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On the geographic genetic variants of the cestode *Echinococcus multilocularis* with reference to the original descriptions from Bowles et al. (1992) and Bowles and McManus (1993), and their use

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Abstract

Alveolar echinococcosis, caused by the larval stages of the tapeworm *Echinococcus multilocularis* (Leuckart, 1863), is of increasing concern in the northern hemisphere. Most cases of alveolar echinococcosis (excluding Alaska) appear to be linked with European and Asian genotypes that highlight the need for a more precise delimiting of their actual distribution and tracing historical episodes of their translocations and introductions into new areas. We have herein summarized previous available research studies, which mentioned firstly described geographic M1/M2 variants of *E. multilocularis* using molecular tools (established by sequencing of mitochondrial genes *cox1*, 366 bp and *nad1*, 471 bp) in an attempt to consolidate their correct affiliations with the geographic origin in sense of the original description from the early 1990´s. Since 2009, inverted designations (M1 named as M2 and vice versa) are being prevailing in research literature (we found ten erroneous vs. three correct classifications) that might bias genetic interpretation of comparative data in specific cases. When comparing M1/M2 profiles to those obtained from mitochondrial evidences over the last decades, the phylogenetic analysis revealed that the M1 strain (described from China, Alaska, North America) grouped with the Asian clade of *E. multilocularis* more recently established, whereas the M2 strain (described from the German vole) had a specific structure, in *cox1* clustering with the North American clade. It is presumed that events of intercontinental expansion and isolation covering glacial and interglacial periods during the late Pleistocene have likely accounted for the transmission of this discrete genotype from Beringia into endemic area of western and central Europe via circumpolar movements of foxes.

Keywords: Echinococcus multilocularis, Genetic variants, Strain designation, Phylogenetic analysis, European-type genotype

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1. Introduction

Human alveolar echinococcosis (AE), caused by the metacestode stage of the cestode *Echinococcus multilocularis* is acknowledged as one of the most pathogenic zoonosis in temperate and arctic regions of the Northern hemisphere. Infected individuals typically develop a slowly-progressing hepatic disease that clinically manifests as a tumour-like, infiltrative aggregates, responsible for tissue destruction [1]. A long asymptomatic incubation period lasting 5-15 years substantially impedes the process of understanding of risk factors for human infections and the associated transmission patterns [2,3].

From the early 1990s, gene sequence data became routinely available and over the past three decades this source has provided a significant contribution to the consolidation of systematic issues in the genus *Echinococcus* Rudolphi, 1801. Early investigations into the genetic diversity of *E. multilocularis* by Bowles and co-workers [4] and Bowles and McManus [5] examined mitochondrial sequence variations in a 366 bp region of cytochrome *c* oxidase subunit 1 (*cox1*) gene and in a 471 bp region of the NADH dehydrogenase subunit 1 (*nad1*) gene. These authors found two nucleotide differences in both mitochondrial genes between the two geographical variants; three human liver isolates from China, Alaska and North America were confined to M1 genotype and one isolate from a naturally infected rodent in Germany to M2 genotype. Later, Okamoto et al. [6] identified *cox1* sequences (fragment size of 391 bp) identical to the M1 variant in five isolates from Japan and in a single isolate from St. Lawrence Island (located west of mainland Alaska). Similar categorization into two genotypes was obtained from sequencing of the nuclear ribosomal DNA containing internal transcribed spacers (ITS1, ITS2) and adjoining rRNA coding region (1295 bp) in 13 screened isolates [7], and from non-coding intron of homeobox gene (331 bp) in 33 isolates [8], with samples from St. Lawrence Island slightly differing from the remaining Eurasian and continental North American samples. These studies implied that genetic structure of *E. multilocularis* is primarily homogeneous across its Holarctic distribution, with the estimated nucleotide diversity about 10 times lower than in *E. granulosus* s.l. [8-10]. Nevertheless, more recent typing procedures employing multiple mitochondrial loci and microsatellite analyses showed greater genetic diversity of the parasite than formerly thought [11-14]. Studies of the tandem repeat EmsB microsatellite polymorphisms revealed fine scale differences between the *E. multilocularis* populations, thus allowing to trace the spatial movements of parasite expansion from endemic areas, and to indicate possible anthropogenic translocations and deeper historical patterns of its spread across Holarctic [e.g. 15-18].

On the account of the increasing attention being paid to the genetic structuring in *E. multilocularis*, the aim of this review is to point to the erroneous designation, which prevails in literature over the last decade, for M1/M2 geographical variants that firstly genetically subdivided the species under consideration.

2. Inconsistencies in designation of M1 and M2 geographic variants of *E. multilocularis*

Using sequencing of 3 complete mitochondrial genes (*cox1*, 1608 sites; cytochrome b - *cob*, 1068 sites; NADH dehydrogenase subunit 2 - *nad2*, 882 sites; in total 3558 sites), Nakao and co-workers [11] reported in 2009 geographically induced partitioning of 76 isolates into three distinct clusters (Europe, Asia, North America), with shallower histories for populations sampled in Eurasia. This classification scheme referring to an evolutionary scenario in which *E. multilocularis* populations derived from glacial refugia have been spread and sustained by indigenous hosts referring to an evolutionary scenario was consecutively broadly adopted in further mitochondrial studies [e.g., 19-22]. A clear genetic subdivision of the species using the sequencing approach had been supplemented by classifying of indigenous human cases in Mongolia as belonging to a separate ´Mongolian´ strain [23]. However, in the above study of Nakao and co-workers [11], the Introduction section contains an erroneously reverse assignment of the two geographic *E. multilocularis* genotypes sensu Bowles et al. [4], with M1 confined to Europe and M2 to China, Alaska and North America, in which the first error in continental allocation implied the second error in geographical classification. In the same paper, the authors also reported the attribution of Japanese isolates published in Okamoto et al (1995) to M2, while the respective sequence exhibited the typical M1 haplotype. Given the great value of Nakao´s study in consolidating a global geographic pattern of parasite genetic variation and received considerable attention among *Echinococcus* working groups, a part of researchers unintentionally used this subdivision in their articles, without comparing data with the original studies of Bowles et al. [4] and Bowles and McManus [5].

To better assess the extent of this mislabeling, we have conducted a literature search from available sources. Retrieved articles including incorrect (inverted) and correct M1/M2 affiliations are listed in Table 1. We have found ten research papers and reviews containing this discrepancy since its first appearance in 2009. This assignment mode has prevailed in studies which mentioned M1/M2 partitioning from this year onwards (only 2 correct citations since publishing of Nakao´s study [11] were retrieved, see Table 1). Two later review papers of Nakao et al. [24] and Knapp et al. [25] from 2013 and 2015, which involved M1/M2 reversals and have high download rates, have likely increased the rate of the mislabeling.

Research papers and reviews with the erroneous classification originated from Asia [five studies; 11,26,27,28,29], North America [three studies; 11,14,20], and Europe [two studies; 25,30].

In light of recent findings of European-type strains (with the presumed higher pathogenicity) in wild canids, humans, a domestic dog, and a captive primate in Canada [14,19,20,31-34], the correct identification of European-type genotypes is especially important regarding estimation of public health risks. However, a study of Gesy et al. [20] provided the incorrect link of *nad1* sequence (395 bp) from coyotes that is, according to the authors, ´99-100% identical to the partial *E. multilocularis* sequence representing the European M1 genotype (GenBank Accession No. AJ237639)´. Nonetheless, a grouping of sequences in further examined genes (*nad2, cob, cox1*) with the European haplotypes has resulted into accurate determination of Canadian wildlife isolates as belonging to the European-like strain in related studies of the research group [19,20,31].

In a European study conducted on Slovak dog isolate, Antolová et al. [30] detected two single nucleotide polymorphisms in the *nad1* target (23[illegible] bp), and included the following incorrect statement in results: ´Isolate is more closely related to European M1 genotype (99.6% similarity) than to M2 genotype (99.2% similarity)´. Nevertheless, the inconsistency had only negligible influence on genetic interpretation of resulted data given the subtle variation. Furthermore, the abstract of that paper does contain essentially the correct note related to variants that ´BLAST analysis of *E. multilocularis nad1* gene revealed that the nucleotide sequence did not exactly match the previously identified M1 (AJ237639) and/or M2 genotype (AJ237640)´.

An accurate classification of M1/M2 genotypes according to the original study was retrieved in 12 literature sources (ten research articles and two reviews) [32,35-45], including self-citations of Bowles and colleagues (Table 1). Until 2009 it was the only way of M1/M2 designation, and since that time only two articles, focused on North American *E. multilocularis* issues [32,45], stated the affiliation of the geographical variants correctly. Coincidentally, sequences deposited for M1/M2 variants in GenBank for *cox1* (366 bp) and *nad1* (471 bp) partial genes (GenBank Accession Nos. M84668, M84669, AJ237639, AJ237640) have not included in ´description of isolate´ features of their geographical origin (except for the latter GenBank annotation), which has probably also contributed to the higher establishment of M1/M2 mislabeling.

3. Phylogenetic view of M1 and M2 variants related to current mitochondrial evidence

A further interesting feature shared with the issue of M1/M2 strains is that the M2 profile established based on *cox1* and *nad1* short sequences derived from the German vole isolate [4,5] does not reflect a major genetic pattern characteristic of European samples, as is obvious after almost three decades of accumulating evidence. As seen in the Bayesian consensus tree and in the maximum parsimony network derived from *cox1* sequences of 366 bp (Figs. 1 and 2), M2 is located apart from European isolates in North American clade with high statistical support (posterior probability value of 0.96). The M2 strain matched N2 reference haplotype sensu Nakao et al. [11] in forming a subclade that has been previously described only in north central region of North America (USA, Canada) in wild canids, rodents and human [11,31,46], not evidenced yet in the Eurasian continent.

The affinity of M2 originating from Europe to the North American assemblage has likely represented another confounding factor contributing to the erroneous strain designations. It was hypothesized that *E. multilocularis* had extended into Europe in the Late Pleistocene Epoch (approx. 130,000-11,700 years ago) through the migration of foxes [11] and its extensive spread may have occurred during the Holocene Epoch (approx. 11.700 years ago until present), in association with historical movements of red fox that had apparently expanded into most of their current range in Europe by the mid Holocene (8.200–4.200 years ago), including a large continuous population across Central Europe and the presence of distinct southern refugial populations [47]. Thus, in addition to recent findings of the N1 haplotype in the East Siberia, Russia [48] and N2-like structure in Austrian patient [49], a deeper historical pattern related to postglacial dispersal of *E. multilocularis* from the Bering land bridge (Beringia) following the last glacial maximum [50], with occasional introductions and persistence of ancestral North American forms in core endemic region of the parasite in Central Europe, might be plausible.

In the *cox1* Bayesian tree, the M1 geographical variant described in the 1990´s [4,5] clustered with the Asian reference haplotypes (sensu Nakao and colleagues) that were beyond Asia detected also in the St. Lawrence Island (A2, A4 haplotypes), situated in the northern Bering Sea between North America and northeastern Asia [11]. The adjacent ´European´ haplogroup clustered in a well-supported clade (posterior probability value of 0.96), and was composed of European-derived samples and recently recorded isolate from a pet dog in the U.S. that constitutes the first finding of a European strain in the U.S. [51].

Phylogenetic evidence from *nad1* (471 bp) sequences (Figs. 3 and 4) also showed M1 clustering with the Asian specimens (China, Japan). The M2 strain had the two additional nucleotide substitutions (230 C/T, 361 A/G), but one base substitution (96 T/C) shared with

the Asian samples and M1 differentiated this subgroup from an ´European´ cluster represented by specimens from Poland, Estonia, Germany and European-type specimens from Canada [14,19,41,52,53]. In addition, the two samples originating from the Xinjiang Province in northwestern China [54] and from northeast Iran (GenBank entry KT033489) also grouped with the European profiles, which indicate that continental differences are not so clear-cut in the short *nad1* fragment than in case of short *cox1* fragment.

Another strongly supported clade in the *nad1* phylogram (posterior probability value of 0.96) was established by samples from Canada (except for Canadian samples producing European-type patterns) examined by Gesy et al. and Schurer et al. [14,32]. Most of members of the cluster shared, interestingly, both additional nucleotide exchanges (230 C/T, 361 A/G) with M2 and in two isolates (CAN-G, CAN-C), the latter exchange was shared. This feature presents further argument to support some adhesion of M2 (originating from Europe) to samples of North American origin. Nevertheless, Canadian isolates possessed several additional nucleotide substitutions that differentiated the ´North American´ haplogroup from Eurasian specimens in the *nad1* partial sequence.

Phylogeny based on the widely used, but relatively short *cox1* and *nad1* sequences published in the 1990´s [4,5] has some limitations in resolving power and there is an increasing trend to use longer sequences and/or higher number of genes for important systematic and phylogeographical inferences in *Echinococcus* spp. [e.g., 55,56]. Notwithstanding, the use of M1/M2 geographical variants for comparative purposes has been revived to a certain extent in several recent Canadian studies employing *nad1* fragments of similar length (395 bp) that has extended into one European study (*nad1*, 235 bp) [14,20,30,31]. Two of these surveys have used M1/M2 references in haplotype network and/or phylogram in Result sections [14,30]. The present study was thus designed to provide a correct basis for further investigation of genetic diversity in *E. multilocularis* to avoid further misuse in haplotype geographic allocations.

4. Conclusions

With respect to the increasing awareness for the diversity of the causative agents of alveolar echinococcosis, we have summarized the current knowledge available on the use of the first genetically established geographical variants of *E. multilocularis* in literature, in an attempt to consolidate their correct affiliation in sense of the original description from the early 1990´s. A consistent M1/M2 linkage with geographical origin is of importance especially in light of the current assumption that European and Asian strains may have greater zoonotic potential

than North American strains. Furthermore, recent findings of a European strain in Canada have evoked interesting issues on possible dispersal of the European genotypes into areas of recognized endemicity of the N2 strain in North America, with concomitant public health hazards. In the phylogenetic analysis, the M1 strain grouped in both genes with the Asian haplotypes described more recently in China, Japan, Russia, Kazakhstan, South Korea, thus covering a similar geographical range similar to that delineated by the initial sequencing studies of Bowles et al. [4], Bowles and McManus [5], and Okamoto et al. [6] from the early 1990´s, unlike the M2 strain with the specific structure resembling North American assemblages, although homoplasy cannot be excluded as a driver for M2/N2 similarity. The continuing inverted designation (if occurs) of M1 and M2 might be therefore confounding for data interpretation in further studies. A noticeably high similarity between the reference M2 haplotype derived from the German vole isolate and from the Austrian patient to North American strains suggests that *E. multilocularis* populations have a common history and ancestral genotypes, peculiar for the given region, may in some extent maintain in historical endemic region where greater genetic structuring is expected and the parasite transmission is more intense.

Conflicts of interest

The authors state that they have no conflict of interest.

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Table 1
List of articles involving M1 and M2 geographical variants of *Echinococcus multilocularis*.

	Reference	Form of the article	Geographical origin; number of studied EM isolates; a way of incorrect designation
Erroneous designation of M1, M2 variants	Nakao et al. (2009)	research paper	Europe (Austria, France, Germany, Belgium, Slovakia), Asia (Kazakhstan, China, Japan), North America (USA - Alaska, Indiana, South Dakota); 76 isolates; reverse assignment of geographical genotypes - the M1 genotype associated with Europe and the M2 genotype with China, Alaska and North America in the Introduction section
	Nakao et al. (2013)	review	Reverse assignment of geographical genotypes - the M1 genotype associated with Europe and the M2 genotype with China, Alaska and North America in the section 5.3. of the review
	Gesy et al. (2013)	research paper	North America (Canada); 19 isolates; M1 genotype with the GenBank Accession No. AJ237539 (*nad1* partial gene) presented as European genotype, its sequence compared to nineteen examined cestodes from 7 coyotes in a 395 bp region of *nad1* in the Result section
	Gesy et al. (2014)*	research paper	North America (Canada); 121 isolates; reverse assignment of geographical genotypes - M1 affiliated with Europe, M2 with Japan, China, Alaska and North America in the Introduction section, correct allocation of the two variants in the Discussion section
	Knapp et al. (2015)	review	Reverse assignment of geographical genotypes - M1 affiliated with Europe, M2 with Japan, China, Alaska and North America in the section 4.1 of the review
	Wu et al. (2017)	research paper	Asia (China); 62 isolates; reverse assignment of geographical genotypes - M1 affiliated with Europe and M2 with China, Alaska and North America in the Background section
	Avcioglu et al. (2017)	research paper	Asia (Turkey); 1 isolate; reverse (inverted) assignment of geographical genotypes - M1 affiliated with Europe and M2 with China, Japan, Alaska and North America in the Introduction section
	Antolová et al. (2018)*	research paper	Europe (Slovakia); 1 isolate; reverse assignment of geographical genotypes - M1 affiliated with Europe and M2 with China, North America, Alaska and Japan in the Introduction section; partial *nad1* sequence (235 bp) of the studied dog isolate linked to the reversely designated reference genotypes, specifically to M1 named as European genotype (99.6% similarity) and to M2

			(99.2% similarity) in the Result section; the correct affiliation of the M2 isolate to Germany (Europe) as the recovery site in the end of the Result section
	Li et al. (2018)	research paper	Asia (China); 39 isolates; reverse assignment of geographical genotypes - the M1 genotype associated with Europe and the M2 genotype with China, Alaska and North America in the Introduction section
	Spotin et al. (2018)	review	Reverse assignment of geographical genotypes (except for Japan corresponding to M1) - M1 affiliated with Europe and Japan, M2 with Alaska, China, and North America in the 1. Introduction section of the review
Correct designation of M1, M2 variants	Bowles et al. (1992)**	research paper	Asia (China), North America (Alaska) (**M1**), Europe (Germany) (**M2**); 3 isolates
	Bowles and McManus (1993a)	research paper	Asia (China), North America (Alaska) (**M1**) Europe (Germany) (**M2**); 4 isolates
	Bowles and McManus (1993b)	review	
	Scott and McManus (1994)	research paper	North America **(M1)**, Europe (Germany) **(M2)**; 2 isolates
	Bowles et al. (1995)	research paper	Asia (China), North America (Alaska) (**M1**), Europe (Germany) (**M2**); 3 isolates
	Gasser et al. (1998a)	research paper	Europe (Germany) (**M2)**; 1 isolate
	Gasser et al. (1998b)	research paper	Europe (Germany, Switzerland) (**M2**); 2 isolates
	van Herwerden et al. (2000)	research paper	North America (Alaska) (**M1**), Europe (Germany), Asia (Japan); 13 isolates
	Kedra et al. (2000)	research paper	Europe (Poland); 4 isolates
	Thompson et al. (2006)	research paper	Asia (China), North America (Alaska) (**M1**), Europe (Germany) (**M2**); 2 isolates
	Šnábel et al. (2006)	research paper	Europe (Slovakia); 12 isolates
	Maillard et al. (2009)	research paper	Asia (China) (**M1**); 2 isolates
	Schurer et al. (2014)	research paper	North America (Canada); 12 isolates
	Massolo et al. (2014)	review	

* studies involving both erroneous and correct designations in different sections of the articles

**original description of M1/M2 genotypes

Table 2
Matching *cox1* sequences represented by node size in the haplotype network of *Echinococcus multilocularis*.

Node label	Matching sequences
M1_M84668	A5_CHN_AB461417 A6_ CHN_AB477011 A7_ CHN_AB477012 CHN_MH259774 CHN_KY354088 JPN_AB385610 SKO_AB780998 EMRUS2_AB688126 EMRUS3_AB688127 EMRUS4_AB688128 EMRUS5_AB688129 EMRUS6_AB688130 EMRUS7_AB688131 EMRUS8_AB688132 EMRUS9_AB688133 EMRUS12_AB777915
M2_M84669	N2_USAIn_AB461419 USA_MIN_AB353729 USA_SD_AB374425 CAN_SK2_KC582621
E1_AUT_AB416412	E3_FRA_AB461413 E5_SVK_AB461414 ROM_MF162280 POL_KY205691 POL_KY205690 EmRUS16_AB777912 USA_MIS_LC380931
N1_USA_AK_ AB461418	EmRUS14_AB777917 EmRUS15_AB777918 AUT_JF774724
CHN_MH259774	CHN_MG674419

Table 3
Matching *nad1* sequences represented by node size in the haplotype network of *Echinococcus multilocularis*.

Node label	Matching sequences
M1_AJ237639	AB018440_JPN MH259778_CHN EU704122_CHN KY094609_CHN
AB668376_GER	JX266825_POL AJ132907_POL AJ132908_POL MH986749_POL AY855918_EST AY389984_CHN KT033489_IRA JF751034_CANeu KF962559_CAN E
KC848462_CAN_W121	KF962555_CAN A KF962563_CAN I
KC848467_CAN_W180	KF962562_CAN H

References

[1] D.P. McManus, W. Zhang, J. Li, P.B. Bartley, Echinococcosis, Lancet 362 (2003) 1295–1304. https://doi-org/10.1016/S0140-6736(03)14573-4.

[2] A. Possenti, R. Manzano-Román, C. Sánchez-Ovejero, B. Boufana, G. La Torre, M. Siles-Lucas, A. Casulli, Potential Risk Factors Associated with Human Cystic Echinococcosis: Systematic Review and Meta-analysis, PLoS Negl. Trop. Dis. 10 (2016) e0005114. https://doi-org/10.1371/journal.pntd.0005114.

[3] P. Kern, A. Menezes da Silva, O. Akhan, B. Müllhaupt, K.A. Vizcaychipi, C. Budke, D. Vuitton, The Echinococcoses: Diagnosis, Clinical Management and Burden of Disease, Adv. Parasitol. 96 (2017) 259–369. https://doi-org/10.1016/bs.apar.2016.09.006.

[4] J. Bowles, D. Blair, D.P. McManus, Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing, Mol. Biochem. Parasitol. 54 (1992) 165–173. https://doi.org/10.1016/0166-6851(92)90109-W.

[5] J. Bowles, D.P. McManus, NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*, Int. J. Parasitol. 23 (1993a) 969–997. https://doi.org/10.1016/0020-7519(93)90065-7.

[6] M. Okamoto, Y. Bessho, M. Kamiya, T. Kurosawa, T. Horii, Phylogenetic relationships within *Taenia taeniaeformis* variants and other taeniid cestodes inferred from the nucleotide sequence of the cytochrome c oxidase subunit I gene, Parasitol. Res. 81 (1995) 451–458.

[7] H. Rinder, R.L. Rausch, K. Takahashi, H. Kopp, A. Thomschke, T. Löscher, Limited range of genetic variation in *Echinococcus multilocularis*, J. Parasitol. 83 (1997) 1045–1050.

[8] K.L. Haag, A.M. Araújo, B. Gottstein, M. Siles-Lucas, R.C. Thompson, A. Zaha, Breeding system in *Echinococcus granulosus* (Cestoda; Taeniidae): selfing or outcrossing? Parasitology 118 (1999) 63–71.

[9] J.M. Bart, I. Breyer, B. Gottstein, T. Romig, R. Piarroux, Development of molecular tools to explore genetic diversity in *Echinococcus multilocularis*, Helminthologia 40 (2003) 117–121.

[10] R.K. Davidson, T. Romig, E. Jenkins, M. Tryland, L.J. Robertson, The impact of globalisation on the distribution of *Echinococcus multilocularis*, Trends Parasitol. 28 (2012) 2239–2247. https://doi.org/10.1016/j.pt.2012.03.004.

[11] M. Nakao, N. Xiao, M. Okamoto, T. Yanagida, Y. Sako, A. Ito, Geographic pattern of genetic variation in the fox tapeworm *Echinococcus multilocularis*, Parasitol. Int. 58 (2009) 384–389. https://doi-org/10.1016/j.parint.2009.07.010.

[12] A. Casulli, J.M. Bart, J. Knapp, G. La Rosa, G. Dusher, B. Gottstein, A. Di Cerbo, M.T. Manfredi, C. Genchi, R. Piarroux, E. Pozio, Multi-locus microsatellite analysis supports the hypothesis of an autochthonous focus of *Echinococcus multilocularis* in northern Italy, Int. J. Parasitol. 39 (2009) 837–842. https://doi.org/10.1016/j.ijpara.2008.12.001.

[13] J. Knapp, J.M. Bart, S. Maillard, B. Gottstein, R. Piarroux, The genomic *Echinococcus* microsatellite EmsB sequences: from a molecular marker to the epidemiological tool, Parasitology 137 (2010) 439–449. https://doi-org/10.1017/S0031182009991612.

[14] K.M. Gesy, J.M. Schurer, A. Massolo, S. Liccioli, R.T. Elkin, R. Alisauskas, E.J. Jenkins, Unexpected diversity of the cestode *Echinococcus multilocularis* in wildlife in Canada, Int. J. Parasitol. Parasites Wildl. 3 (2014) 81–87. https://doi-org/ 10.1016/j.ijppaw.2014.03.002.

[15] J.M. Bart, J. Knapp, B. Gottstein, F. El-Garch, P. Giraudoux, M.L. Glowatzki, H. Berthoud, S. Maillard, R. Piarroux, EmsB, a tandem repeated multi-loci microsatellite, new tool to investigate the genetic diversity of *Echinococcus multilocularis*, Infect. Genet. Evol. 6 (2006) 390–400. https://doi.org/10.1016/j.meegid.2006.01.006.

[16] J. Knapp, J.M. Bart, P. Giraudoux, M.L. Glowatzki et al., Genetic Diversity of the Cestode *Echinococcus multilocularis* in Red Foxes at a Continental Scale in Europe, PLOS Negl. Trop. Dis. 3 (2009) e452. https://doi-org/10.1371/journal.pntd.0000452.

[17] J. Knapp, S. Staebler, J.M. Bart, A. Stien, N.G. Yoccoz, C. Drögemüller, B. Gottstein, P. Deplazes, *Echinococcus multilocularis* in Svalbard, Norway: microsatellite genotyping to investigate the origin of a highly focal contamination, Infect. Genet. Evol. 12 (2012) 1270–1274. https://doi-org/10.1016/j.meegid.2012.03.008.

[18] G. Umhang, J. Knapp, V. Hormaz, F. Raoul, F. Boué, Using the genetics of *Echinococcus multilocularis* to trace the history of expansion from an endemic area, Infect. Genet. Evol. 22 (2014) 142–149. https://doi.org/10.1016/j.meegid.2014.01.018.

[19] E.J. Jenkins, A.S. Peregrine, J.E. Hill, C. Somers, K. Gesy, B. Barnes, B. Gottstein, L. Polley, Detection of European strain of *Echinococcus multilocularis* in North America [letter], Emerg. Infect. Dis. 18 (2012) 1010–1012. https://doi-org/10 10.3201/eid1806.111420.

[20] K. Gesy, J.E. Hill, H. Schwantje, S. Liccioli, E.J. Jenkins, Establishment of a European-type strain of *Echinococcus multilocularis* in Canadian wildlife, Parasitology 140 (2013) 1133–1137. https://doi-org/10.1017/S0031182013000607.

[21] V. Šnábel, C. Calma, S.O. Georgescu, S. Cavallero, S. D'Amelio, Z. Vasilková, A. Ito, Genetic survey of alveolar and cystic echinococcoses in Romania: First molecular evidence of *Echinococcus multilocularis* in humans in the country, Helminthologia 54 (2017) 189–198. https://doi.org/10.1515/helm-2017-0025.

[22] J. Karamon, K. Stojecki, M. Samorek-Pierog, E. Bilska-Zajac, M. Rozycki, E. Chmurzynska, J. Sroka, J. Zdybel, T. Cencek, Genetic diversity of *Echinococcus multilocularis* in red foxes in Poland: the first report of a haplotype of probable Asian origin, Folia Parasitol. (Praha) 64 (2017) 007. https://doi-org/10.14411/fp.2017.007.

[23] A. Ito, G. Agvaandaram, O.E. Bat-Ochir, B. Chuluunbaatar et al., Histopathological, serological, and molecular confirmation of indigenous alveolar echinococcosis cases in Mongolia, Am. J. Trop. Med. Hyg. 82 (2010) 266–269. https://doi-org/10.4269/ajtmh.2010.09-0520.

[24] M. Nakao, A. Lavikainen, T. Yanagida, A. Ito, Phylogenetic systematics of the genus *Echinococcus* (Cestoda: Taeniidae), Int. J. Parasitol. 43 (2013) 1017-1029. https://doi-org/10.1016/j.ijpara.2013.06.002.

[25] J. Knapp, B. Gottstein, U. Saarma, L. Millon, Taxonomy, phylogeny and molecular epidemiology of *Echinococcus multilocularis*: From fundamental knowledge to health ecology, Vet. Parasitol. 213 (2015) 85–91. https://doi-org/10.1016/j.vetpar.2015.07.030.

[26] C. Wu, W. Zhang, B. Pan, L. Fan et al., Genetic variation of mitochondrial genes among *Echinococcus multilocularis* isolates collected in western China, Parasit. Vectors 10 (2017) 265. https://doi-org/10.1186/s13071-017-2172-y.

[27] H. Avcioglu, E. Guven, I. Balkaya, R. Kirman, M.M. Bia, H. Gulbeyen, A. Kurt, S. Yaya, S. Demirtas, First detection of *Echinococcus multilocularis* in rodent intermediate hosts in Turkey, Parasitology 144 (2017) 1821–1827. https://doi-org/10.1017/S0031182017001226.

[28] J.Q. Li, L. Li, Y.L. Fan, B.Q. Fu, X.Q. Zhu, H.B. Yan, W.Z. Jia, Genetic Diversity in *Echinococcus multilocularis* From the Plateau Vole and Plateau Pika in Jiuzhi County, Qinghai Province, China, Front. Microbiol. 9 (2018) 2632. https://doi-org/10.3389/fmicb.2018.02632.

[29] A. Spotin, B. Boufana, E. Ahmadpour, A. Casulli, M. Mahami-Oskouei, S. Rouhani, A. Javadi-Mamaghani, F. Shahrivar, P. Khoshakhlagh, Assessment of the global pattern of

genetic diversity in *Echinococcus multilocularis* inferred by mitochondrial DNA sequences, Vet. Parasitol. 262 (2018) 30–41, https://doi:10.1016/j.vetpar.2018.09.013.

[30] D. Antolová, B. Víchová, J. Jarošová, V. Gál, B. Bajužík, Alveolar echinococcosis in a dog; analysis of clinical and histological findings and molecular identification of *Echinococcus multilocularis*, Acta Parasitol. 63 (2018) 486–494. https://doi-org/ 10.1515/ap-2018-0058.

[31] K.M. Gesy, E.J. Jenkins, Introduced and Native Haplotypes of *Echinococcus multilocularis* in Wildlife in Saskatchewan, Canada, J. Wildl. Dis. 51 (2015) 743–748. https://doi-org/10.7589/2014-08-214.

[32] J.M. Schurer, K.M. Gesy, B.T. Elkin, E.J. Jenkins, *Echinococcus multilocularis* and *Echinococcus canadensis* in wolves from western Canada, Parasitology 141 (2014) 159–163. https://doi-org/10.1017/S0031182013001716.

[33] E.F. Christiansen, C.G. Himsworth, J.E. Hill, M. Haulena, Infection of a Goeldi's monkey (*Callimico goeldii*) with a European strain of *Echinococcus multilocularis* in a Canadian institution, J. Zoo. Wildl. Med. 46 (2015) 378-381. https://doi-org/ 10.1638/2014-0123R1.1.

[34] A. Massolo, C. Klein, K. Kowalewska-Grochowska, S. Belga et al., European *Echinococcus multilocularis* Identified in Patients in Canada, N. Engl. J. Med. 381 (2019) 384-385. https://doi-org/10.1056/NEJMc1814975.

[35] J. Bowles, D.P. McManus, Molecular variation in *Echinococcus*, Acta Trop. 53 (1993b) 291–305, https://doi.org/10.1016/0001-706X(93)90035-A.

[36] J.C. Scott, D.P. McManus, The random amplification of polymorphic DNA can discriminate species and strains of *Echinococcus*, Trop. Med. Parasitol. 45 (1994) 1–4.

[37] J. Bowles, D. Blair, D.P. McManus, A molecular phylogeny of the genus *Echinococcus*, Parasitology 110 (1995) 317-328.

[38] R.B. Gasser, X. Zhu, D.P. McManus, Display of sequence variation in PCR-amplified mitochondrial DNA regions of *Echinococcus* by single-strand conformation polymorphism, Acta Trop. 71 (1998a), 107–115. https://doi-org/10.1016/S0001-706X(98)00052-7.

[39] R.B. Gasser, X. Zhu, D.P. McManus, Dideoxy fingerprinting: application to the genotyping of *Echinococcus*, Int. J. Parasitol. 28 (1998b), 1775–1779. https://doi.org/10.1016/S0020-7519(98)00126-X.

[40] L. van Herwerden, R.B. Gasser, D. Blair, ITS-1 ribosomal DNA sequence variants are maintained in different species and strains of *Echinococcus*, Int. J. Parasitol. 30 (2000) 157–169. https://doi.org/10.1016/S0020-7519(00)00002-3.

[41] A.H. Kedra, Z. Swiderski, V.V. Tkach, B. Rocki, J. Pawlowski, Z. Pawlowski, Variability within NADH dehydrogenase sequences of *Echinococcus multilocularis*, Acta Parasitol. 45 (2000), 353–355.

[42] R.C. Thompson, A.C. Boxell, B.J. Ralston, C.C. Constantine, R.P. Hobbs, T. Shury, M.E. Olson, Molecular and morphological characterization of *Echinococcus* in cervids from North America, Parasitology 132 (2006) 439–447. https://doi.org/10.1017/S0031182005009170.

[43] V. Šnábel, M. Miterpáková, S. D´Amelio, M. Busi, D. Hartková, Ľ. Turčeková, C. Maddox-Hyttel, P. Skuce, P. Dubinský, Genetic structuring and differentiation of *Echinococcus multilocularis* in Slovakia assessed by sequencing and isoenzyme studies, Helminthologia 43 (2006) 196–202. https://doi.org/10.2478/s11687-006-0037-6.

[44] S. Maillard, B. Gottstein, K.L. Haag, S. Mir, I. Colovic, M.C. Benchikh-Elfegoun, J. Knapp, R. Piarroux, The EmsB tandemly repeated multilocus microsatellite: a new tool to investigate genetic diversity of *Echinococcus granulosus* sensu lato, J. Clin. Microbiol. 47 (2009) 3608–3616. https://doi.org/10.1128/JCM.00938-09.

[45] A. Massolo, S. Liccioli, C. Budke, C. Klein, *Echinococcus multilocularis* in North America: the great unknown, Parasite 21 (2014) 73. https://doi-org/10.1051/parasite/2014069.

[46] H. Yamasaki, M. Nakao, K. Nakaya, P.M. Schantz, A. Ito, Genetic analysis of *Echinococcus multilocularis* originating from a patient with alveolar echinococcosis occurring in Minnesota in 1977, Am. J. Trop. Med. Hyg. 79 (2008) 245–247.

[47] R. Sommer, N. Benecke, Late-Pleistocene and early Holocene history of the canid fauna of Europe (Canidae), Mamm. Biol. 70 (2005) 227–241, https://doi.org/10.1016/j.mambio.2004.12.001

[48] S.V. Konyaev, T. Yanagida, M. Nakao, G.M. Ingovatova et al., Genetic diversity of *Echinococcus* spp. in Russia, Parasitology 140 (2013) 1637–1647. https://doi.org/ 10.1017/S0031182013001340.

[49] V. Šnábel, T. Sréter, B. Gottstein, J. Gawor, T. Romig, M. Miterpáková, Genetic diversity of *Echinococcus multilocularis* cestodes in Europe as determined by mitochondrial and nuclear sequences, in: F.N. Indyuhova (Ed.), Theory and Practice of

Parasitic Disease Control,. All Russian Scientific Research Institute of Fundamental and Applied Parasitology of Animals and Plants, Moscow, 2019, pp. 754 -758.

[50] E.P. Hoberg, K.E. Galbreath, J.A. Cook, S.J. Kutz, L. Polley, Northern host-parasite assemblages: history and biogeography on the borderlands of episodic climate and environmental transition, Adv. Parasitol. 79 (2012) 1–97. https://doi.org/10.1016/B978-0-12-398457-9.00001-9.

[51] K. Kuroki, *Echinococcus Multilocularis* in a Pet Dog, MU VMDL Newsletter, Summer 2018 (2018), p. 2.

[52] M. Dybicz, P.K. Borkowski, M. Padzik, W. Baltaza, L. Chomicz, Molecular determination of suspected alveolar echinococcosis requiring surgical treatment in human cases from Poland, Ann. Parasitol. 64 (2018) 339–342. https://doi.org/10.17420/ap6404.169.

[53] E. Moks, U. Saarma, H. Valdmann, *Echinococcus multilocularis* in Estonia, Emerg. Infect. Dis. 11 (2005) 1973–1974. https://doi: 10.3201/eid1112.050339

[54] Y.R. Yang, M.C. Rosenzvit, L.H. Zhang, J.Z. Zhang, D.P. McManus, Molecular study of *Echinococcus* in west-central China, Parasitology 131(Pt 4) (2005) 547–555. https://doi.org/10.1017/S0031182005007973.

[55] T. Romig, D. Ebi, M. Wassermann, Taxonomy and molecular epidemiology of *Echinococcus granulosus* sensu lato, Vet. Parasitol. 213 (2015) 76-84. https://doi: 10.1016/j.vetpar.2015.07.035.

[56] L. Kinkar, T. Laurimäe, C. Acosta-Jamett, V. Andresiuk et al., Global phylogeography and genetic diversity of the highly zoonotic tapeworm *Echinococcus granulosus* sensu stricto genotype G1, Int. J. Parasitol. 48 (2018) 729-742. https://doi: 10.1016/j.ijpara.2018.03.006.

Figure captions

Fig. 1. Bayesian consensus tree showing phylogenetic relationships for M1 and M2 geographic variants of *Echinococcus multilocularis* sensu Bowles et al. (1992), using mitochondrial sequences of cytochrome *c* oxidase subunit 1 (*cox1*, 366 bp) in comparison to available GenBank sequences. *Echinococcus granulosus* sensu stricto (G1 genotype) was used as the outgroup. Posterior probability values are shown next to relevant nodes.
Reference isolates included in the phylogram: M1 (China, Alaska, North America), M2 (Germany), N1 (St. Lawrence Island, Alaska, USA), N2 (Indiana, USA), E1 (Austria), E3 (France), E5 (Slovakia), A1 (Kazakhstan), A5 (China). Geographic affiliations of the screened isolates: CAN (Canada), RUS (Russia), CHN (China), JPN (Japan), SKO (South Korea), KAZ (Kazakhstan), MON (Mongolia), AUT (Austria), GER (Germany), POL (Poland), ROM (Romania), FRA (France).

Fig. 2. The maximum parsimony network depicting the mutational relationships for M1 and M2 geographic variants of *Echinococcus multilocularis* sensu Bowles and McManus (1993a) using mitochondrial sequences of cytochrome *c* oxidase subunit 1 (*cox1*, 366 bp). Labeled circles represent distinct haplotypes, transversal bars at branches represent point mutations, the size of the circles correlates with haplotype frequency. Shared sequences with haplotype representatives labeled in circles are listed in Table 2.

Fig. 3. Bayesian consensus tree showing phylogenetic relationships for M1 and M2 geographic variants of *Echinococcus multilocularis* sensu Bowles et al. (1992), using mitochondrial sequences of NADH dehydrogenase subunit 1 (*nad1*, 471 bp) in comparison to available GenBank sequences. *Echinococcus granulosus* sensu stricto (G1 genotype) was used as the outgroup. Posterior probability values are shown next to relevant nodes.
Reference isolates included in the phylogram: M1 (China, Alaska, North America); M2 (Germany); CAN-eu, a European type strain detected by Jenkins et al. (2012) in a dog in western Canada; CAN-E, most common haplotype E found in a study of Gesy et al. (2014) in wild canids in western Canada resembling the European structure.
Geographic affiliations of the screened isolates: CAN (Canada), CHN (China), JPN (Japan), IRA (Iran), AUT (Austria), GER (Germany), POL (Poland), EST (Estonia).

Fig. 4. The maximum parsimony network depicting the mutational relationships for M1 and M2 geographic variants of *Echinococcus multilocularis* sensu Bowles and McManus (1993a) using mitochondrial sequences of NADH dehydrogenase subunit 1 (*nad1*, 471 bp). Labeled circles represent distinct haplotypes, transversal bars at branches represent point mutations, the size of the circles correlates with haplotype frequency. Shared sequences with haplotype representatives labeled in circles are listed in Table 3.

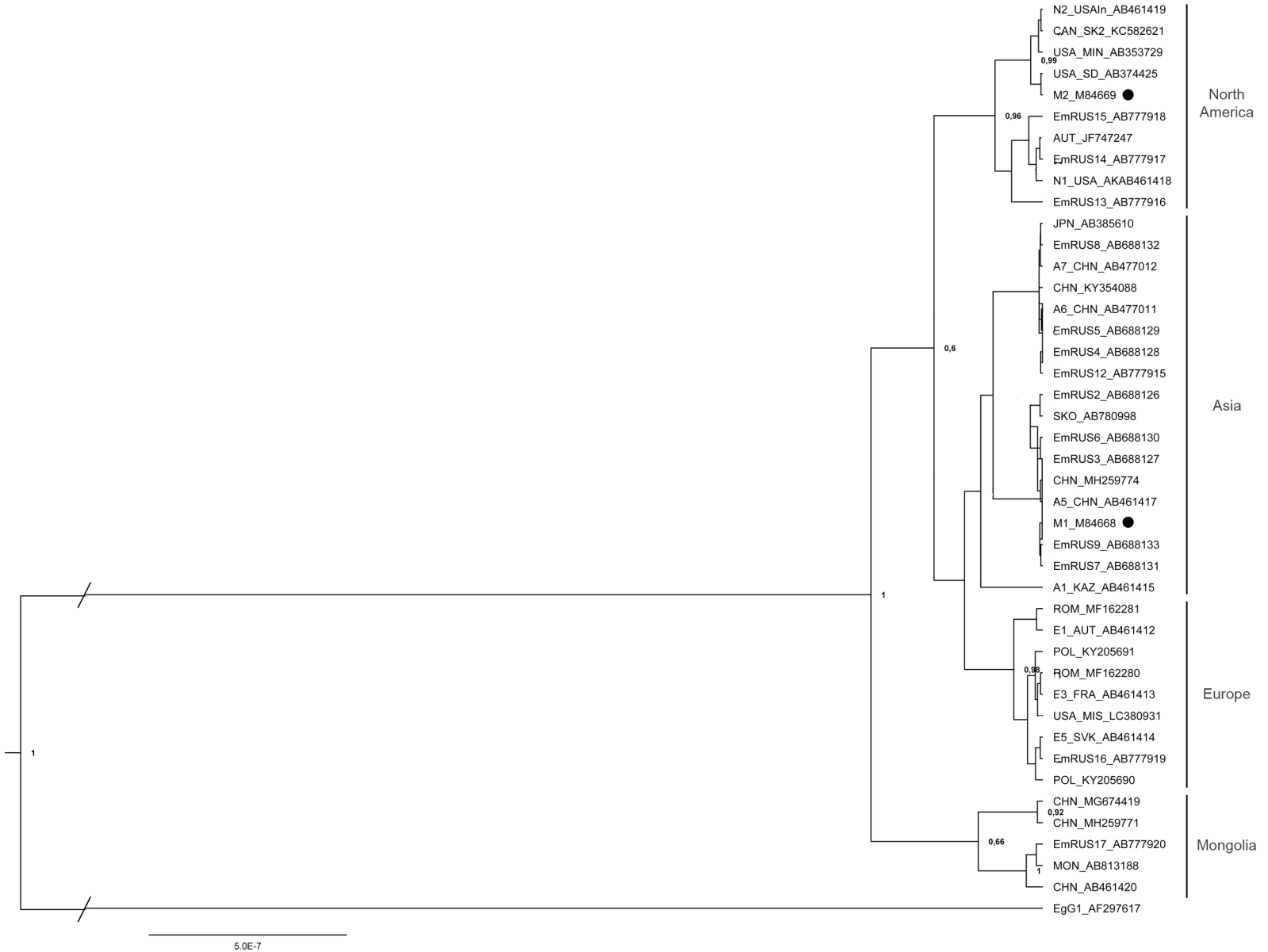
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Figure 1

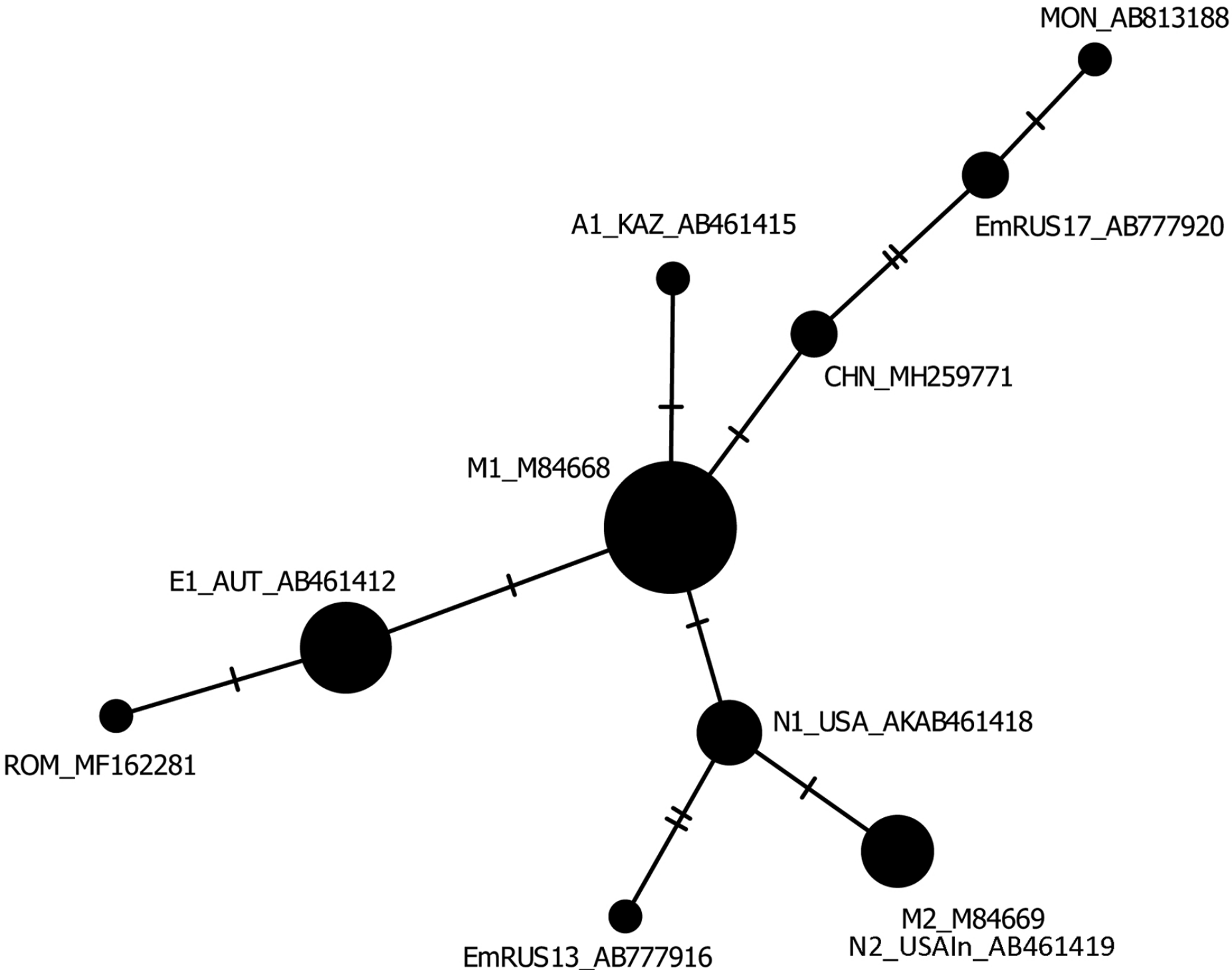
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Figure 2

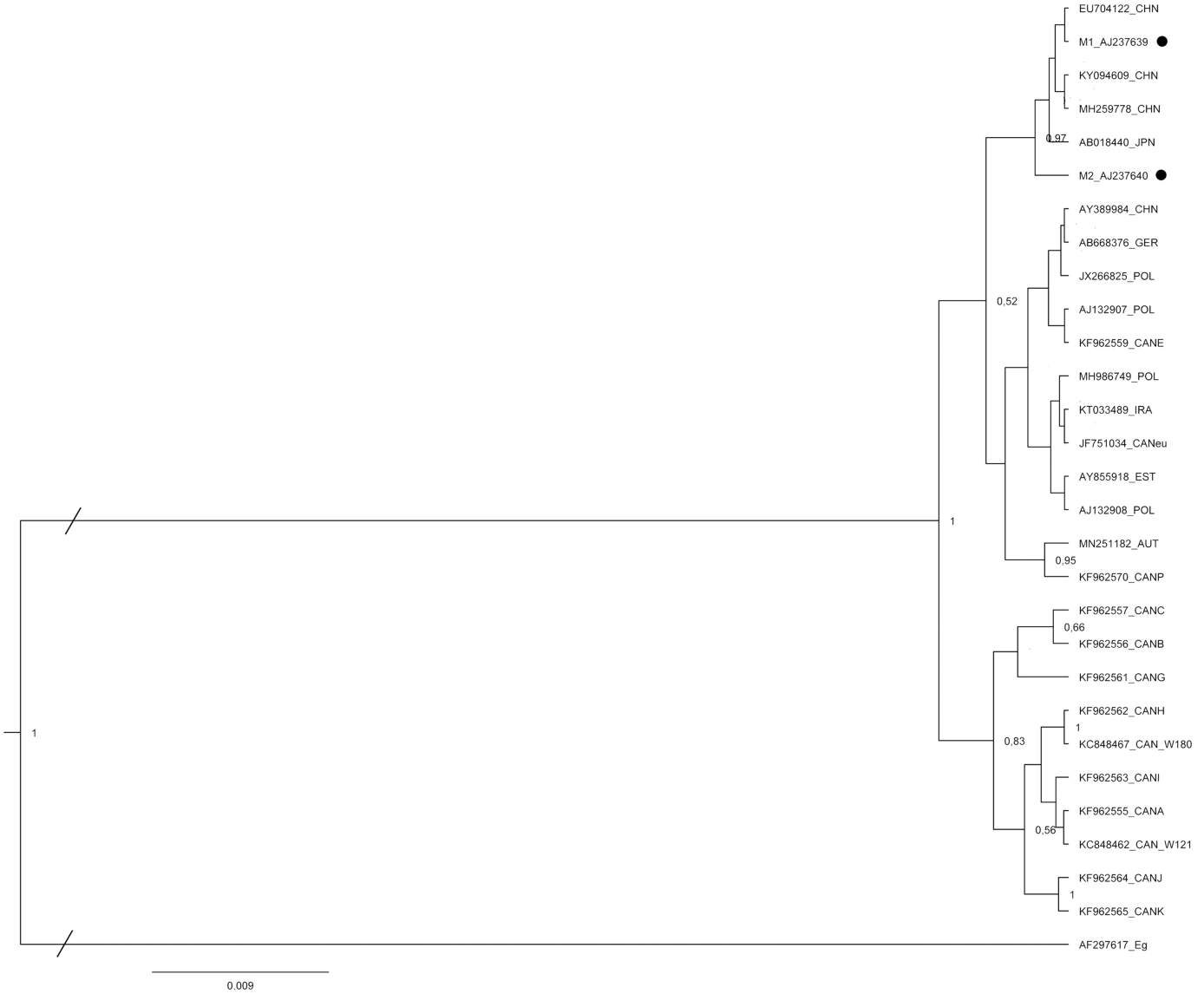
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Figure 3

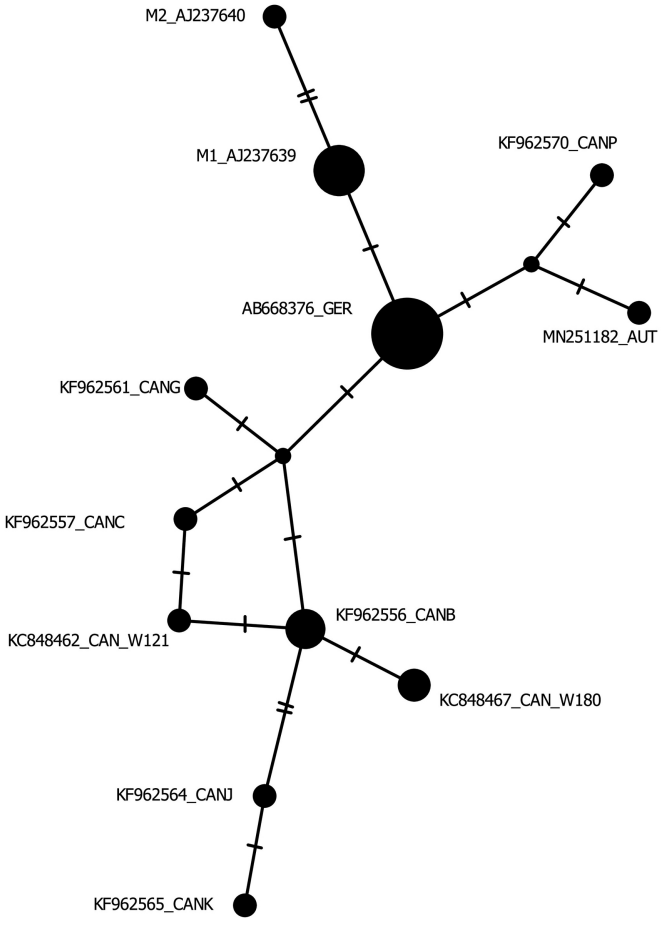
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Figure 4