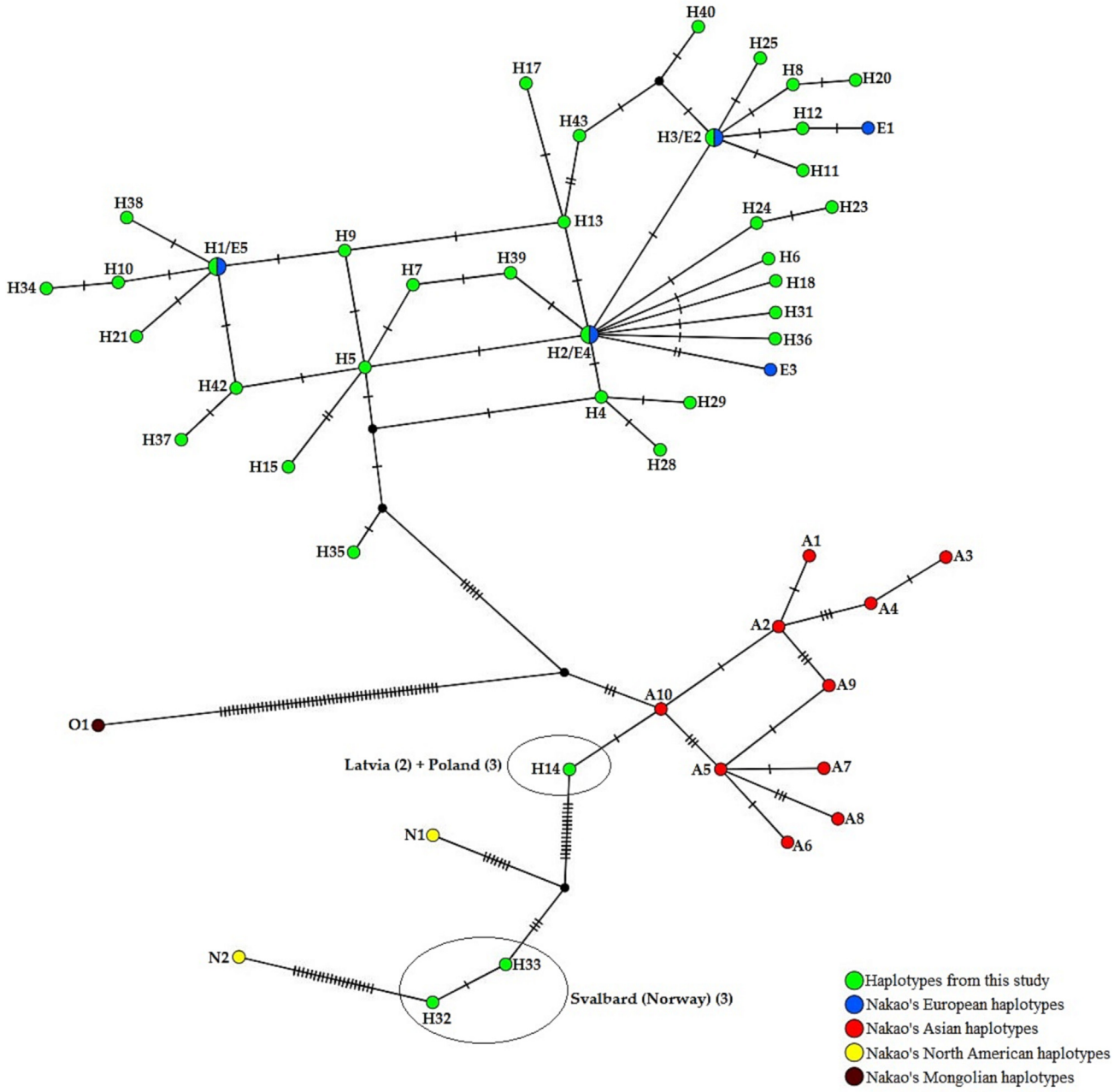


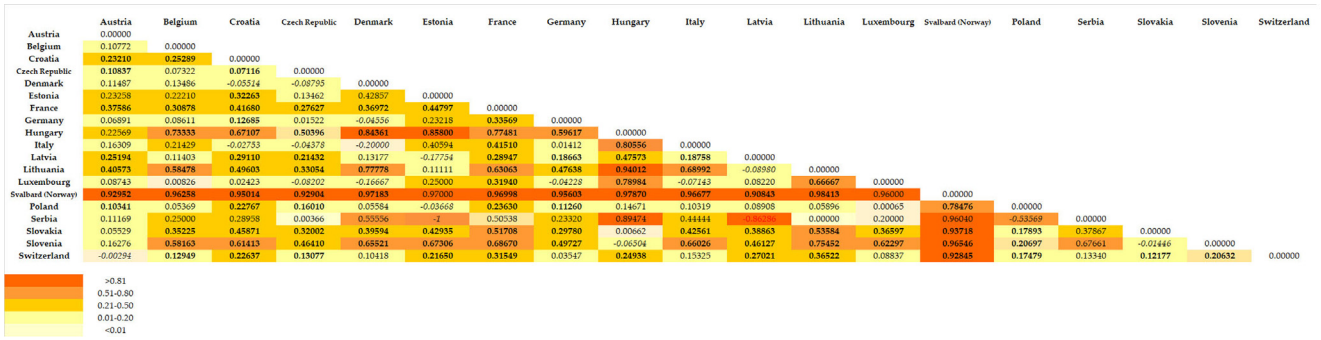
Fig. 3. Haplotype network sensu Nakao. TCS network representing the relationship between the 36 *Echinococcus multilocularis cob* + *nad2* + *cox1* haplotypes (3,558 bp) detected in this study (in green) and the Nakao historical haplotypes, designated as European, Asian, North American and Mongolian. In this network, former H16, H19, H22 and H27 are incorporated in H2, H41 is incorporated in H3, H30 is incorporated in H4 and H26 are incorporated in H24. Each haplotype is represented by a circle, with the area of the circle proportional to its frequency. Hatch marks depict single mutational steps and small dots display hypothetical haplotypes. A colour version of this figure is available on-line.

and Poland (corresponding to haplotype H14) placed within the Asian cluster, as was the case for one Austrian isolate (AUS24) in both *cob* and *atp6*, and one Hungarian isolate (HU5) in *nad1*. In this Asian cluster, some sequences from Canada and some European reference sequences were also found (Polish sequences for *cob*, *nad2* and *cox1*). While there was limited divergence in *atp6* and *nad1* markers, Svalbard (Norway) haplotypes were in proximity with haplotypes from the USA (St. Lawrence Island, Indiana and South Dakota) by means of *cob* and *nad2* markers. In *cox1*, Svalbard haplotypes were 3–7 mutational steps apart from the USA haplotypes but were identical to one *cox1* haplotype from Russia (Yakutia). It must be pointed out that for this analysis Russian sequences from Altai Krai, Yakutia and Irkutsk Oblast were considered as "Asian" while the single sequence from Moscow (Russia) was considered as "European", since the Ural Mountains are conventionally considered a natural boundary between the European and the Asian territories.

The Svalbard isolates were highly differentiated from all the others (Fst = 0.78–0.97). Beyond Svalbard, significant Fst values varied from 0.94 between Hungary and Lithuania to 0.07 between Croatia and the Czech Republic ($P < 0.05$) (Fig. 4).

4. Discussion

In the last three decades, *E. multilocularis* infections in red foxes have been recorded outside the historical endemic regions of Aus-



	Austria	Belgium	Croatia	Czech Republic	Denmark	Estonia	France	Germany	Hungary	Italy	Latvia	Lithuania	Luxembourg	Svalbard (Norway)	Poland	Serbia	Slovakia	Slovenia	Switzerland
Austria	0.00000																		
Belgium	0.10772	0.00000																	
Croatia	**0.23210**	**0.25289**	0.00000																
Czech Republic	**0.10857**	0.07322	**0.07116**	0.00000															
Denmark	0.11487	0.13486	*-0.05514*	*-0.08795*	0.00000														
Estonia	0.23258	0.22210	**0.32263**	0.13462	**0.42857**	0.00000													
France	**0.37586**	**0.30878**	**0.41680**	**0.27627**	**0.36972**	**0.44797**	0.00000												
Germany	0.06891	0.08611	**0.12685**	0.01522	*-0.04556*	**0.23218**	**0.33569**	0.00000											
Hungary	0.22569	**0.73333**	**0.67107**	**0.50396**	**0.84361**	**0.85800**	**0.77481**	**0.59617**	0.00000										
Italy	0.16309	0.21429	*-0.02733*	*-0.04378*	*-0.20000*	**0.40594**	**0.41510**	0.01412	**0.80556**	0.00000									
Latvia	**0.25194**	0.11403	**0.29110**	**0.21432**	0.13177	*-0.17754*	**0.28947**	**0.18603**	**0.47573**	**0.18758**	0.00000								
Lithuania	**0.40573**	**0.58478**	**0.49603**	**0.33054**	**0.77778**	0.11111	**0.63063**	**0.47638**	**0.94012**	**0.68992**	*-0.08980*	0.00000							
Luxembourg	0.08743	0.00826	0.02423	*-0.08202*	*-0.16667*	**0.25000**	**0.31940**	*-0.04228*	**0.78984**	*-0.07143*	0.08220	**0.66667**	0.00000						
Svalbard (Norway)	**0.92952**	**0.96258**	**0.98014**	**0.92904**	**0.97183**	**0.97000**	**0.96998**	**0.95603**	**0.97870**	**0.96677**	**0.90843**	**0.98413**	**0.96000**	0.00000					
Poland	**0.10341**	0.05369	**0.22767**	**0.16010**	0.05584	*-0.03668*	**0.23630**	**0.11260**	0.14671	0.10319	0.08908	0.05896	0.00065	**0.78476**	0.00000				
Serbia	0.11169	**0.25000**	0.28938	0.00366	0.55556	*-1*	0.50538	0.23320	**0.89474**	0.44444	*-0.36586*	0.00000	0.20000	**0.96040**	*-0.53569*	0.00000			
Slovakia	0.05529	**0.35225**	**0.45871**	**0.32002**	**0.39594**	**0.42935**	**0.51708**	**0.29780**	0.00662	**0.42561**	**0.38863**	**0.53584**	**0.36597**	**0.93718**	**0.17893**	**0.37867**	0.00000		
Slovenia	0.16276	**0.58163**	**0.61413**	**0.46410**	**0.65521**	**0.67306**	**0.68670**	**0.49727**	*-0.06504*	**0.66026**	**0.46127**	**0.75452**	**0.62297**	**0.96546**	**0.20697**	**0.67661**	*-0.01446*	0.00000	
Switzerland	*-0.00294*	**0.12949**	**0.22637**	**0.13077**	0.10418	**0.21650**	**0.31549**	0.03547	**0.24938**	0.15325	**0.27021**	**0.36522**	0.08837	**0.92845**	**0.17479**	0.13340	**0.12177**	**0.20632**	0.00000

>0.81
0.51-0.80
0.21-0.50
0.01-0.20
<0.01

Fig. 4. Fixation index (Fst) values. Matrix of pairwise Fst values among 19 populations. Significant ($P < 0.05$) Fst values are in bold. Negative Fst values are in italics. NO, Norway. A colour version of this figure is available on-line.

tria, France, Germany, and Switzerland (e.g. Eckert et al., 2000; Henttonen et al., 2001; Casulli et al., 2005, 2010; Moks et al., 2005; Bružinskaitė et al., 2007; Osterman Lind et al., 2011; Ćirović et al., 2012; Karamon et al., 2014; Maas et al., 2014; Beck et al., 2018; Jarošová et al., 2020; Herzig et al., 2021). Indeed, the increase in red fox populations, as a consequence of rabies eradication campaigns, and the colonization of urban environments by red foxes, contributed to the increase in infection extensity in animals and then the incidence of human AE cases (Laurimaa et al., 2015; Delcourt et al., 2022; ECDC, 2022). At the same time, major sampling efforts over time may partly explain this observed increasing trend. Therefore, molecular epidemiology is fundamental to understanding the distribution, spread, colonization and introduction of *E. multilocularis*. Moreover, molecular clinical studies, taking into account both the genetic variability of *E. multilocularis* and the clinical condition of AE patients, would be valuable in identifying a possible correlation between strains and the severity of disease. Based on limited current knowledge, no particularly pathogenic or less pathogenic *E. multilocularis* strains have been currently identified (Bohard et al., 2023).

Most genotyping studies have been conducted using the DNA microsatellite marker EmsB, at regional or national scales, in Denmark (Knapp et al., 2019), Estonia (Laurimaa et al., 2015), France (Knapp et al., 2008), Germany (Herzig et al., 2021), Hungary (Casulli et al., 2010), Italy (Casulli et al., 2009), Svalbard (Norway) (Knapp et al., 2012), Poland (Umhang et al., 2017, 2021a), Sweden (Knapp et al., 2019) and Switzerland (Knapp et al., 2021) and at a continental level (Knapp et al., 2009; Umhang et al., 2021b). Indeed, the first known European-scale study exploring the genetic diversity of *E. multilocularis* was based upon the differentiation of EmsB profiles from red fox samples collected in historical endemic and more peripheral areas of central Europe (Knapp et al., 2009). Results suggested for the first time, a mainland-island transmission model spreading from the northern Alps with a genetic flow from historical to newly endemic regions. A greater genetic variability has been noticed in the first compared with the latter areas, and the presence of similar genetic profiles in both zones suggested a founder effect (Knapp et al., 2009). This model was further supported by the study of Umhang and colleagues that provided a larger sampling area in eastern Europe (Umhang et al., 2021b).

No studies, on a similar scale, have been proposed using *E. multilocularis* mitochondrial markers so far. Surveys based on the analysis of *E. multilocularis* mitochondrial genes at a regional or national scale in Europe generally observed patterns defined as the "European" clade *sensu* Nakao (Laurimaa et al., 2015; Karamon et al., 2017; Jarošová et al., 2020; Herzig et al., 2021). In a study conducted in Russia, based only on the *cox1* gene, most *E. multilocularis* isolates corresponded to the "Asian" (82 %), "North American" (10 %) and "Mongolian" (6 %) clades. However, *E. multilocularis* isolates from this study were all sampled in eastern Russian territories; just a single isolate from a captive primate reared in the Moscow Zoo was classified as "European" but genetic comparison led to the hypothesis that the source of infection originated in eastern Europe (Konyaev et al., 2013; Umhang et al., 2021b). In another study conducted in Poland, based on the concatenated *cob*, *nad2* and *cox1* sequences, most *E. multilocularis* isolates were grouped in the "European" clade, while one haplotype grouping several samples from the northeastern part of the country resembled the "Asian" clade from Japan and Kazakhstan. According to the authors, this finding in Poland represented at the time "the westernmost location of any member of the Asian cluster of haplotypes in Europe" (Karamon et al., 2017).

Finally, it is worth mentioning the results obtained from 25 *E. multilocularis* isolates from eight contiguous European countries (Austria, France, Germany, Hungary, Latvia, Poland, Slovakia, Switzerland) by sequencing partial *nad1*, *cox1*, *rrnS*, *atp6* and *actII* genes (2,715 bp, Šnábel et al., 2019). In this study, aside the finding of the North American strain (N1) (Nakao et al., 2009) in an Austrian patient, the π was significantly higher in the historical endemic countries, represented by Austria, eastern France, Germany, and Switzerland, compared with the remaining sampling area. According to the authors, these results were in line with the mainland-island model defined with the EmsB microsatellite data (Knapp et al., 2009).

In the present study, the highest frequency haplotypes (H1, H2 and H3) were found both in the historical endemic countries and in those considered to be new endemic areas, with slight differences (Figs. 1 and 2). Haplotype H1 was prevalent in the Alpine area (Switzerland and Austria) and in neighbouring areas including Slovakia, Hungary, and Slovenia, present at lower prevalence in Germany and Latvia, and absent from the other countries. Haplotype H2 was dominant in Belgium and Germany, and its distribution ranged from the northern (Estonia) to the southern edges of the sampling area (Italy and Croatia) (excluding Svalbard), while it was absent only from Hungary, Lithuania and Serbia. A similar, but more restricted, distribution was observed for haplotype H3, which was absent from Baltic countries (Estonia, Latvia, and Lithuania), Hungary, Poland and Serbia. Finally, haplotype H4 was predominant in the Baltics and was found also in Poland (northeastern provinces) and Serbia, suggesting a distribution limited to the northeastern part of Europe (Figs. 1 and 2).

Overall, two of the dominant haplotypes (H1 and H4) were inversely distributed from north-eastern to south-western Europe, while haplotypes H2 and H3 filled the space between and around. Haplotypes H2 and H3 link H1 and H4 in the haplotype network, as intermediate genetic variants (Fig. 1).

The picture from this study therefore suggests the presence of three main haplotype groups, the first comprising most of western,

central, and eastern European countries, the second Baltic countries and northeastern Poland, and the third the Svalbard archipelago (Figs. 1–4). The presence of the Baltic countries and Poland group was due to two concomitant events: the dominance here of the European haplotype H4, which is absent from the rest of Europe except for Serbia, and the presence of one Asian-like haplotype (H14).

The phylogeography of the red fox could be helpful in interpreting the genetic difference of *E. multilocularis* in the northeastern territories observed in this study. It is known that during the Last Glacial Maximum (LGM, 22,000–18,000 BC), many terrestrial species (both plants and animals) in Europe were forced into three main southern refugia, represented by Iberian, Italian and Balkan peninsulas. After the northward recolonization during the interglacial periods, many species have maintained the genetic patterns acquired during the period of isolation in the southern refugia (Taberlet et al., 1998). While this was confirmed for species such as the brown bear (Sommer and Benecke, 2005), the situation is more complex for the red fox. Earlier studies did not find evidence of a genetic structure for the red fox at the European level, but recently, the acquisition of genome-wide data showed that some geographic patterns were indeed present and resulted from post-glacial admixture and isolation events (Teacher et al., 2011; McDevitt et al., 2022). In fact, red foxes from central Europe exhibited a genetically homogeneous pattern, while those from the Baltic region and northeastern Poland showed similar ancestry, mixed between that of central Europe and Scandinavia (McDevitt et al., 2022). We cannot exclude that these evolutionary pressures on red foxes during glacial and interglacial periods could have also involved its parasite, *E. multilocularis*.

Nakao and colleagues (2009) dated the presence of *E. multilocularis* in Europe prior to the LGM and suggested that it was presumably isolated and maintained by the red fox in the southern European glacial refugia during the LGM, shaping the European clade of *E. multilocularis* (Nakao et al., 2009). It is worth noting that the red fox, according to extension of the permafrost during the Weichselian and Würm glaciations (115,000–11,700 years ago) was confined in the European glacial refugia (Iberian, Italian and Balkan peninsulas) where *E. multilocularis* is partly or completely absent at present (Taberlet et al., 1998). Whether *E. multilocularis* became extinct from these refugia after the LGM (for both climatic and ecological reasons), or whether other hypotheses might explain its absence from these areas, is unknown based on the current knowledge from paleo-parasitological data. However, the southern European glacial refugia alone could not have shaped the diversity of *E. multilocularis* observed in Europe. The recognition of other refugia, named cryptic, allowed the survival of adaptable, temperate carnivore species such as the brown bear and the red fox at northern latitudes during the glacial periods (Sommer and Nadachowski, 2006; Provan and Bennett, 2008; Edwards et al., 2012). One of the prime candidates is the Carpathian refuge area (Saarma et al., 2007) situated in the vicinity of the contemporary endemic centre for *E. multilocularis*. On the other hand, potential eastern refuge areas should also be considered (Davison et al., 2011; Anijalg et al., 2018). Another efficient definitive host species to be considered is the Arctic fox. It cannot be excluded that the Arctic fox was an important reservoir of *E. multilocularis* during the last glacial period(s), since both host and parasite are well adapted to cold temperatures. Some biological features of *E. multilocularis*, such as the metacestode viability after deep freezing, suggest that this is a species well adapted to cold ecosystems (Laurimäe et al., 2020). Moreover, both the spatial distribution and population size of the Arctic fox were greater during the glaciation period (Stewart et al., 2010). Fossil findings demonstrated that the red fox and the Arctic fox co-existed in some areas during the Late Pleistocene – Early Holocene (15,000–9500 BC). After this period, as the temperature increased, the Arctic fox populations were more and more fragmented both due to the altitudinal expansion of red foxes and to reduced Arctic fox habitats, with significant population loss and extinction as consequences (Dalén et al., 2007). It is possible to speculate that the red fox inherited *E. multilocularis* from the Arctic fox during its northward recolonization, as they were interfacing and sharing prey species along the retreating ice line. The genetic diversity of *E. multilocularis* maintained by the Arctic fox in the Alps (i.e., Alpine area of Austria, France, Germany, and Switzerland) and in northeastern Europe (Baltic countries and northern Poland), both under ice sheets isolated from each other during the glacial period, could therefore have been inherited by the red fox that colonized these habitats when they became ice-free and the Arctic fox became extinct. In this scenario, two foci, one located in the Baltic territories and the other corresponding to the Alpine area, can be inferred from our analyses. A true gradient of genetic diversity, sustaining mainland-island models, cannot be inferred by our data due to the uneven number of samples available from each country.

This picture differs, to some extent, from that suggested by studies based on microsatellite markers (Knapp et al., 2009; Umhang et al., 2021b), likely due to the different markers analysed. Mitochondrial genes, in contrast with microsatellite markers, are only maternally inherited, therefore they lack the recombination mechanisms of the nuclear genes and accumulate mutations at a different rate. Combining the resolution power of both the markers may lead to concordant (Santa et al., 2023), or complementary results (Shang et al., 2021; Umhang et al., 2021a).

To our knowledge, this study used the largest mitochondrial marker dataset of *E. multilocularis* conducted to date.

Regarding the network *sensu* Nakao, three Asian-like isolates have been identified in the present study from northeastern Poland. This finding is consistent with the previous identification of one Asian haplotype from Poland in the northeastern provinces of Warmińsko-Mazurskie and Mazowickie in 2017 (Karamon et al., 2017), even though the Asian-like haplotype found in Poland in the present study differed by one mutation from the one detected in 2017. The two Asian-like isolates in Latvia were reported from the Rēzekne and Ugāle parish districts, at opposite borders of the country. Overall, the five Asian-like isolates belonged to the same haplotype. Observing the *cob* and the *atp6* networks, one isolate from Austria (AUS24), was identified as Asian-like as well, even though for this isolate, sequences were not complete for all five genes (Supplementary Figs. S1–S5). This Austrian isolate, however, was obtained from a human patient whose nationality was Kyrgyz, therefore the infection was likely acquired in Asia. It is not easy to establish what evolutionary events determined this presence, but the multiple findings of Asian haplotypes in animal isolates in Latvia and Poland in this and in previous studies, would suggest a spread of *E. multilocularis* genetic variants from Asia to Europe. The lack of baseline data makes it impossible to determine how long this process has been going on. This uncertainty applies to Turkey, where the presence of both European and Asian clades of *E. multilocularis* has been detected by EmsB microsatellites (Umhang et al., 2021b). While the red fox is considered an indigenous species and likely the main definitive host for *E. multilocularis* in Europe, the role of the invasive raccoon dog (*Nyctereutes procyonoides ussuriensis*), considered "one of the most successful alien carnivores in Europe" (Kauhala and Kowalczyk, 2011), in introducing zoonotic pathogens has received attention in the last decade (Sutor et al., 2014). In fact, more than 9,000 individuals of *N. p. ussuriensis*, originating from Amur and Usuri regions of Siberia and eastern China, were introduced into European parts of former Soviet Union (USSR) in 1929–1955. As a result, this species was first noticed in Fennoscandia, Baltic countries and Poland in the 1950s and subsequently spread to other parts of Europe (Kauhala

and Kowalczyk, 2011). Therefore, the hypothesis of raccoon dogs being responsible for introducing Asian haplotype/s of *E. multilocularis* into northeastern Europe may not be excluded. Additional data from northeastern Europe and western Asia could assist further clarification of the molecular epidemiology of *E. multilocularis*.

Interestingly, genes *atp6* and *nad1* have not been so informative in this wide-scale study. More specifically, they have only partially contributed to depiction of the genetic diversity of *E. multilocularis*, since their absence from the study would decrease the number of haplotypes from 43 to 36. Accordingly, from a time and cost saving perspective, *cob* + *nad2* + *cox1* could be considered a sufficient set of markers, especially for large-scale studies.

The haplotypes identified in the Svalbard isolates from Arctic foxes were markedly separated from the European groups and barely related to the Asian and North American genetic variants. The Svalbard *cox1* sequences were identical to *cox1* sequences from Yakutia, eastern Russia (Supplementary Figs. S1–S5). Sequences of other markers were not available from this region. Svalbard is the only Norwegian territory where *E. multilocularis* is found. Here, Arctic foxes and sibling voles (*Microtus levis*) are the only definitive and intermediate hosts (Fuglei et al., 2008). Recent molecular analyses revealed that the parasite was likely spread by Arctic foxes migrating from Siberia through the Arctic Sea ice (Knapp et al., 2012), and was able to establish its life cycle due to the presence of the sibling vole introduced from Europe (Henttonen et al., 2001). Molecular identity with Russian sequences strongly supports this route; moreover, the same dynamic was described for the introduction of rabies (Mørk et al., 2011), and Arctic foxes are known to cover extensive stretches of sea ice and glaciers (Fuglei and Tarroux, 2019).

Another interesting feature, revealed by the network analyses of *cob*, *nad2* and *cox1* (Supplementary Figs. S1–S5), was the similarity of some North American sequences (from the USA and Canada) to those from Europe (from this study or previously deposited in GenBank) and Asia. The presence of European-like *E. multilocularis* variants in North America has been reported since 2009, in wildlife, domestic dogs and then in humans (Jenkins et al., 2012; Gesy et al., 2013; Massolo et al., 2019; Polish et al., 2021; Santa et al., 2023). The authors hypothesized that multiple and recent introduction events from Europe may have caused the establishment of European-like variants in North America (Jenkins et al., 2012; Santa et al., 2023). The results from this study are not in contrast with this hypothesis. However, we cannot exclude that natural movements of definitive hosts may have contributed, to some extent, to the spread of *E. multilocularis* strains worldwide. Latitudinal dispersion of Arctic foxes in the polar or subpolar zones, but also longitudinal dispersion of red foxes in temperate zones, may have played a role in the current distribution of European, North American and Asian clades. In fact, eastward expansion of the Asian clade could not be excluded, since coexistence of Asian and North American haplotypes in the St. Lawrence Island (western Alaska) was documented (Nakao et al., 2009).

In conclusion, our study suggested the presence of three main clusters in Europe represented by most western, central and eastern European countries, the Baltic countries and northeastern Poland, and Svalbard (Norway), respectively. In this context we propose that the genetic diversity present in the first two clusters may have been inherited from isolated *E. multilocularis* populations present in ice-covered areas during the glacial period, in particular those located in the Alpine and the Baltic areas.

According to the current data, the discontinuity between these two clusters can be identified in Poland with a buffer zone between the South (dominance of H2 and H10 haplotypes) and the North-East of Poland.

Future studies using larger numbers of samples and a combination of mitogenomes and nuclear genomic data may provide a better insight into the population dynamic of *E. multilocularis* at a European scale. Additional sampling of grey-zone areas between Europe and Asia, and of European countries where little or no data are currently available (Belarus, Hungary, Ukraine, Romania, Serbia, and Sweden), will also be relevant in testing the different hypotheses on the population dynamic of *E. multilocularis*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2024.01.003.

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