



Specific status of *Echinococcus canadensis* (Cestoda: Taeniidae) inferred from nuclear and mitochondrial gene sequences [☆]



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ABSTRACT

The specific status of *Echinococcus canadensis* has long been controversial, mainly because it consists of the mitochondrial lineages G6, G7, G8 and G10 with different host affinity: G6 (camel strain) and G7 (pig strain) with domestic cycles and G8 (cervid strain) and G10 (Fennoscandian cervid strain) with sylvatic or semi-domestic cycles. There is an argument whether the mitochondrial lineages should be recognised as separate species which correspond to the biological or epidemiological aggregation. In the present study, the specific status of *E. canadensis* was investigated using mitochondrial DNA and single copy nuclear DNA markers. Nucleotide sequences of complete mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and partial nuclear phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*) were determined for 48 isolates of *E. canadensis* collected from different hosts in a wide range of regions. The mitochondrial phylogeny of *cox1* showed that all the isolates were clearly divided into three clades corresponding to G6/G7, G8 and G10. Five and three alleles were confirmed at *pepck* and *pold* loci, respectively. These alleles were generally divided into two groups corresponding to G6/G7 or G8 and G10. However, allele sharing was confirmed among individuals belonging to different lineages. The allele sharing occurred primarily in regions where different mitochondrial DNA lineages were found in sympatry. The resultant nuclear mitochondrial discordance suggests the genetic exchangeability among *E. canadensis* isolates belonging to different lineages. An apparently mosaic parasite fauna that reflects faunal mixing due to natural and anthropogenic disturbance, including introductions and invasion, precludes us from designating each of G6/G7, G8 and G10 into a different species.

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1. Introduction

Echinococcosis or hydatidosis is one of the most serious helminthic zoonoses with a worldwide distribution. It affects various internal organs, particularly liver, of terrestrial mammals including livestock, wildlife and humans. The causative agents are the cestodes belonging to the genus *Echinococcus* Rudolphi, 1801 (Cestoda: Taeniidae). *Echinococcus* spp. utilise canids or felids as

definitive hosts and a variety of ungulates, rodents or lagomorphs as intermediate hosts.

Taxonomy of *Echinococcus* has continuously been revised based on morphology, ecological features (hosts) and molecular phylogeny (Nakao et al., 2013a, b; see review in Lymbery, 2017). *Echinococcus granulosus* sensu lato (s.l.) is the most prevalent morphospecies in the genus with a worldwide distribution. It causes unilocular hydatid cysts in a variety of ungulate intermediate hosts, and hence the species complex has been divided into the 'strains' based to a large degree on the host affinity (Eckert et al., 2001; Thompson and McManus, 2002). The taxonomic status of these strains has been verified by molecular analyses, and nine mitochondrial DNA (mtDNA) lineages (G1–G8 and G10) have been

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demonstrated and formally recognised (Bowles et al., 1992, 1994; Bowles and McManus, 1993; Lavikainen et al., 2003). These mitochondrial lineages have conventionally been referred as “genotypes”. In addition to *E. granulosus* sensu stricto (s.s.) (for G1 and G2 sheep strains and G3 buffalo strain), nominal taxa currently considered as valid and characterised by typical unilocular hydatid cysts include *Echinococcus equinus* (G4 horse strain), *Echinococcus ortleppi* (G5 cattle strain), *Echinococcus canadensis* (G6 camel strain, G7 pig strain, G8 cervid strain and G10 Fennoscandian cervid strain) and *Echinococcus felidis* (lion strain) (see review in Nakao et al., 2013a). Although taxonomic diversity within the genus has mostly been settled following intensive molecular phylogenetic and phylogeographic analyses using parasite isolates from a variety of hosts worldwide, the status of *E. canadensis* remains a topic of debate (Nakao et al., 2013a, 2015; Lymbery et al., 2015a,b; Lymbery 2017; Ito et al., 2017).

Echinococcus canadensis was originally established and described as a variant of *E. granulosus* with hydatids in Eurasian reindeer, *Rangifer tarandus tarandus*, from an introduced population at Aklavik, Northwest Territories, Canada (Cameron, 1960), and later validated as a subspecies of *E. granulosus* (Sweatman and Williams, 1963). Concurrently, another subspecies of *E. granulosus* was described in free-ranging and endemic cervid intermediate hosts (moose, *Alces alces gigas*) from northern Ontario, Canada (Sweatman and Williams, 1963). These two North American subspecies were formally recognised as *Echinococcus granulosus canadensis* and *Echinococcus granulosus borealis*, occurring in a semi-domestic wolf/dog-reindeer cycle and a wolf-moose sylvatic cycle, respectively (Sweatman and Williams, 1963). Cestodes attributable to these subspecies apparently were prepared as stained, whole-mounted, specimens that were used as the basis for descriptions and differential diagnosis. However, as far as we examined, these slides have not been located in North America or in New Zealand where these studies were conducted. The only remaining specimens were formalin-fixed and ethanol-preserved strobilate tapeworms from domestic dogs experimentally infected with metacestodes of reindeer or moose (Sweatman and Williams, 1963). These fluid specimens were labelled as types of *E. granulosus canadensis* (USNPC 59725) and *E. granulosus borealis* (USNPC 59724), and archived in the former United States National Parasite Collection (USNPC).

Although the validity of these subspecies was denied by Rausch (2003) due to their assumed sympatric distribution, *E. canadensis* was subsequently resurrected as a putative species (containing *E. g. canadensis* and *E. g. borealis*) consisting of the G6, G7, G8 and G10 of *E. granulosus* based on molecular phylogenetic analysis using mtDNA genome sequences (see review in Nakao et al., 2013a). There still remains, however, a debate whether the mitochondrial lineages should be treated as 2–3 separate species with different biological and epidemiological features, or as a complex of poorly differentiated populations within a single species influenced by widespread translocation, introduction and gene flow at some geographic localities and in regions of primary and secondary contact (Nakao et al., 2013a,b, 2015; Lymbery et al. 2015a,b; Ito et al., 2017).

Resolution of taxonomic status in this assemblage has been hindered by the absence of conclusive evidence. For example, the putative genetic relationships among *E. canadensis* mitochondrial lineages have been evaluated primarily based on sequences derived from mtDNA and generally there has been an absence of sequence data from known strobilate adults linked to voucher specimens. To draw definitive conclusions on the specific status of *E. canadensis*, it is essential to use nuclear DNA (nDNA) markers for the investigation of genetic exchangeability among *E. canadensis* lineages, especially in the regions where different lineages are distributed in sympatry, such as in the Russian Far East, North

America and Mongolia (Thompson et al., 2006; Moks et al., 2008; Himsworth et al., 2010; Ito et al., 2013, 2017; Konyaev et al., 2013; Schurer et al., 2013). In the present study, genetic relationships among the mitochondrial lineages of *E. canadensis* were investigated using mtDNA and single copy nDNA markers. Isolates of *E. canadensis* collected from a variety of host animals in a wide range of regions were examined. Furthermore, a molecular identification was attempted to clarify the mitochondrial lineages of the archival type specimens of *E. granulosus canadensis* and *E. granulosus borealis* deposited by Sweatman and Williams (1963).

2. Materials and methods

2.1. Geographic isolates and DNA sequencing

A total of 48 isolates of *E. canadensis* were examined for the two nuclear gene markers in this study. The specimens were collected from various animals, including humans, in a wide range of geographic areas (Table 1). Some of them were previously used for the mtDNA analyses and the detailed data have already been published. Parasite specimens were preserved in 70–100% ethanol until DNA extraction. The genomic DNA of each parasite isolate was extracted from a single cyst or an adult tapeworm, by using a spin column purification kit (DNA Mini kit or DNeasy Tissue kit, Qiagen, Germany). Alkaline lysates were also made for some tapeworms as reported previously (Hüttner et al., 2008). Extracted DNA or the crude lysate was used as a template for PCR. For the mtDNA gene marker, cytochrome *c* oxidase subunit I (*cox1*) was amplified by PCR and the complete *cox1* gene sequences were determined using previously reported primer pairs under the same conditions (Hüttner et al., 2008). For nDNA markers, a part of phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*) were selected, for their single-copy presence in *Echinococcus multilocularis* (*Echinococcus* genome project, Wellcome Trust Sanger Institute, UK, <http://www.sanger.ac.uk/>) and usefulness in differentiating *E. canadensis* lineages (Knapp et al., 2011). These nDNA gene markers were amplified by previously reported and newly designed primers: *pepck* F1 (5'-ACC GAG ACC AAG CTC ACA-3') and *pepck* R (5'-AGC CCA TTC AAT CAC ACG GAT ATT-3') (Knapp et al., 2011), *pold* F1 (5'-CTA AGC GGA ATT ATC ATT CC-3') and *pold* R1 (5'-CAA CGA CTA ACA GTA TTG ACA-3'). The primers were designed to amplify regions including introns to increase the chance of finding variations. PCR was performed in 20 µL containing 0.5 units of ExTaq Hot Start Version (TaKaRa, Japan), 0.2 mM of dNTP, 1 × Ex Taq Buffer with a final MgCl₂ concentration of 2.0 mM, 15 pmol of each primer and 1.0 µL of genomic DNA. PCR amplification consisted of initial denaturation of 94 °C for 2 min, 40 cycles of 94 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s, and a terminal extension at 72 °C for 1 min. PCR products were treated with illustra ExoProStar (GE Healthcare, UK) to remove excess primers and dNTPs, and subsequently sequenced directly with a BigDye Terminator v3.1 and a 3500 DNA sequencer (Life Technologies, USA). When double peaks were confirmed in the sequencing of nuclear genes, purified PCR products were ligated into a pGEM-T plasmid vector (Promega, USA) and subsequently introduced into *Escherichia coli* DH5α. At least 10 colonies were picked up from agar plates and their insert DNAs were sequenced directly to confirm allelic polymorphism.

2.2. Archival analysis of the museum specimens

Formalin-fixed adult worms of *E. granulosus canadensis* and *E. granulosus borealis*, deposited by Sweatman and Williams (1963) in the USNPC curated by the United States Department of Agriculture (now Smithsonian Institution, National Museum of Natural

Table 1
Isolates of *Echinococcus ortleppi* and *Echinococcus canadensis* mitochondrial DNA lineages used for molecular analyses.

Mitochondrial lineages	Localities	Parasite stages ^a (hosts)	Nuclear gene alleles		<i>cox1</i> haplotypes	Accession No. of <i>cox1</i>	References for <i>cox1</i> sequences
			<i>pepck</i>	<i>pold</i>			
G5 (<i>E. ortleppi</i>)	Argentina		<i>E. ortleppi</i>	<i>E. ortleppi</i>	<i>E. ortleppi</i>	AB235846	Nakao et al. (2007)
G6	Kazakhstan	L (camel)	A	A	EcG6	AB208063	Nakao et al. (2007)
G6	Mongolia	A (wolf)	A	A	EcMGL1	AB813182	Ito et al. (2013)
G6	Mongolia	A (wolf)	A	A	EcMGL2 (=EcRUS1)	AB813183	Ito et al. (2013)
G6	Mongolia	A (wolf)	A	A	EcMGL2 (=EcRUS1)	AB813183	Ito et al. (2013)
G6	Mongolia	A (wolf)	A	A	EcMGL2 (=EcRUS1)	AB813183	Ito et al. (2013)
G6	Asian Russia	L (human)	A	A	EcRUS1 (=EcMGL2)	AB688142	Konyaev et al. (2012)
G6	Asian Russia	A (wolf)	A	A	EcRUS2	AB777909	Nakao et al. (2013b)
G6	Asian Russia	A (wolf)	E	n.d.	EcRUS1 (=EcMGL2)	AB688142	Nakao et al. (2013b)
G6	Far East Russia	L (reindeer)	C	C	EcRUS1 (=EcMGL2)	AB688142	Nakao et al. (2013b)
G6	Ethiopia	L (camel)	A	A	EcETH2	AB777923	Nakao et al. (2013b)
G6	Ethiopia	L (camel)	A	A	EcETH1	AB777922	Nakao et al. (2013b)
G6	Mongolia	L (camel)	A	A	EcMGL16	LC184603	This study
G6	Peru	L (goat)	A	A	EcRUS1 (=EcMGL2)	AB688142	Nakao et al. (2013b)
G6	Peru	L (goat)	A	A	EcRUS1 (=EcMGL2)	AB688142	Nakao et al. (2013b)
G6	Peru	L (goat)	A	A	EcRUS1 (=EcMGL2)	AB688142	Nakao et al. (2013b)
G6	Peru	L (goat)	A	A	EcRUS1 (=EcMGL2)	AB688142	Nakao et al. (2013b)
G7	Peru	L (pig)	B	A	EcPER1	AB777924	Nakao et al. (2013b)
G7	Peru	L (pig)	B	A	EcPER2	AB777925	Nakao et al. (2013b)
G7	Poland	L (pig)	B	A	EcG7	AB235847	Nakao et al. (2007)
G8	Far East Russia	L (moose)	E	C	EcRUS3	AB777910	Nakao et al. (2013b)
G8	Far East Russia	L (moose)	E	C	EcRUS3	AB777910	Nakao et al. (2013b)
G8	Far East Russia	A (wolf)	E	C	EcRUS3	AB777910	Nakao et al. (2013b)
G8	European Russia	L (moose)	C	B	EcRUS8	LC184604	This study
G8	European Russia	L (moose)	C	B	EcRUS8	LC184604	This study
G8	European Russia	L (moose)	C	B	EcRUS8	LC184604	This study
G8	Alaska, USA	L (moose)	C	B	EcG8	AB235848	Nakao et al. (2007)
G10	Mongolia	A (wolf)	A	A	EcMGL3	AB813184	Ito et al. (2013)
G10	Mongolia	A (wolf)	A	A	EcMGL3	AB813184	Ito et al. (2013)
G10	Mongolia	A (wolf)	C/E	C	EcMGL4	AB813185	Ito et al. (2013)
G10	Mongolia	A (wolf)	C/E	C	EcMGL4	AB813185	Ito et al. (2013)
G10	Far East Russia	L (moose)	E	B/C	EcRUS4	AB777911	Nakao et al. (2013b)
G10	European Russia	L (moose)	C	B	EcG10	AB745463	This study
G10	European Russia	L (moose)	C	B	EcG10	AB745463	This study
G10	European Russia	L (moose)	C	B	EcG10	AB745463	This study
G10	European Russia	L (moose)	C	B	EcG10	AB745463	This study
G10	European Russia	L (moose)	C	B	EcRUS9	LC184605	This study
G10	European Russia	L (moose)	C	B	EcG10	AB745463	Nakao et al. (2013b)
G10	European Russia	L (moose)	C	B	EcG10	AB745463	Nakao et al. (2013b)
G10	Finland	A (wolf)	C	B	EcG10	AB745463	This study
G10	Finland	A (wolf)	C	B	EcG10	AB745463	This study
G10	Finland	L (moose)	C	B	EcG10	AB745463	Nakao et al. (2013b)
G10	Finland	L (moose)	C	B	EcG10	AB745463	Nakao et al. (2013b)
G10	Finland	L (moose)	C	B	EcG10	AB745463	Nakao et al. (2013b)
G10	Alaska, USA	L (moose)	D	B	EcUSA1	AB777926	This study
G10	Alaska, USA	L (caribou)	D	B	EcUSA3	LC184606	This study
G10	Alaska, USA	L (moose)	D	B	EcUSA1	AB777926	Nakao et al. (2013b)
G10	Alaska, USA	L (moose)	C	B	EcUSA2	AB777927	Nakao et al. (2013b)
G10	Alaska, USA	L (moose)	C/D	B	EcUSA2	AB777927	Nakao et al. (2013b)

pepck, partial nuclear phosphoenolpyruvate carboxykinase gene; *pold*, DNA polymerase delta gene; *cox1*, mitochondrial cytochrome c oxidase subunit 1; n.d., not determined.

^a Parasite stages as L, larva; A, adult.

History, USA), were subjected to molecular analysis. Specimen numbers representing tapeworms among designated types included USNPC 59724 for *E. granulosus borealis*, and USNPC 59725 for *E. granulosus canadensis*. Five worms from each specimen vial were used for DNA extraction. Parasite DNAs were extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen). Extracted DNA was used for the subsequent PCR. Due to the degradation of DNA, it is difficult to obtain long fragments of DNA from formalin-preserved specimens. Therefore, PCR primers were designed to amplify short (100–200 bp) fragments, based on the *cox1* sequences of *E. canadensis*. Primer information is provided in the Supplementary Table S1. These fragments were expected to include nucleotide positions which were variable among different mitochondrial lineages and able to distinguish them. PCR amplification consisted of initial denaturation of 94 °C for 2 min, 40 cycles of 94 °C for 15 s, 45–50 °C for 15 s and 72 °C for 30 s,

and a final extension at 72 °C for 1 min. PCR products were visualised by electrophoresis using 2.0% agarose gel. DNA of *E. canadensis* G10 was used as a positive control in every trial.

2.3. DNA analysis

Nucleotide sequences were edited by using Geneious Pro software ver. 7.0.4 (created by Biomatters, available from <http://www.geneious.com/>). A BLAST search was performed for *cox1* gene sequences to identify the mtDNA lineage of each isolate. Then, the sequences were aligned by Clustal W 2.0 (Larkin et al., 2007) with the *cox1* gene sequences of *E. canadensis* and *E. ortleppi* in DDBJ/EMBL/GenBank databases by MEGA 6 (Tamura et al., 2013) and a phylogenetic tree was inferred by neighbour-joining method under the Kimura's two-parameter model (Kimura, 1980) with the gamma setting of 0.5 using PAUP 4.0b (Swofford, 2002). The tree

robustness was tested by bootstrapping with 1000 replicates. A *cox1* gene sequence of *E. multilocularis* was used as an outgroup. For the nuclear gene markers, obtained sequences were aligned and the allelic polymorphism was examined. Nucleotide sequences of *pepck* and *pold* alleles confirmed in *E. canadensis* were compared with those of other *Echinococcus* spp. (DNA database accession Nos. FN567985–FN567992 for *pepck* and FN568356–FN568363 for *pold*) (Knapp et al., 2011). All the alignment files are available online (doi:10.17632/cs45cv8bg.1).

3. Results

3.1. mtDNA phylogeny

In the present study, 48 isolates of *E. canadensis* collected from different hosts and regions were examined (Table 1). All specimens were used for the sequencing of the nuclear genes (*pepck* and *pold*). Mitochondrial lineages were determined for 13 new specimens; others had already been identified in previous studies. Among the 48 specimens, 19, seven and 22 isolates were identified as G6/G7, G8 and G10 mtDNA, respectively (G6 and G7 are hereinafter referred to as G6/G7 due to their sequence resemblance). Four new mtDNA haplotypes were obtained in the present study and two of them were identified as G10, and one each as G6/G7 and G8. The phylogenetic analysis of the *cox1* sequences shows that the mtDNA haplotypes of *E. canadensis* can be clearly divided into three monophyletic clades corresponding to G6/G7, G8 and G10, in which G6/G7 and G10 are the sister lineages (Fig. 1) as previously demonstrated (Lavikainen et al., 2006; Moks et al., 2008; Nakao et al., 2013a, b). Maximum pairwise divergence values within each mitochondrial lineage (G6/G7, G8 and G10) were 0.5%, 1.1% and 0.7%, respectively. On the other hand, pairwise divergence values were 1.8–2.2% between G6/G7 and G10, 2.7–3.5% between G6/G7 and G8, and 2.7–3.6% between G8 and G10. Although the monophyly of the mtDNA haplotypes obtained from pigs in Poland and Peru (EcG7, EcPER 1 and EcPER2) was shown, the clade was nested within the larger monophyletic clade consisting of G6 and G7. Among G10 haplotypes, EcMGL3 collected from a wolf in Mongolia was sister to all the other haplotypes, which were divided into Alaskan and Eurasian clades.

3.2. Nuclear DNA analysis

3.2.1. Sequence variations in nDNA markers

In the *pepck* and *pold* loci, five (*pepck* A–E) and three (*pold* A–C) alleles were confirmed and the lengths of the nucleotide sequence of the alleles were 525–528 bp and 689 bp, respectively (Figs. 2 and 3). These alleles were not identical to any of other *Echinococcus* spp. in the database, although only one allele was available for each species (Supplementary Figs. S1 and S2). The nucleotide positions 269–349 in *pepck* and 1–105 and 480–689 in *pold* are introns. The nucleotide sequence of *pold* was not determined for the isolate in wolf from Asian Russia (Altai region in West Siberia), because PCR did not yield amplicons (Table 1). At the *pepck* locus, mutations were found both in the exons and intron, and all except one were synonymous substitutions (Fig. 2). At the *pold* locus, all mutations were in the introns (Fig. 3).

3.2.2. Characteristics of *pepck* locus

Among the *pepck* alleles, only one nucleotide difference was observed between *E. ortleppi* and *pepck* A, between *pepck* B and E, and between *pepck* C and D (Fig. 2). *Peppck* B was obtained only from the isolates collected from pigs, namely G7, in Peru in Poland (Fig. 4a, Table 1). *Peppck* D was exclusively confirmed in the isolates of G10 in moose (*Alces alces gigas*) and barren-ground caribou (*Ran-*

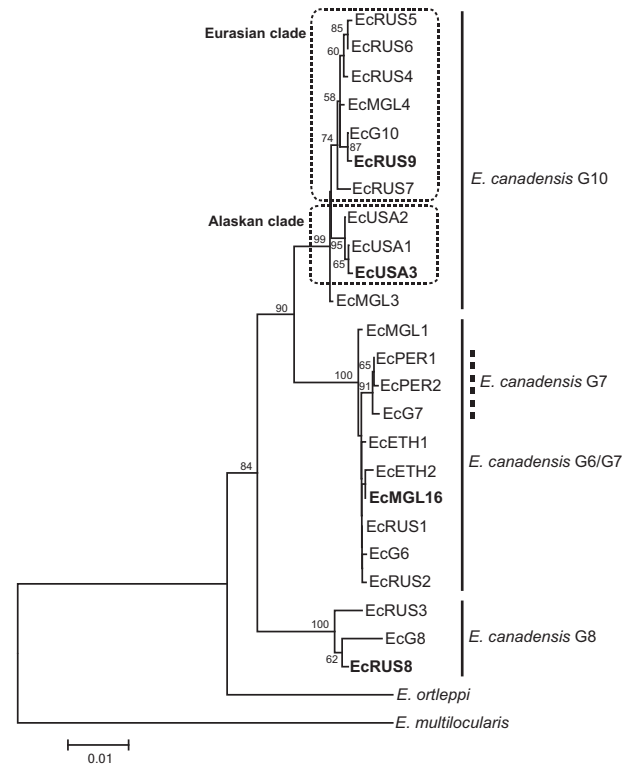


Fig. 1. A neighbour-joining tree of *Echinococcus canadensis* mitochondrial lineages inferred based on the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene sequences. The names of haplotypes newly confirmed in this study are shown in bold. Numbers on the nodes are the bootstrap values.

gifer tarandus granti) from Alaska. While *pepck* B and D were unique to G7 and G10, respectively, and focally distributed in a region or a host species, *pepck* A, C and E were shared by different mtDNA lineages. *Peppck* A was shared among most of G6 from a variety of hosts worldwide, and two isolates of G10 in wolves from Mongolia. *Peppck* C showed a Holarctic distribution among G6, G8 and G10 in moose, reindeer and wolves. *Peppck* E was also shared among G6, G8 and G10 in moose and wolves from the Palearctic. In Mongolia, three alleles (*pepck* A, C and E) were sympatrically distributed in the Zavkhan province. Two adult worms in wolves from Mongolia and a hydatid cyst in a moose in Alaska proved to be heterozygous, both the adult worms with C/E alleles, and the cyst with C/D alleles.

3.2.3. Characteristics of *pold* locus

Among the *pold* alleles, *pold* A was distributed worldwide (Fig. 4b, Table 1). It was shared among G6, G7 and G10 from humans, domestic ungulates and wild wolves. *Pold* B showed a Holarctic distribution (Alaska and northern Europe) among G8 and G10, and *pold* C was confirmed in G6, G8 and G10 from Mongolia and the Russian Far East. Both alleles were mostly shared among the parasite isolates collected from wild animals such as moose, reindeer/caribou and wolves.

3.2.4. Combined data of *pepck* and *pold* loci

When two genes were combined, seven nDNA haplotypes were confirmed. The notation of the haplotypes was made by the combination of the alphabetical numbers of *pepck* and *pold* alleles (e.g., AA = *pepck* A and *pold* A). Most of the G6 examined in this study possessed the haplotype AA (Fig. 4c, Table 1). This haplotype was found from livestock intermediate hosts (camel and goat), wild wolves, a patient with cystic echinococcosis (CE) in the Altai

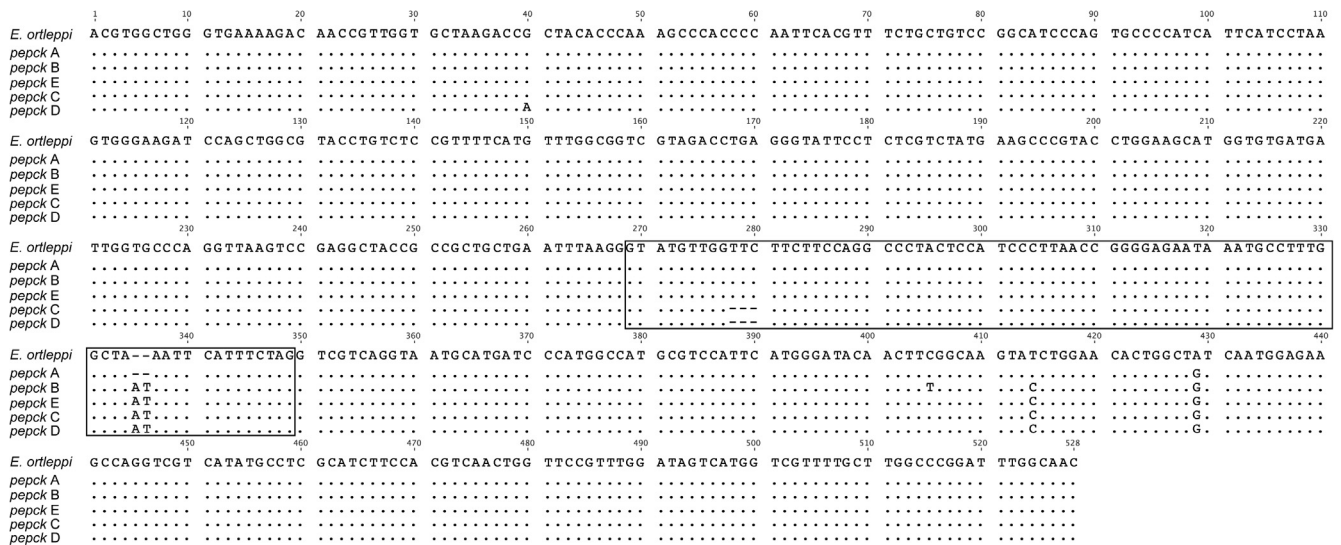


Fig. 2. Nucleotide alignment of the partial nuclear phosphoenolpyruvate carboxykinase (*pepck*) gene in five alleles of *Echinococcus canadensis* and *Echinococcus ortleppi*. An intron is located at nucleotide positions 269–349 (a framed box in the alignment).



Fig. 3. Nucleotide alignment of the partial DNA polymerase delta (*pold*) gene in three alleles of *Echinococcus canadensis* and *Echinococcus ortleppi*. Introns are located at nucleotide positions of 1–105 and 480–689 (framed boxes in the alignment).

region of Russia and a reindeer in the Russian Far East. Haplotype BA was unique to G7 in pig, and confirmed in geographically separated Poland and Peru. Haplotype CB and EC were both shared by G8 and G10 obtained from moose and wolves, but the geographical distribution was markedly different. CB was distributed in Alaska and the northern Europe, while EC was found in the Far East Russia and Mongolia. Haplotypes DB and EB were obtained only from G10, and their distributions were confined to Alaska and the Russian Far East, respectively.

3.3. Retrospective analysis of archival specimens

To clarify the specific status of *E. canadensis* and *E. borealis*, we attempted sequencing to provide genetic information from formalin-fixed archival specimens held in the former USNPC. Separate lots of strobilate adult cestodes represent the type specimens attributed to *E. granulosus borealis* and *E. granulosus canadensis* des-

ignated by [Sweetman and Williams \(1963\)](#). Two lots with numerous specimens are held, and represent the outcome of experimental infections in domestic dogs based on natural infections of hydatid metacestodes derived, respectively, from moose (Ontario, Canada) and semi-domestic reindeer (Aklavik, Northwest Territories, Canada). More than 10 combinations of the primers ([Supplementary Table S1](#)) were examined for the PCR using template DNAs extracted from the archival specimens, but none of them could yield amplicons except for the positive control.

4. Discussion

Due to the diversity of mitochondrial lineages, and in the absence of clear morphological attributes, the taxonomy of *E. canadensis* remains a controversial issue, i.e. whether this taxon, as currently understood, consists of a single or multiple species.

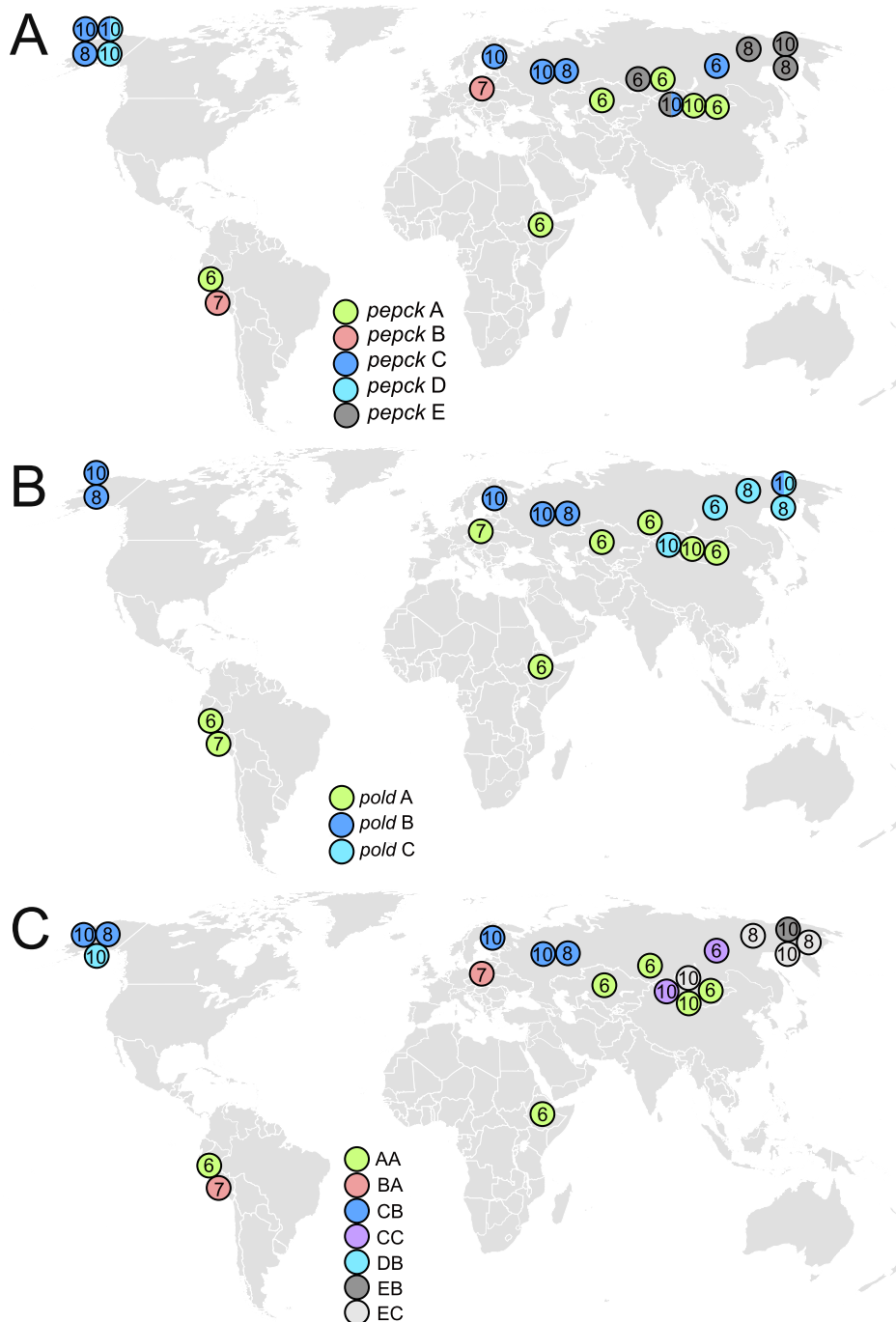


Fig. 4. Geographic distribution of the nuclear DNA alleles and haplotypes of *Echinococcus canadensis*. (A) Distribution of partial nuclear phosphoenolpyruvate carboxykinase (*pepck*) alleles. (B) Distribution of the DNA polymerase delta (*pold*) alleles. (C) Distribution of the nDNA haplotypes (*pepck* + *pold*). The combination of the alphabetical numbers of *pepck* and *pold* alleles are used to show the combined data of both loci (e.g., AA = *pepck* A and *pold* A). Numbers in circles show the mtDNA lineages.

In the present study, we attempted to subject specimens designated in the type series of *E. granulosus canadensis* and *E. granulosus borealis* to mtDNA analysis to shed light on the specific status of *E. canadensis*. Unfortunately, we could not obtain any positive results from PCR, due to the possible degradation of DNA during the long-term preservation in formalin and recently in ethanol. Therefore, our approach was to infer the specific status of *E. canadensis* by the analysis of recently obtained specimens using a combination of mtDNA and nDNA markers.

Phylogenetic analysis based on the complete *cox1* sequences clearly showed that the *E. canadensis* isolates from a variety of

hosts were divided into three monophyletic mtDNA clades, which correspond to G6/G7, G8 and G10. It was also clear that G6/G7 and G10 are sister to each other. Thus, from a molecular cladistic viewpoint, *E. canadensis* can be treated as two (G6/G7 and G10 versus G8) or three (G6/G7 versus G10 versus G8) maternal lineages. The genetic distance between G6/G7 and G10 (1.8–2.2%) is not markedly smaller than that between G6/G7 and G8 (2.7–3.5%) or G10 and G8 (2.7–3.6%), supporting the three maternal lineages.

Because G6/G7 and G10 shared a most recent common ancestor, parphyly of the lineages utilising northern free-ranging wild cervids as the intermediate hosts (G8 and G10) was demonstrated

again (Lavikainen et al., 2006; Moks et al., 2008; Nakao et al., 2013a, b). The isolates obtained from pigs, representing G7 (pig strain), clustered in a monophyletic clade, but it was nested among the isolates of G6. The intermediate host ecology is different between G6/G7 (camel, goat and pig; domestic phylogenetically unrelated ungulates) versus G10 and G8 (wild or semi-domestic cervids), although there is some overlap since G6 has been reported in reindeer (Konyaev et al., 2013).

Lymbery et al. (2015a) proposed to designate the three lineages as *Echinococcus intermedius* (G6/G7), *Echinococcus borealis* (G8) and *E. canadensis* (G10). The resurrection and application of *E. intermedius* was rejected by Nakao et al. (2015) based on the arbitrary nature of this nomenclatural proposal, an absence of clear provenance, and lack of unequivocal type or voucher specimens archived in any global museum repository. In response to this opinion, Lymbery et al. (2015b) stressed the importance of considering the differences in morphology, life-history and evolutionary trajectories for respective mitochondrial lineages within *E. canadensis*. We agree that the phenotypic and mtDNA phylogenetic evidence shows that the lineages had experienced divergent evolutionary histories, but the status as discrete taxa has not been unequivocally resolved and requires clear criteria for species delimitation which combines phylogenetic (historical), ecological and biogeographic components (e.g., Brooks and McLennan, 2002). Lymbery (2017) proposed the application of the evolutionary species concept, which consists of two steps. The first step is the phylogenetic reconstruction to identify putative species which should be monophyletic and exclusive. The second step is to assess whether the members of the putative species possess genetic exchangeability or ecological exchangeability. Conversely, lack of any degree of exchangeability among groups in sympatry would indicate that those groups are different species. Thus, to elucidate the specific status of *E. canadensis*, genetic characterisation using nDNA markers is essential.

In the present study, two single copy nDNA gene markers were examined. Both *pepck* and *pold* genes have previously been used as genetic markers to infer the phylogeny of taeniid tapeworms (Knapp et al., 2011). In addition, the *pold* gene has been demonstrated to be useful to evaluate hybridisation in taeniid tapeworms (Yamane et al., 2013; Yanagida et al., 2014). Based on our current evaluations, from a total of 48 *E. canadensis* isolates, five and three alleles were identified at *pepck* and *pold*, respectively, and each allele generally showed affinity to a particular spectrum of host animals. None of these alleles was confirmed in the other *Echinococcus* spp., but only one isolate was available for each species. When we focus on the parasite isolates collected from the intermediate hosts, nDNA alleles were clearly classified to the alleles obtained from domestic animals such as camel, goat and pig (“southern domestic alleles”: *pepck* A, *pepck* B and *pold* A) and those from wild or semi-wild cervids (“northern sylvatic alleles”: *pepck* C, D, E and *pold* B and C) (Table 1). Although it is supposed that the two nuclear loci are not necessarily related directly to the host specificity, these results suggest the existence of genetic lineages adapted to certain intermediate hosts and life cycles, or may reflect other historical factors involved in isolation or connectivity. Concurrently most of the alleles, however, were shared among the parasite isolates belonging to the different mtDNA lineages. Some of them (*pepck* C, E and *pold* C) were found in G6, G8 and G10. The allele sharing among the different mitochondrial lineages was more evident where the different mtDNA lineages occur in sympatry, such as in Mongolia and Asian Russia, the Russian Far East and, concerning G8 and G10, across the Holarctic. Besides, in Mongolia, the Russian Far East and Alaska, heterozygotes were found at either locus.

These results strongly suggest that cross-fertilisation between individual adult worms belonging to the different mitochondrial lineages occurred in the past, after the divergence of the lineages. Cestodes are hermaphroditic, and both self- and cross-fertilisation are possible. In the case of *E. granulosus*, the frequency of outcrossing was estimated to be as high as 74% (Haag et al., 2011). Hybridisation among closely related species or intraspecific genetic lineages has been demonstrated in other taeniid tapeworms such as between *Taenia asiatica* and *Taenia saginata* (Okamoto et al., 2010; Yamane et al., 2012, 2013), and between two genotypes of *Taenia solium* (Yanagida et al., 2014). G6, G8 and G10 of *E. canadensis* have common definitive hosts, wolves and dogs, and thus genetic mixing is quite possible in the regions where the domestic or semi-domestic livestock and wildlife coexist. It is likely that these genetic lineages possess both genetic and ecological exchangeability.

The most intriguing specimen, G6 from a reindeer in the Russian Far East, possessed “sylvatic alleles” at both *pepck* and *pold* loci. The mechanism of host specificity in *Echinococcus* spp. is not known, but it is reasonable to presume that it is under the control of nDNA. Therefore, although the mtDNA lineages generally correspond to their intermediate host affinity, this result indicates the potential risk of making a wrong estimation in the transmission ecology of CE caused by *E. canadensis*, when only using mtDNA genotyping. In the present study, nuclear-mitochondrial discordance was also observed in the adult worms collected from wolves in Mongolia and Asian Russia (Altai region). Two worms of G10 in Mongolia had “domestic alleles” at both nuclear loci. In contrast, “sylvatic allele” at the *pepck* locus was found from a worm of G6 in Asian Russia. Given that the above-mentioned G6 isolate with “sylvatic alleles” was found in reindeer, it is suggested that the G10 isolate with “domestic alleles” and the G6 isolate with “sylvatic alleles” were acquired by the wolves through the consumption of intermediate hosts which did not correspond to ungulates estimated by the mtDNA genotyping.

The present nDNA data show that *pepck* and *pold* alleles are shared among the isolates of G8 and G10 in Alaska and northern Europe, whereas the different alleles were confirmed from the population in the Russian Far East (Fig. 4). Based on the mtDNA phylogeny, it is obvious that G8 and G10 have demonstrated divergent trajectories and a shallow history of common ancestry in the Holarctic. Considering both mtDNA and nDNA data obtained in this study, the divergence between G8 and G10 probably occurred by geographic isolation in the Arctic across the Beringian nexus since the Last Glacial Maximum (LGM) and termination of the Pleistocene no deeper than 11 kiloyears before present (e.g., Hoberg et al., 2012, 2017). Geographic expansion and establishment may have been mediated by canid definitive hosts, or ungulates (particularly moose) which arrived very late in the Nearctic to become initially established in Alaska, and subsequently further eastward through post-glacial expansion following the LGM (Hundertmark et al., 2002). Although continuity of Eurasian and Nearctic populations of these parasites may extend deeper into the late Pleistocene, considerably greater levels of genetic divergence might be predicted in such a situation.

Events of natural expansion associated with free-ranging assemblages of hosts and parasites account for some component of the current distribution. Subsequently, it is apparent that anthropogenic drivers and introduction with reindeer from Palearctic sources during the last century have secondarily influenced the structure of a mosaic parasite fauna, now with extensive overlap in North America (e.g., Hoberg, 2010). Further, that G6/G7 are identical based on hydatid material from Poland and Peru is indicative of the role of long-range translocation and introduction

either with dogs, or with infected ungulate intermediate hosts following European contact in the 1500s, or later. Generally, this history is consistent with the role of trade and European exploration in widespread dissemination, introduction and establishment of *Echinococcus* genetic lineages and species globally.

When a new species is erected or the validity of a known species is confirmed, the evidence for species boundaries or limits, which are linked to morphology, ecology, biogeography, genetics, and evolution should be demonstrated (e.g., Brooks and McLennan, 2002). In the case of morphologically indistinguishable cryptic species complexes, molecular phylogeography and population genetics play a key role in judging the validity of species. Based on an evolutionary species concept, Lymbery et al. (2015a) argued that *E. canadensis* should be split into three species, depending on their host specificities and distributional patterns. However, based on the exploration of mtDNA and nDNA in the current study, it is likely that *E. canadensis* individual tapeworms of G6/G7, G8 and G10 possess genetic exchangeability and that hybridisation occurs when they occur in sympatry, resulting from natural or anthropogenic drivers. Besides, it is natural to consider that there is an ecological exchangeability between G8 and G10 because they share both definitive and intermediate hosts distributed in sympatry (Lavikainen et al., 2003; Thompson et al., 2006; Schurer et al., 2013). Further, there is no basis for recognition of different lineages of North American and Eurasian origin since both G8 and G10 are widely distributed across the Holarctic. Therefore, it is not appropriate to treat each mitochondrial lineage of *E. canadensis* as an independent species. Herein, we propose to treat all G6/G7, G8 and G10 as a single species, *E. canadensis*. However, we also agree that there is a continuing necessity for differentiating the genetic lineages of the species showing different biological features, as stressed by Lymbery et al. (2015b). For epidemiological purposes, the traditional use of both mtDNA lineages and strains can be continued. However, “Fennoscandian cervid strain” seems to be an inappropriate name for G10 as it is distributed across the Holarctic and it is unlikely that it originates in northwestern Europe. At present, mtDNA lineages of G6, G7, G8 and G10, and “camel and goat strain”, “pig strain” and “cervid strain” are preferable terms.

Although it is likely that the nuclear-mitochondrial discordance confirmed in this study reveals a genetic exchangeability among the mitochondrial lineages of *E. canadensis*, another explanation is also possible. If the nDNA allele polymorphism occurred before the divergence of the *E. canadensis* lineages or even earlier, some nuclear alleles could be shared by different lineages or *Echinococcus* spp. However, it is reasonable to assume that the divergence of “domestic alleles” and “sylvatic alleles” resulted from the divergence of G6/G7 and G10, and could be associated with processes of domestication of dogs or ruminants starting nearly 14–15 thousand years ago (Shannon et al., 2015; Orland, 2016), because these alleles generally show affinities to particular hosts. Considering the mtDNA phylogeny, separation of G8 should have preceded the divergence of “domestic” and “sylvatic” alleles. Therefore, it is not likely that the current allele polymorphism directly reflects the ancient polymorphism before the divergence of the lineages. However, to demonstrate the genetic exchangeability among the mitochondrial lineages of *E. canadensis* more objectively, statistical analyses using a greater number of nDNA gene markers and parasite isolates are needed. Lymbery (2017) also proposed the application of a coalescent-based approach to species delimitation among *E. ortleppi* and *E. canadensis* lineages. However, in the present study, we could not acquire a sufficient number of parasite isolates to conduct objective statistical analyses at any localities. Genomic data is not available for *E. canadensis*, however, these data for *E. granulosus* and *E. multilocularis* can facilitate establishment of new nDNA markers. Further intensive sampling of parasite isolates

and investigation of the nDNA allelic polymorphism of both *E. canadensis* and *E. ortleppi* are essential in arriving at a complete understanding of the history of distribution and evolution for the former species. The history of *E. canadensis*, and identification of the factors linked to distribution and host association, have been complex, reflecting considerable faunal mixing extending across ecological time and concurrently influenced by natural expansion and human-caused invasions and introductions. If the genetic exchangeability among the mitochondrial lineages of *E. canadensis* is completely demonstrated, the morphological discrimination of the *E. canadensis* mitochondrial lineages (e.g., Lymbery et al., 2015a) will be ineffective in diagnosing discrete species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpara.2017.07.001>.

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