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# *Echinococcus multilocularis* genetic diversity based on isolates from pigs confirmed the characteristic haplotype distribution and the presence of the Asian-like haplotype in Central Europe

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## Abstract

**Introduction:** The aim of the study was to determine the genetic diversity of *Echinococcus multilocularis* in pigs in highly endemic areas in Poland, as well as to attempt to confirm the occurrence and geographical distribution of haplotypes characteristic for these areas, which were previously described on the basis of examination of adult tapeworms isolated from foxes. **Material and Methods:** Twenty samples of *E. multilocularis* larval forms were obtained from pigs' livers in four provinces of Poland. Genetic analyses were conducted on sequences of two mitochondrial genes: *cox1* and *nad2*. **Results:** Seven haplotypes were found for the *cox1* gene (OQ874673–OQ874679) and four haplotypes for *nad2* (OQ884981–OQ884984). They corresponded to the haplotypes described earlier in foxes in Poland (some of them differing only in one nucleotide). The analysis showed the presence of the Asian-like haplotype in both the *cox1* and *nad2* genes. The remaining haplotypes were grouped in the European clade. The geographical distribution of haplotypes identified in the pig samples was noticed to bear a similarity to the distribution of haplotypes previously isolated from foxes in the same regions. **Conclusion:** The characteristic geographical distribution of *E. multilocularis* haplotypes in Central Europe (including the presence of the Asian-like haplotype) previously described in the population of definitive hosts (foxes) has now been confirmed by the analysis of samples from non-specific intermediate hosts (pigs).

**Keywords:** *Echinococcus multilocularis*, pigs, haplotypes, genetic diversity.

## Introduction

Alveolar echinococcosis is a parasitic zoonosis dangerous to human health and life, which is caused by larval forms of the *E. multilocularis* tapeworm. A typical definitive host, in the intestines of which adult forms of this tapeworm develop, is the red fox. Other rare definitive hosts are raccoon dogs, arctic foxes, golden jackals, wolves and also dogs and cats. Adult tapeworms produce eggs that are excreted into the environment and are a source of infection for intermediate hosts, which are typically rodents (31). However, eggs can also infect humans and other animal species (*e.g.* pigs and horses) acting as non-specific (aberrant) intermediate hosts. In the tissues of these hosts, the larva develops in an unusual way and most often it does not produce protoscolices or degenerate (31).

In recent years, much attention has been devoted to the genetic diversity of *E. multilocularis*. The studies have been carried out in two main ways, namely by analysing the sequence of selected genes (usually mitochondrial genes) (6, 11, 27) and by an innovative method of analysing tandemly repeated microsatellites (1, 20, 34). They revealed the divisions between the main clades grouping the most common genotypes according to the geographical distribution of parasites.

Thus the genotypes typical of Europe, Asia and North America were described (20, 27). This genetic diversity research also made it possible to trace the migration of parasites with their hosts and to determine the primary foci of occurrence and peripheral areas colonised by parasites later (on the pattern of the mainland-island model). Knowledge of genetic diversity provided the means to observe the mixing of genotypes characteristic for one continent with those characteristic for others. Example of this are the finding of Asian-like haplotypes in Europe (18, 37) or European-like examples in North America (12, 32).

Poland's geographical location and relatively high prevalence of *E. multilocularis* in red foxes (15, 17) make it an interesting site for the analysis of the genetic diversity of this tapeworm. There is a specific mixing of western (European) and eastern (probably Asian) genetic pools. Recent studies in Poland conducted on adult worms isolated from red foxes showed the presence of dominant haplotypes characteristic for this area of Europe. What is most interesting is that the presence of the Asian-like haplotype was also found (18, 37).

The good availability of genetic material of tapeworms isolated from the intestines of definitive hosts dictates that a lot of data on genetic diversity come from studies of such – most often from red fox tapeworms (8, 18, 35, 36). However, samples from other animal species are also investigated, including some from pigs, which may act as non-specific intermediate hosts in the life cycle of *E. multilocularis*. There is small number of studies on the prevalence of *E. multilocularis* in pigs. The only ones that refer to a wider population come from Switzerland, where the prevalence was estimated at 0.009% in 2017–2018 (26), and from Japan, where *E. multilocularis* larvae were identified in 0.0002% of slaughtered pigs in 2005–2008 (19). Despite the relatively low prevalence, this animal species can be a specific indicator of environmental contamination with *E. multilocularis* eggs, which is an important element in assessing the risk of human infection in a given region. Recently, extensive studies were carried out in a pig population in France using analysis of the tandemly repeated microsatellite EmsB, confirming the possibility of monitoring this species as an additional indicator of the risk of human infection, as well as one by which to observe the genetic diversity of these tapeworms. In Poland, the first detections of larval forms in pigs have already been described (16), but no wide investigation has been conducted on the genetic diversity of *E. multilocularis* based on material obtained from this host.

The aim of the study was to determine the genetic diversity of *E. multilocularis* in pigs in highly endemic areas of Poland, as well as to attempt to confirm the occurrence and geographical distribution of haplotypes characteristic for this region, which were previously described on the basis of examination of adult tapeworms isolated from red foxes.

## Material and Methods

**Samples of *Echinococcus multilocularis.*** *Echinococcus multilocularis* larval forms were obtained in 2011–2016 from pigs' livers in four provinces of Poland: Warmińsko-Mazurskie and Podlaskie (north-east) and Podkarpackie and Małopolskie (south-east). Fragments of livers with lesions were collected individually by vets in slaughterhouses, and after freezing were sent to the laboratory of the National Veterinary Research Institute in Puławy, Poland. Samples were first investigated macroscopically to ascertain the content of lesions and microscopically using a stereo microscope at 50× magnification to detect the protoscolices. Extraction of DNA was carried out using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. That DNA matched the *E. multilocularis* species was confirmed molecularly by a nested PCR (10) with modifications (16). Overall, 20 DNA samples of *E. multilocularis* were used for genetic analysis.

**PCR and sequencing.** Two mitochondrial genes, *nad2* and *cox1*, were amplified by PCR according to Nakao *et al.* (27). Sequencing was performed at a commercial company (Genomed, Warsaw, Poland) using Sanger dideoxy sequencing.

**Data analysis.** Phylogenetic analyses were conducted separately for each molecular marker (*nad2* and *cox1*). The forward and reverse sequences were analysed, aligned and trimmed using the Geneious Alignment algorithm in the Geneious Prime bioinformatics software platform (Biomatters, Auckland, New Zealand). The obtained consensus sequences were aligned with sequences from GenBank using the BLAST nucleotide algorithm. Phylogenetic analysis was also conducted using sequences available in GenBank as outgroups. A phenogram was created by applying the Tamura–Nei genetic distance model and the neighbour-joining building method with 1,000 bootstrap replications in Geneious Prime. The nucleotide sequences obtained in this study were submitted to the GenBank database under the accession numbers OQ874673–OQ874679 (*cox1*) and OQ884981–OQ884984 (*nad*2). To estimate the phylogenetic position of the Polish isolates, homologous mitochondrial DNA sequences obtained in previous research (12, 13, 18, 22–25, 27, 30, 38) were retrieved from GenBank and used in analyses.

## Results

The macroscopic examination demonstrated the following types of lesions: in 15 samples there were white, thick-walled cysts 1–10 mm in diameter deeply recessed in the liver tissue with whitish degenerated mass or thick fluid, and in 5 samples white nodular forms 2–4 mm in diameter were found on the surface of liver tissue (Fig. 1). Protoscolices were not found microscopically in any of the examined samples.

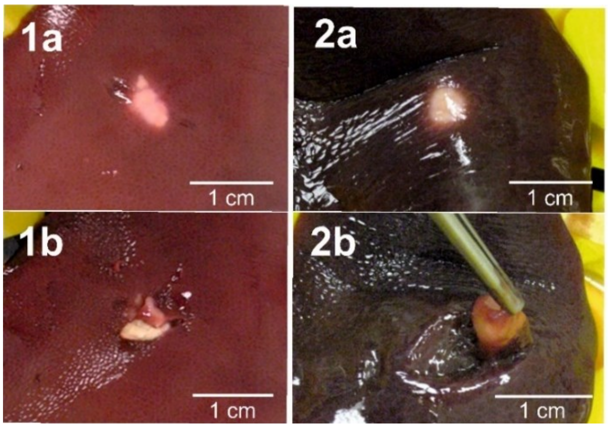

**Fig. 1.** Two examples of *Echinococcus multilocularis* lesions found in pigs' livers
1a and 2a – before cutting; 1b and 2b – after cutting

All 20 samples gave specific products in PCRs for *cox1* and *nad2*. Good-quality sequences were obtained for 19 *cox1* and 19 *nad2* products, but one sample only had a *cox1* sequence and another sample only had a *nad2* sequence. One *cox1* sequence (EmPL_cox_E) was incomplete and lacked its first 207 nucleotides.

In the analysis of the *cox1* gene, seven haplotypes were found (GenBank accession numbers OQ874673–OQ874679) (Fig. 2). Five of them, EmPL_cox_A, EmPL_cox_B, EmPL_cox_E, EmPL_cox_F and EmPL_cox_G, corresponded exactly to the haplotypes described previously in foxes in Poland (KY205683, KY205691, KY205685, KY205689 and KY205690) (18). The other two, EmPL_cox_B2 and EmPL_cox_G2, differed by only one nucleotide from the previously described ones.

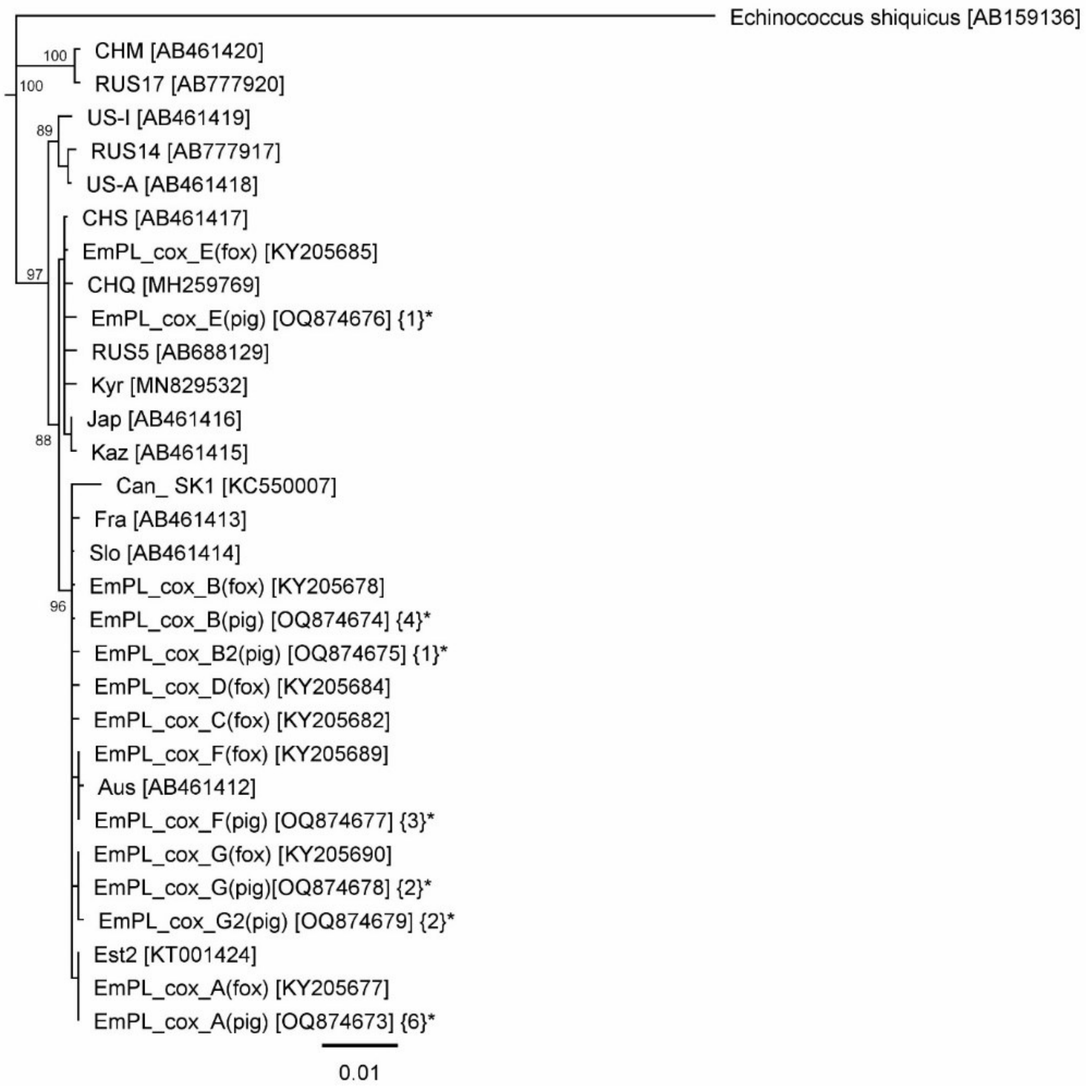

**Fig. 2.** The phylogenetic tree of *Echinococcus multilocularis* based on the *cox1* gene
EmPL_cox_A–EmPL_cox_G – Polish haplotypes (* – sequences of this study, numbers of isolates in each haplotype are shown in curly brackets); Aus – Austria; Can_SK1 – Canada; CHM – China (Inner Mongolia); CHQ – China (Quinhai); CHS – China (Sichuan); Est2 – Estonia; Fra – France; Jap – Japan, Kaz – Kazakhstan; Kyr – Kyrgyzstan; RUS5, RUS14, RUS17 – Russia; Slo – Slovakia. US-A – USA (Alaska - St. Lawrence Island); US-I – USA (Indiana). Values on the tree nodes are bootstrap proportions (%)

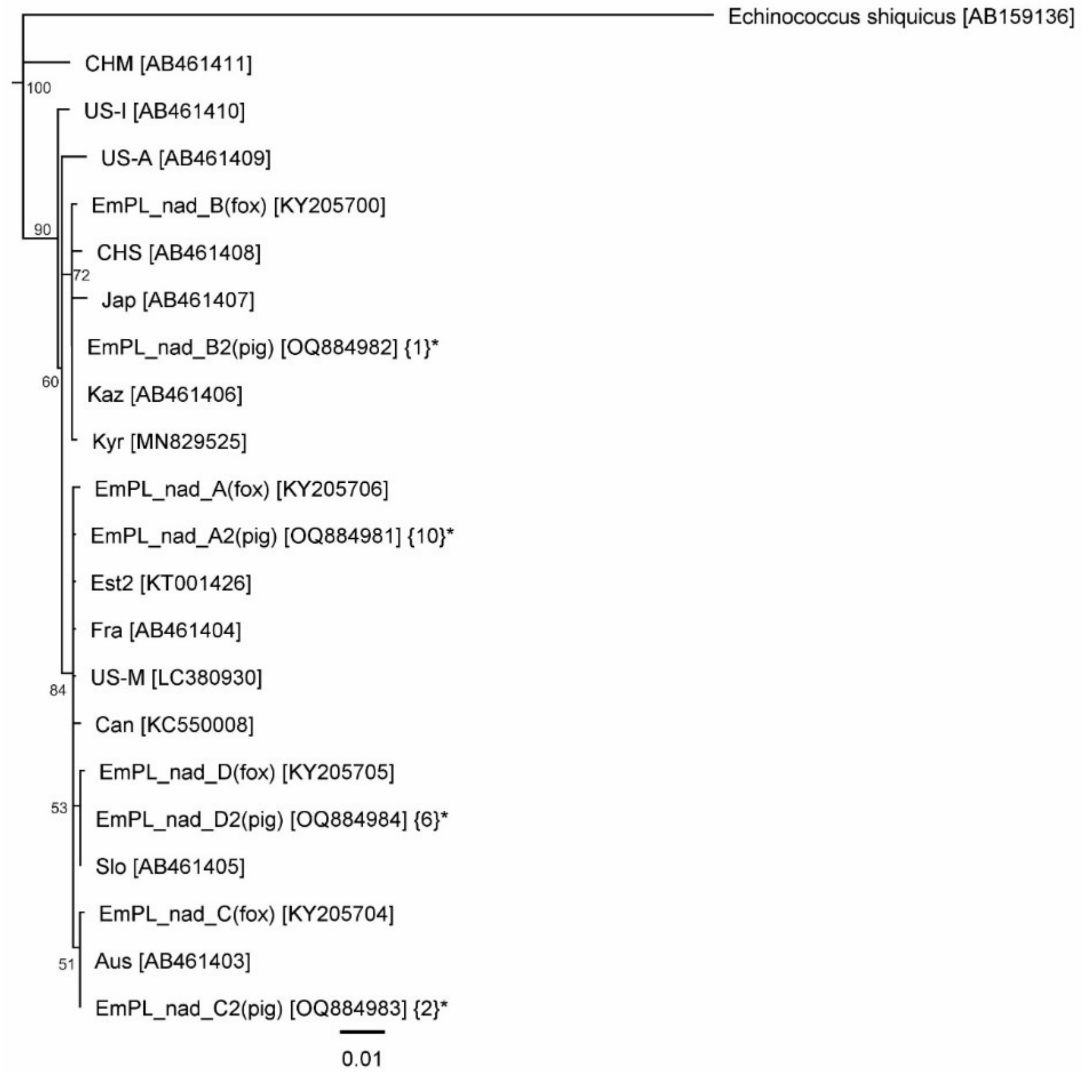

**Fig. 3.** The phylogenetic tree of *Echinococcus multilocularis* based on the *nad2* gene
EmPL_nad_A–EmPL_nad_D – Polish haplotypes (* – sequences of this study, numbers of isolates in each haplotype are shown in curly brackets); Aus – Austria; Can – Canada; CHM – China (Inner Mongolia); CHS – China (Sichuan); Est2 – Estonia; Fra – France; Jap – Japan, Kaz – Kazakhstan; Kyr – Kyrgyzstan; Slo – Slovakia. US-A – USA (Alaska - St. Lawrence Island); US-I – USA (Indiana); US-M – USA (Missouri). Values on the tree nodes are bootstrap proportions (%)

The *nad2* gene analysis revealed four haplotypes (GenBank accession numbers OQ884981–OQ884984) (Fig. 3). They were EmPL_nad_A2, EmPL_nad_B2, EmPL_nad_C2 and EmPL_nad_D2, and all of them corresponded to similar haplotypes described previously in foxes in Poland (18), EmPL_nad_A, EmPL_nad_B, EmPL_nad_C and EmPL_nad_D (GenBank accession numbers KY205706, KY205700, KY205704 and KY205705), with a difference of only one nucleotide.

The analysis showed the presence of the Asian-like haplotype, to which one sample's genetic material affiliated, in both the *cox1* (EmPL_cox_E) (OQ874676) and *nad2* (EmPL_nad_B) (OQ884982) genes. These genes' sequences were similar to those previously identified in foxes in Poland (KY205670 and KY205700) (18). The remaining haplotypes were grouped in the European clade.

Comparing the geographical distribution of haplotypes identified in the pig samples, a similarity could also be noticed with the distribution of haplotypes previously isolated from foxes. This is particularly evident in the more diverse *cox1* gene and the EmPL_cox_A and E haplotypes. From both foxes and pigs these were present, or were in significant predominance, only in the north of the country. In contrast, the EmPL_cox_F, G and B haplotypes from both pig and fox samples were found only in the south (Fig. 4). A resemblance could also be observed in the distributions of pig and fox *nad2* haplotypes – similar groups of haplotypes dominated in the same regions in pigs and foxes (Fig. 5).

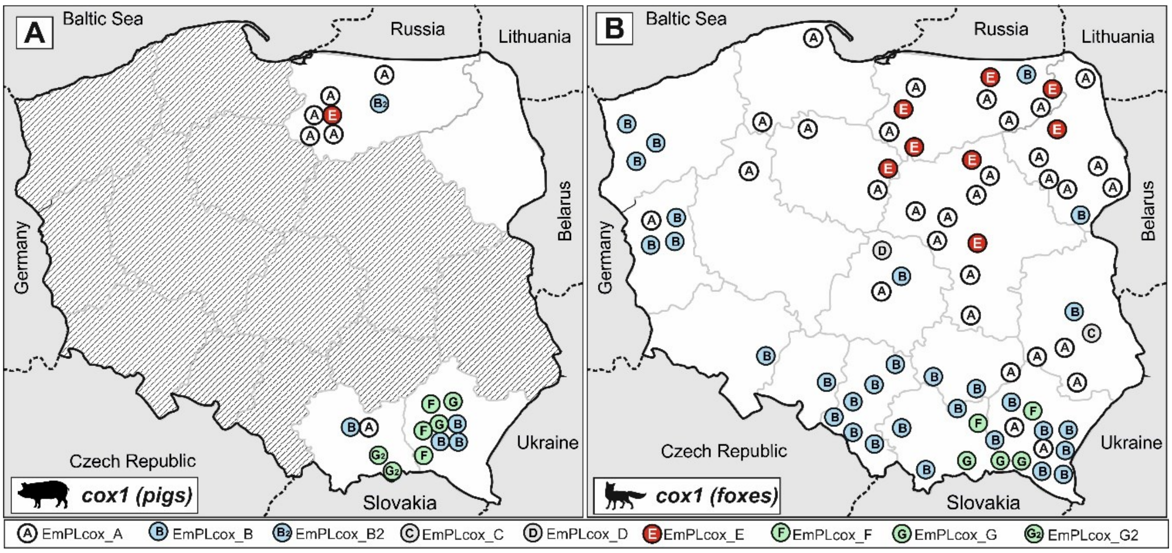

**Fig. 4.** Geographic distribution of *Echinococcus multilocularis cox1* gene haplotypes in isolates
A – in pigs in this study; B – in foxes according to Karamon *et al*. (18)

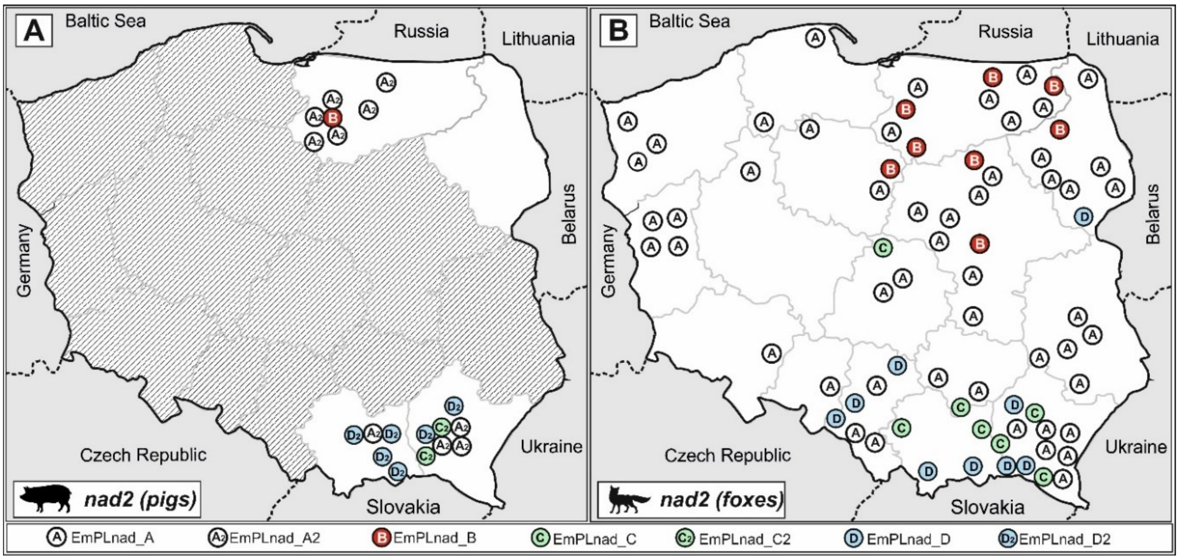

**Fig. 5.** Geographic distribution of *Echinococcus multilocularis nad2* gene haplotypes in isolates
A – in pigs in this study; B – in foxes according to Karamon *et al*. (18)

## Discussion

In pigs, the lesions caused by *E. multilocularis* are often characteristic of the early phase of larval development. In our studies, these were usually small nodules/cysts embedded in the liver parenchyma, often filled with caseous matter indicating degeneration of the larva. This is consistent with the description of lesions observed in the first case of *E. multilocularis* in pigs in Poland (16). These lesions are also comparable to those caused by *E. multilocularis* larvae found in commercially reared pigs and described in the few published reports on the subject. Kimura *et al.* (19) identified six lesions positive for *E. multilocularis* in Japan among 109 slaughtered pigs with whitish nodules in the liver. In Switzerland, sharply demarcated dense white nodules 5–15mm in diameter were observed (33). Larvae of *E. multilocularis* as nodular foci (from 3 to 22 mm in diameter) consisting of white capsules surrounding whitish to light yellow material with paste consistency were noted in Germany (3). Necrotic and calcified *E. multilocularis* lesions were found in Lithuania (7). Similar lesions were also obtained during experimental infections of pigs with *E. multilocularis* eggs (9, 28), and the same forms were also observed in infected wild boar livers (4, 29). Characteristic for all the described studies was the lack of protoscolices. As a contrasting finding, the infectivity of larval forms in pigs or wild boars was confirmed by inoculation of gerbils with parasitic tissue material isolated from the livers of infected animals, as a result of which characteristic cysts containing protoscolices were obtained in gerbils immediately after the first inoculation (14) or after the second passage (29).

The genetic diversity of *E. multilocularis* is highly dependent on geographical location. In the early 1990s, two different genotypes of *E. multilocularis* were characterised: M1 among isolates from China, Alaska and North America and M2 as an isolate from Europe (5, 6). With more isolates from different parts of the world becoming available, more extensive investigations were carried out using mitochondrial genes, and in them genotypes characteristic for Asia, Europe and North America, and specific to selected areas of China were distinguished (27). The study of genetic diversity also involved the analysis of microsatellite tandem repetitions (1), which facilitated the identification of genetic profiles characteristic for individual regions of Europe and the tracking of the routes of infection spread from originally endemic areas (the "core region") to peripheral areas (20, 36). The type of spread of this infection has been theorised to be the mainland-island model of parasite transmission. It assumes greater genetic diversity of *E. multilocularis* in the "core region" or "mainland", which in Europe is southern Germany, Switzerland and south-eastern France, from which the parasites gradually spread to create areas with less genetic diversity ("islands") (20). Therefore, according to this theory, it is believed that the genetic groups of tapeworms detected in central-eastern Europe reached there *via* hosts from the historical "core region" referred to above. On the other hand, the results obtained first in Russia (100 km from Moscow) (22) and later in central-eastern Europe (Poland) (18) suggested that the genetic diversity of *E. multilocularis* on the continent is also significantly influenced by parasites migrating with hosts from Asia. This was implied by these studies' confirmation of the presence of a haplotype belonging to the Asian clade. The probable Asian origin of this haplotype in Poland was further confirmed by EmsB analysis (37). In studies in Poland, all Asian-like isolates contained one haplotype from the Asian clade, which suggests a relatively early arrival of these parasites from Asia (18). Poland is an interesting area where parasite genetic diversity manifests the mixed influences of western and southern Europe and Asia. This is also observed in the case of other parasites, *e.g. Trichinella*: in western Poland *T. spiralis* was significantly dominant in wild boars (70–85% of infected animals), while in the eastern part of the country approximately half of the infected wild boars showed infection with *T. britovi*, a species more characteristic of areas located east of Poland (2).

The research carried out in pigs described in this article confirmed the presence of an Asian-like haplotype in Poland similar to that previously described in red foxes, and did so by analysing the *cox1* (EmPL_cox_E) and *nad2* (Em_PL_nad_B) genes. Other genetic diversity studies conducted with swine-derived material in Switzerland using EmsB only showed the presence of different genetic profiles typical of Europe (21).

The geographical distribution of haplotypes found in pigs in Poland corresponds to the characteristic distribution of them in the red fox population in the country, which was particularly visible in the analysis of the more diverse *cox1* gene. The *E. multilocularis* isolates from both red foxes and pigs in the south of the country were dominated by EmPL_cox_F, G and B haplotypes (similar to the haplotypes found in Slovakia, bordering Poland to the south). Similarly, the EmPL_cox_A haplotype (also found in Estonia (24)) was in the majority in the isolates from the north-east of Poland in both host species. The Asian-like haplotype was also detected in pigs in similar areas to where it was identified in red foxes.

Unusual geographical locations of *E. multilocularis* genotypes in terms of the continent have also recently been observed in North America, where the presence of genotypes characteristic for Europe was noted in adult tapeworms detected in coyotes (11) and in larvae derived from a dog (13). This was also confirmed by recent studies conducted in red foxes and coyotes in Canada, where various European-like haplotypes were found among North American haplotypes (32). Phylogenetic analysis showed some differences among European-like haplotypes isolated in North America, which suggests different scenarios with multiple introductions of European strains of *E. multilocularis* to Canada, probably occurring over several hundred years (*e.g.* through dogs originally imported from Europe) (32).

Summarising, the original division into continental clades was previously decided on the results given by a limited number of samples (27). Subsequent studies using more samples revealed further interesting facts probably related to the intercontinental migration of parasites with their hosts and their actual characteristic location.

In conclusion, studies on the genetic diversity of *E. multilocularis* deepen the knowledge of the ways in which this parasite spreads across continents. The results obtained in our present investigation conducted on samples of larval forms complement and confirm our previous studies conducted on material isolated from mature parasites. This is important due to the complex life cycle of tapeworms. The characteristic geographical distribution of haplotypes in this part of Europe, and most interestingly the presence of the haplotype from the Asian clade, which had previously been described in the population of definitive hosts (red foxes), have now been confirmed by the analysis of samples from accidental intermediate hosts (pigs).

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