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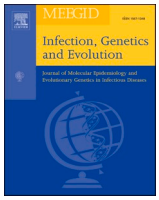

Genetic diversity of *Echinococcus multilocularis* from red foxes and humans in northern and northeastern Poland investigated using the microsatellite EmsB

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ABSTRACT

Background: Echinococcus multilocularis is the causative agent of alveolar echinococcosis (AE). We explored the diversity of EmsB profiles of *E. multilocularis* from red foxes and humans in northern and northeastern Poland, with a particular focus on autochthonous Asian variants.
Methods: We investigated 263 adult tapeworms from 59 red foxes hunted in selected districts of three voivodships and ten metacestodes extracted from AE patients who never visited Asia. We conducted hierarchical clustering of the obtained EmsB profiles combined with a custom Asian reference dataset and interpreted the resulting phenogram by applying the standard genetic distance threshold (GDT) of 0.08 and Dynamic Tree Cut (DTC).
Results: The GDT divided the 273 profiles into six units, with Pol-B being the most frequent (220/273, 81 %) and widespread variant. DTC grouped the profiles into three phenons (PH). Eight out of ten people got infected with the predominant variant, PH-2/Pol-B. Among the 273 samples, thirty-six (13 %) matched the Asian reference set, including metacestodes from a Lithuanian patient and a Polish patient.
Conclusions: Genetically extra-European variants reach as far north as Pomorskie Voivodship. They likely come from East Asia. The autochthonous AE case with an Asian profile confirms that such tapeworms have penetrated the synanthropic cycle.

1. Introduction

Ingestion of viable *Echinococcus multilocularis* Leuckart, 1863, eggs by humans may result in the development of a tumour-like hepatic lesion. Treatment options include resection and prolonged benzimidazole therapy, often burdened with side effects (Wen et al., 2019). Eggs are spread by canids — mainly red foxes and raccoon dogs in Europe — which prey on infected rodents in areas where people grow and collect fruit, vegetables, and mushrooms (Lass et al., 2015; Umhang et al., 2024). Post-glacial expansion, influenced by human-mediated host translocations — for example, from Europe to Western Canada (Santa et al., 2023) and the US (Conlon et al., 2023), or from St. Lawrence Island and Sichuan to Hokkaido (Hayashi et al., 2023) — has shaped a complicated pattern of *E. multilocularis* genetic diversity.

Microsatellite EmsB is an *Echinococcus* spp. target in chromosome 5, comprised of two tandem-repeat regions, one on the forward and one on the reverse strand (Valot et al., 2015). It owes its superior discriminatory power to its complexity (Knapp et al., 2007). There are many possible allele combinations, rendering the probability of homoplasy low. That and its stability over time make EmsB fit for spatiotemporal transmission tracking of *E. multilocularis* and identification of infection sources (Mohammadi and Harandi, 2024; Umhang et al., 2016). EmsB profiling linked human alveolar echinococcosis cases to sympatric canine populations in Europe (Bohard et al., 2023; Knapp et al., 2020) and China

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(Shang et al., 2021) and provided proof that Europeans contract Asian variants autochthonously (Gładysz and Lass, 2023).

EmsB data is used for hierarchical clustering (tree diagram creation), a classification method that reflects *E. multilocularis* genetic diversity in a phenetic sense (Sneath and Sokal, 1973). An algorithm utilising the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) returns a tree diagram — a phenogram demonstrating the degree of overall similarity between profiles. It is important to stress that such a diagram does not represent evolutionary relationships.

A crucial methodological aspect of EmsB profiling is the process of identifying individual branches (tree cutting) in UPGMA phenograms. The majority of studies use the genetic distance threshold (GDT), a straight line delimiting clusters of profiles (metaprofiles). The assumption is that samples share an identical profile when the Euclidean distance between them falls below a certain value. Originally, the threshold was based on an experiment in which two strains of *E. multilocularis* were cultivated in mouse passages for a year (strain A) and seven years (strain B) (Bart et al., 2006). DNA was extracted at time point zero, three months, and one year in the case of strain A and at time point zero and seven years in the case of strain B, and the samples were profiled. The genetic distance between isolates of each strain did not exceed 0.11, and the researchers fixed the threshold at 0.2.

Subsequently, the threshold was calculated according to an arbitrary formula $x + 3\sigma$, where x is the average Euclidean distance between the strain A samples and σ is the standard deviation (Knapp et al., 2007). The resulting value equalled 0.08 and has been used internationally in research based on wild definitive and intermediate hosts ever since (e.g., France (Knapp et al., 2008; Umhang et al., 2015); Italy (Casulli et al., 2009); Hungary (Casulli et al., 2010); Svalbard, Norway (Knapp et al., 2012); Estonia (Laurimaa et al., 2015); Poland (Umhang et al., 2017); Denmark and Sweden (Knapp et al., 2019); Canada (Santa et al., 2023)), with a notable exception of a Brandenburg study in which the authors discarded GDT and applied alternative methods (Herzig et al., 2021). In a paper on human metacestodes, the threshold was raised to 0.1 to avoid over-discrimination presumed based on a visual assessment of profiles (Knapp et al., 2020). The GDT of 0.1 became the default value in research on human-derived or mixed material, creating double standards for the analysis of equivalent data (Bohard et al., 2023; Knapp et al., 2021; Sacheli et al., 2023; Shang et al., 2021). Metaprofile discrimination by independent teams should not be a matter of individual preference if large-scale monitoring efforts are to be dependable and comparable. Additionally, cutting a phenogram either at 0.08 or 0.1 leaves some samples unassigned to any cluster. Such outliers pose an explanatory challenge because thresholds lack firm justification. Also, an outlier at 0.08 may not stand out at 0.1.

EmsB has revealed a surprising blend of European and Asian lineages in Poland. Warmińsko-Mazurskie Voivodship is one of the voivodships where autochthonous Asian mitochondrial haplotypes were first detected in the European Union, in red foxes and pigs (Karamon et al., 2017, 2023). This observation was further investigated using EmsB and led to the discovery of Euro-Asian hybrids (Umhang et al., 2021b). One possible explanation for the presence of Asian variants in Poland is their introduction by wild canids migrating from the east (Umhang et al., 2021b). Another scenario involves introductions of infected raccoon dogs by the Soviets from the Soviet Far East to the European parts of the Soviet Union between the late 1920s and the late 1950s (Kauhala and Kowalczyk, 2011; Santoro et al., 2024). Little, though, is known about profile diversity in Asia: there have been relatively few samples collected from geographically limited areas, and the studies are fragmented (Afonso et al., 2015; Knapp et al., 2007; Shang et al., 2021; Umhang et al., 2021a). The majority of this vast continent remains terra incognita.

This study aimed to explore the diversity of EmsB profiles of *E. multilocularis* from red foxes and humans in northern and northeastern Poland, using a larger worm collection relative to the study area than collections used in previous reports (Umhang et al., 2017, 2021b), with a particular focus on Asian variants.

2. Methods

2.1. Metacestodes of E. multilocularis

Ten *E. multilocularis* metacestodes infecting humans, excised between 2001 and 2018 from AE patients of the University Centre for Maritime and Tropical Medicine in Gdynia, living in Pomorskie (three samples) and Warmińsko-Mazurskie Voivodships (seven samples), were processed as in (Gładysz and Lass, 2023) (Fig. 1, Table 1). The sampled patients were aged 30 to 69 (median age 51) and exhibited mild to advanced disease stages, from II to IV (Kern et al., 2006). For detailed information on the metacestode samples and resulting profiles, see Tables 1–2 in (Gładysz and Lass, 2023) and Additional File 1: Table S1 and Table S2 attached to this paper.

2.2. Mature specimens of E. multilocularis

Adult tapeworms were collected from the intestines of red foxes hunted between 2022 and 2024 in several districts of Pomorskie and Warmińsko-Mazurskie Voivodships and Augustów District, Podlaskie Voivodship (Fig. 1), using the sedimentation and counting technique (Hofer et al., 2000). The number of tapeworms (T) and the number of red foxes (F) collected in each district were as follows (T/F): Puck, Wejherowo, Kartuzy, Kościerzyna: 17/4; Bartoszyce: 55/11; Kętrzyn: 65/14; Węgorzewo: 25/5; Gołdap: 53/14; Augustów: 48/11 (Table 1). In total, we examined 263 tapeworms from 59 red foxes.

We found mature *E. multilocularis* tapeworms in four red foxes from Pomorskie Voivodship out of 182 collected during two ceremonial displays of the game killed on hunts organised by local hunting clubs (Gładysz et al., 2025). With 19 individuals positive in a copro-PCR test, the most likely explanation for this undersampling is worm digestion in corpses kept outdoors for several days before proper storage and necropsy. To prevent this issue, the individuals from Warmińsko-Mazurskie Voivodship and Augustów District were hunted systematically and stored at −20 °C before sanitary transportation and necropsy.

Up to ten tapeworms per red fox were washed in physiological saline in a Petri dish (Karamon et al., 2017). Total genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany, cat. no. 69506) (elution in 100 µl of Buffer ATE).

2.3. PCR amplification of EmsB

We processed up to five random tapeworms per red fox. To amplify microsatellite alleles, PCR reactions were performed in a 25-µl reaction mixture containing 10–12 ng of DNA, 12.5 µl of PCR Mix Plus HGC (A&A Biotechnology, Gdańsk, Poland, cat. no. 2005-100G) and primers forward (5′-(FAM)-GTGTGGATGAGTGTGCCATC-3′) and reverse (5′-CCACCTTCCCTACTGCAATC-3′) (Knapp et al., 2007) added to the final concentration of 0.4 µM. PCR conditions were as follows: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min; 72 °C for 30 min.

2.4. Capillary electrophoresis of PCR products

Half a microlitre of a PCR product was mixed with 0.5 µl of Orange DNA Size Standard (MCLAB, San Francisco, CA, USA, cat. no. DSMO-100) and 9 µl of Super-DI™ Formamide (MCLAB, San Francisco, CA, USA, cat. no. SDI-100). Samples were incubated at 95 °C for 3 min and immediately moved to a cooler to retain the single-stranded structure. Capillary electrophoresis (injection voltage = 3.0 kV, injection time = 2 s) was performed on an Applied Biosystems® 3130 Genetic Analyzer (Life Technologies, Foster City, CA), with a synthetic calibrating sequence to scale the readings (Gładysz and Lass, 2023; Knapp et al., 2017). The results were analysed in GeneMapper 3.1. Raw profiles were

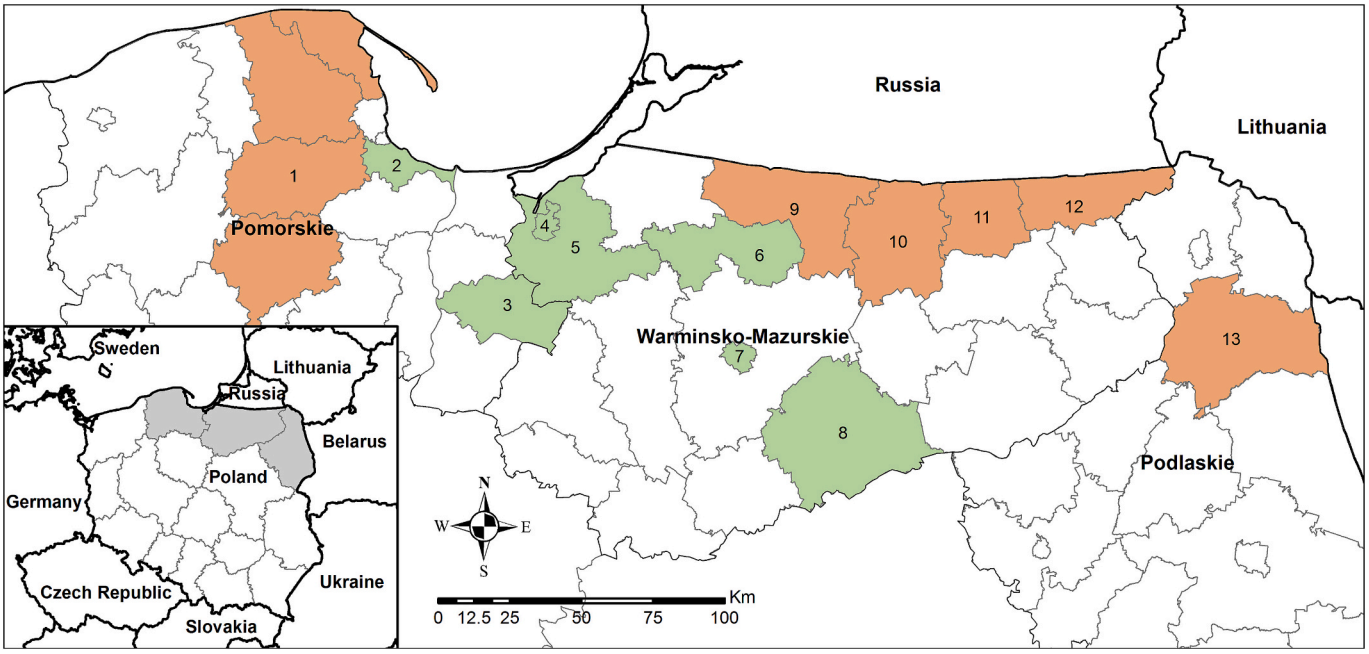

Fig. 1. Map of the study area. Districts where red foxes were hunted are shown in orange. Districts where AE patients resided, but where no red foxes were sampled, are shown in green. 1 – Puck, Wejherowo, Kartuzy, Kościerzyna; 2 – City of Gdańsk; 3 – Sztum; 4 – City of Elbląg; 5 – Elbląg; 6 – Lidzbark; 7 – City of Olsztyn; 8 – Szczytno; 9 – Bartoszyce; 10 – Kętrzyn; 11 – Węgorzewo; 12 – Gołdap; 13 – Augustów. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Origin and number of samples analysed in this study. Counts include metacestodes infecting humans. Districts are numbered as in Fig. 1.

No.	District	Red Foxes	Adult Tapeworms	Metacestodes
1	Puck, Wejherowo, Kartuzy, Kościerzyna	4	17	–
2	City of Gdańsk	–	–	1
3	Sztum	–	–	2
4	City of Elbląg	–	–	1
5	Elbląg	–	–	1
6	Lidzbark	–	–	1
7	City of Olsztyn	–	–	1
8	Szczytno	–	–	1
9	Bartoszyce	11	55	–
10	Kętrzyn	14	65	1
11	Węgorzewo	5	25	1
12	Gołdap	14	53	–
13	Augustów	11	48	–
	Total	**59**	**263**	**10**

Table 2
Counts of red-fox and human samples (combined) from districts where red foxes were hunted: for each metaprofile delimited by the genetic distance threshold of 0.08 (Knapp et al., 2007).

District	Pol-A	Pol-B	Pol-C	Pol-D	Pol-E	Pol-F	Total
Augustów	–	43	5	–	–	–	**48**
Bartoszyce	10	32	–	–	2	11	**55**
Gołdap	–	47	–	–	–	6	**53**
Kętrzyn	–	54	2	3	3	4	**66**
Puck, Wejherowo, Kartuzy, Kościerzyna	–	12	–	–	–	5	**17**
Węgorzewo	–	26	–	–	–	–	**26**
Total	**10**	**214**	**7**	**3**	**5**	**26**	**265**

processed and normalised as in Knapp et al. (2017).

2.5. Hierarchical clustering (HC)

We combined the obtained profiles with the Asian records from the EmsB Website for Echinococcus Typing (EWET, https://ewet-db.univ-fcomte.fr/), including Polish ones identified as Asian in previous studies. To create the most up-to-date set of Asian profiles for HC, we also gathered previously reported data absent from EWET by directly contacting the corresponding authors. As a result, our phenogram includes extra-European profiles collected in Shiqu and Seda Counties, West Sichuan Province, China (Shang et al., 2021), Poland (Umhang et al., 2017, 2021b), Armenia, Mongolia, Russia, and Turkey (Umhang et al., 2021a).

Hierarchical clustering using Euclidean distance and the UPGMA (Legendre and Legendre, 2012) was performed with the package 'pvclust' (Suzuki et al., 2019) in R version 4.3.2 (Posit team, 2024). Approximately unbiased (AU) *p*-values were computed by multiscale bootstrap resampling (1000 replicates) to assess cluster stability (Shimodaira, 2002, 2004).

We established clusters using Dynamic Tree Cut (DTC), a top-down algorithm that relies only on a phenogram and respects the order of clustered objects (see Langfelder et al. (2008) for a summary and a detailed outline). For this purpose, we used the package 'dynamicTreeCut' (Langfelder et al., 2016). This method identifies branches based on their shape rather than absolute height. It is a desirable property given that dendrogram structure depends on profile variation. Compared to cutting with the genetic distance threshold, DTC is adaptive, capable of identifying nested clusters, and suitable for automation.

To avoid any further terminological confusion (Mohammadi and Harandi, 2024), we propose to refer to the DTC clusters as 'phenons' after Sneath and Sokal (1973), pheneticists who intended this general term 'to cover the groups produced by any form of cluster analysis or from any form of similarity coefficients'. To ensure comparability with previous studies, we also analysed the phenogram with the standard genetic distance threshold of 0.08 (Knapp et al., 2007).

3. Results

The genetic distance threshold of 0.08 divided the full set (new data plus reference profiles) into 29 metaprofiles, 14 of which were outliers represented by a single sample. Due to the size of the phenogram, we present it in full in Additional File 2. The GDT grouped the 273 profiles obtained in this study into six metaprofiles, Pol-A to Pol-F. Pol-B was the most represented and widespread metaprofile, comprising 214 tapeworms from all districts where red foxes were hunted. Pol-A and Pol-D were the rarest metaprofiles, with ten tapeworms from Bartoszyce District and three tapeworms from Kętrzyn District, respectively. Pol-C was detected only in Augustów and Kętrzyn Districts (five and two tapeworms, respectively), while Pol-E was detected only in Bartoszyce and Kętrzyn Districts (two and three tapeworms, respectively) (Table 2).

DTC grouped all profiles into six phenons, PH-1 to PH-6, without leaving any profile unassigned. Mongolian and Kyrgyz profiles formed distinct branches, PH-1 and PH-4. The remaining extra-European samples clustered into PH-6, predominantly Chinese, and PH-5, containing all other Asian profiles. Tapeworms and metacestodes from this study were sorted into three units, namely PH-2, PH-3, and PH-6 (Table 3). PH-2, corresponding to Pol-ABC, was the most numerous phenon, present in all districts where red foxes were hunted. PH-3, corresponding to Pol-D, was the rarest phenon, detected only in three tapeworms from Kętrzyn District. PH-6 tapeworms, corresponding to Pol-EF, originated from Pomorskie Voivodship and Bartoszyce, Gołdap, and Kętrzyn Districts, Warmińsko-Mazurskie Voivodship.

We managed to analyse five tapeworms per red fox for 46 individuals. The majority of red foxes (40 individuals, 87 %) contained tapeworms of one metaprofile. One red fox from Gołdap District and three red foxes from Kętrzyn District contained tapeworms of two metaprofiles. Single individuals with three different metaprofiles came from Bartoszyce and Kętrzyn Districts (Table 4).

DTC produced a slightly different division of samples in the phenogram than the GDT. As a result, 41 red foxes (89 %) contained tapeworms belonging to one phenon. Five red foxes from Bartoszyce, Kętrzyn, and Gołdap Districts were infected with tapeworms of two phenons: one European and one Asian (PH-2 and PH-6 or PH-2 and PH-3) (Table 5, Additional File 3).

In humans, eight out of ten patients got infected with the predominant variant, PH-2/Pol-B. The two remaining metacestodes, i.e. co-1857 from a Lithuanian patient living in the city of Olsztyn and cz-1925 from a Polish patient living in Szczytno District, Warmińsko-Mazurskie Voivodship, belonged to PH-6/Pol-F, an Asian grouping (Table 6).

In total, 36 samples clustered with the Asian reference profiles, indicating their extra-European origin: 34 tapeworms from nine red foxes from Pomorskie Voivodship and Bartoszyce, Gołdap, and Kętrzyn Districts (PH-3/Pol-D, PH-6/Pol-E or F) and two metacestodes (PH-6/Pol-F) (Fig. 2). Five red foxes — one from Bartoszyce District, one from Gołdap District, and three from Kętrzyn District — harboured tapeworms of mixed origin, i.e. European and Asian (Additional File 3).

Table 3
Counts of red-fox and human samples (combined) from districts where red foxes were hunted: for each phenon delimited by Dynamic Tree Cut (Langfelder et al., 2008).

District	Phenon 2	Phenon 3	Phenon 6	Total
Augustów	48	–	–	**48**
Bartoszyce	42	–	13	**55**
Gołdap	47	–	6	**53**
Kętrzyn	56	3	7	**66**
Puck, Wejherowo, Kartuzy, Kościerzyna	12	–	5	**17**
Węgorzewo	26	–	–	**26**
Total	**231**	**3**	**31**	**265**

Table 4
Counts of red foxes from districts where red foxes were hunted: infected with tapeworms of one, two, or three different metaprofiles. Metaprofiles were delimited by the genetic distance threshold of 0.08 (Knapp et al., 2007). Calculations include only red foxes with five tapeworms profiled (46 out of 59 individuals).

District	One Metaprofile	Two Metaprofiles	Three Metaprofiles	Total
Augustów	8	–	–	**8**
Bartoszyce	10	–	**1**	**11**
Gołdap	6	1	–	**7**
Kętrzyn	8	3	**1**	**12**
Puck, Wejherowo, Kartuzy, Kościerzyna	3	–	–	**3**
Węgorzewo	5	–	–	**5**
Total	**40**	**4**	**2**	**46**

Table 5
Counts of red foxes from districts where red foxes were hunted: infected with tapeworms of one or two different phenons. Phenons were delimited by Dynamic Tree Cut (Langfelder et al., 2008). Calculations include only red foxes with five tapeworms profiled (46 out of 59 individuals).

District	One Phenon	Two Phenons	Total
Augustów	8	–	**8**
Bartoszyce	10	1	**11**
Gołdap	6	1	**7**
Kętrzyn	9	3	**12**
Puck, Wejherowo, Kartuzy, Kościerzyna	3	–	**3**
Węgorzewo	5	–	**5**
Total	**41**	**5**	**46**

4. Discussion

Little EmsB data comes from Eastern European countries (sensu United Nations Statistics Division (2024)). Expanding the global collection of profiles is crucial for large-scale reasoning and tracing the expansion of *E. multilocularis*, both past and future. Initial efforts involving Poland concentrated on the assessment of the EmsB suitability for spatial distribution tracking at the continental level (Knapp et al., 2007), the investigation of *E. multilocularis* colonisation of Europe from the historical endemic area (Knapp et al., 2009), and on northern Italy (Casulli et al., 2009). In each of these objectives, a small number of Polish samples served only as a part of the European background composed of samples from various countries.

A nationwide investigation of profile diversity among 301 worms from 87 red foxes, collected between 2009 and 2013 across all but one voivodship, revealed 29 clusters. Cluster Pol19 was found everywhere except for Małopolskie Voivodship and accounted for 44.9 % of the tapeworms (Umhang et al., 2017). Three and 16 variants were exclusive for the western and eastern parts of the country, respectively, and ten variants were shared by all voivodships. The six most common metaprofiles (but for Pol19) had already been noted in other parts of the continent, but none corresponded to any of the most common European variants, suggesting that Poland has been colonised relatively recently due to a founder event (Knapp et al., 2009).

The study confirmed that Poland is embedded in a still obscure network of the parasite's genetic variation in Europe, since regions directly adjacent to Germany, Czechia, and Slovakia shared EmsB variants with the neighbouring areas abroad. With 45 tapeworms from 15 red foxes from Warmińsko-Mazurskie Voivodship and 14 tapeworms from four red foxes from Pomorskie and Zachodniopomorskie Voivodships, the findings of Umhang et al. (2017) provided a basis for a detailed investigation in northern and northeastern Poland.

Among 14 tapeworms from four red foxes from Zachodniopomorskie and Pomorskie Voivodships, Umhang et al. (2017) found six

Table 6
Classification of metacestodes infecting humans based on the genetic distance threshold of 0.08 (Knapp et al., 2007) and Dynamic Tree Cut (Langfelder et al., 2008). Patient 1857 most likely acquired the infection before moving to Poland (Gładysz and Lass, 2023).

Sample	Patient's Nationality	Voivodship	District	EmsB Metaprofile (GDT)	EmsB Phenon (DTC)	Origin
cg-1791	Polish	Pomorskie	City of Gdańsk	Pol-B	PH-2	European
sz-1841	Polish	Pomorskie	Sztum	Pol-B	PH-2	European
sz-1920	Polish	Pomorskie	Sztum	Pol-B	PH-2	European
ce-795	Polish	Warmińsko-Mazurskie	City of Elbląg	Pol-B	PH-2	European
dz-853	Polish	Warmińsko-Mazurskie	Lidzbark	Pol-B	PH-2	European
e-843	Polish	Warmińsko-Mazurskie	Elbląg	Pol-B	PH-2	European
k-1664	Polish	Warmińsko-Mazurskie	Kętrzyn	Pol-B	PH-2	European
w-525	Polish	Warmińsko-Mazurskie	Węgorzewo	Pol-B	PH-2	European
co-1857	Lithuanian	Warmińsko-Mazurskie	City of Olsztyn	Pol-F	PH-6	Asian
cz-1925	Polish	Warmińsko-Mazurskie	Szczytno	Pol-F	PH-6	Asian

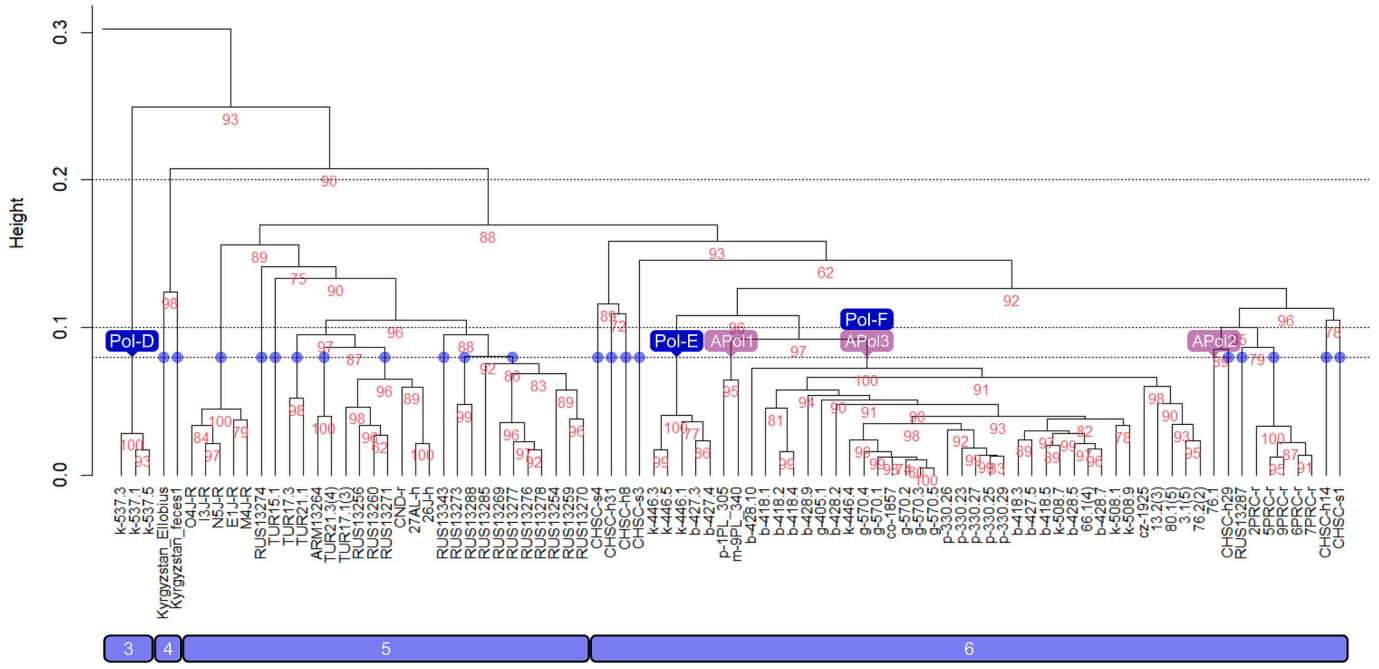

Fig. 2. Asian subsection of the UPGMA phenogram in Additional File 2 (Mongolia excluded). The phenogram comprises the obtained EmsB profiles combined with Asian records from the EmsB Website for Echinococcus Typing and relevant papers, including Polish profiles identified as Asian in previous studies: Shiqu and Seda Counties, West Sichuan Province, China (Shang et al., 2021); Poland (Umhang et al., 2017, 2021b); Armenia, Mongolia, Russia, and Turkey (Umhang et al., 2021a). Tapeworms with the same profile, extracted from one red fox, were pooled (the number of parasites in brackets). Approximately unbiased *p*-values (in %, in red) were calculated by multiscale bootstrap resampling (1000 replicates). Phenons 3–6, identified by Dynamic Tree Cut (Langfelder et al., 2008), are indicated with bars below the leaves. Genetic distance thresholds of 0.08, 0.1, and 0.2 used in previous EmsB studies (Bart et al., 2006; Knapp et al., 2007, 2020) are represented with dotted lines. Metaprofile clusters delimited by the standard genetic distance threshold of 0.08 are marked at points where branches intersect the threshold line. Metaprofile clusters of samples obtained in this study were labelled Pol-D through to Pol-F. APol1, APol2, and APol3 are metaprofiles first identified by Umhang et al. (2021b). District codes in the leaves: b – Bartoszyce; g – Gołdap; k – Kętrzyn; co – City of Olsztyn; cz – Szczytno; p – Puck, Wejherowo, Kartuzy, Kościerzyna. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metaprofiles, four of which were represented by a single sample (GDT of 0.08). In Warmińsko-Mazurskie Voivodship, they detected 12 metaprofiles, six of which were represented by a single sample. Thirty-six tapeworms from nine red foxes from Podlaskie Voivodship were divided into four metaprofiles. The contrast with our results most likely stems from technical factors associated with PCR efficiency and capillary electrophoresis (the practical importance of template concentration was mentioned in Knapp et al. (2021)). However, both studies identified a European metaprofile predominant in the sampled regions (Pol19 or Pol-B, respectively) — a symptom of low marker diversity typical of a recent founder event (Templeton, 2008).

Though much genetic diversity in the Asian genetic landscape awaits discovery, the broadened reference dataset allowed us to look deeper into the genetic provenance of the Asian variants observed in Poland. A larger number of tapeworms relative to the area than in previous studies (Umhang et al., 2017, 2021b) gave us access to more Asian samples distributed in this region of Europe. Groupings in the Asian part of the phenogram were consistent with the hierarchical clustering conducted by Umhang et al. (2021b), with notable exceptions. Unit PH-3/Pol-D, comprising three worms from Kętrzyn District, is a new addition and clustered externally with other Asian samples (AU *p*-value of 93 %). Metaprofile AS8 sensu Umhang et al. (2021a) — comprising six human samples and one red-fox sample from Altai Krai, and three rodent samples from Ryazan Oblast, Russia — was divided into two metaprofiles. The new metaprofile Pol-E contained five tapeworms from two red foxes hunted in the neighbouring Kętrzyn and Bartoszyce Districts.

Metaprofile APol1 sensu Umhang et al. (2021b) contained two samples, each from a different Polish red fox, and APol2 was represented by tapeworm 76.1 from the same study. None of our samples joined either of these two clusters. In contrast, APol3/Pol-F expanded, with 28 tapeworms joining this grouping. APol3/Pol-F included samples from Bartoszyce, Kętrzyn, and Gołdap Districts, adjacent to Kaliningrad

Oblast, as well as five tapeworms from red fox p-330 shot in Pomorskie Voivodship. Importantly, APol3/Pol-F contained two human metacestodes from Warmińsko-Mazurskie Voivodship: one from a Lithuanian patient living in the city of Olsztyn and one from a Polish patient living in Szczytno District. The former has already been identified as extra-European and was the first autochthonous European case of an Asian variant detected in humans (Gładysz and Lass, 2023). The latter is new, further substantiating the hypothesis that Asian metaprofiles have penetrated the synanthropic cycle.

Seven metaprofiles were each represented by a single West Sichuanese sample, as reported by Shang et al. (2021); no Polish tapeworms joined these units below the 0.08 threshold. However, a broader look beyond the GDT reveals that West Sichuanese sample CHSC-h29 clustered with tapeworm 76.1 from a red fox from Kujawsko-Pomorskie Voivodship (AU *p*-value of 89 %). The entire PH-6 contained only one non-Sichuanese Asian sample: metacestode RUS13287 from a patient from Altai Krai, a federal subject of Russia bordering northeastern Kazakhstan. The remaining PH-6 leaves come from either Poland or West Sichuan. Four phenons formed geographically well-defined units representing Mongolia (PH-1, AU *p*-value of 100 %), Kyrgyzstan (PH-4, AU *p*-value of 98 %), China and Poland (PH-6, AU *p*-value of 93 %), and the rest of the Asian continent (PH-5, AU *p*-value of 89 %). The geographic origin of PH-3 is unknown due to the scarcity of reference samples. The similarity of Polish and Chinese profiles adds credibility to the hypothesis of human-mediated introductions of Asian *E. multilocularis* tapeworms with raccoon dogs transported from the Russian territory bordering Heilongjiang Province, China, in the 20th century (Santoro et al., 2024).

Besides reference sample 1PL_305 from Puck (EWET code EWET-767-POL, first reported in Knapp et al. (2009)), all five tapeworms from red fox p-330 were grouped in the Asian cluster, joining those detected previously in Warmińsko-Mazurskie, Podlaskie, Mazowieckie, Kujawsko-Pomorskie, and Lubuskie Voivodships (Umhang et al., 2021b). This means that an extra-European variant is present as far north as Pomorskie Voivodship. In total, 34 out of 263 red-fox tapeworms had an Asian profile (12.5 %), all from the voivodships of Warmińsko-Mazurskie (29 tapeworms from eight red foxes) and Pomorskie (five tapeworms from one red fox).

One should bear in mind that the insight provided by microsatellites is limited to the nuclear genome. Examination of heterogeneous *E. multilocularis* populations should preferably combine hierarchical clustering of EmsB profiles with mitochondrial sequencing to account for differences in mutation rates, modes of inheritance, and evolutionary histories of mitochondrial and nuclear targets. Such a dual perspective reveals hybrids and increases the resolution of regional studies. Thus, in a follow-up phylogenetic investigation, the collected red-fox samples will serve as templates for cytochrome *c* oxidase subunit 1 gene amplification and sequencing.

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CRediT authorship contribution statement

Paweł Gładysz: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Małgorzata Samorek-Pieróg:** Investigation. **Jacek Karamon:** Writing – review & editing, Resources. **Krzysztof Rębała:** Supervision. **Małgorzata Sulima:** Resources. **Dariusz Zadrożny:** Resources. **Anna Lass:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare no competing interests.

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Data availability

Raw FSA files (fsa_files), normalised EmsB profiles of *Echinococcus multilocularis* metacestodes from humans and adult tapeworms from red foxes hunted in selected districts of Pomorskie, Warmińsko-Mazurskie, and Podlaskie Voivodships (profiles.txt), and an R script generating a UPGMA phenogram cut with genetic distance thresholds and/or Dynamic Tree Cut (hierarchical_clustering.R) are available at Mendeley Data, DOI: 10.17632/zcfb7k3jn8.1.

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