Int J Clin Exp Pathol 2018;11(2):795-801
www.ijcep.com /ISSN:1936-2625/IJCEP0070758

Original Article

Sequence analysis of mitochondrial cytochrome c oxidase 1 and cytochrome b genes of echinococcus multilocularis from human patients

Abuduaini Abulizi[1], Hao Wen[1,2], Chuanshan Zhang[2], Liang Li[2], Bo Ran[1], Tiemin Jiang[1], Tuerganaili Aji[1], Yingmei Shao[1]

[1]*Hepatobiliary & Hydatid Department of Digestive and Vascular Surgery Centre, Xinjiang Key Laboratory of Echinococcosis, First Affiliated Hospital, Xinjiang Medical University, Urumqi, Xinjiang, China;* [2]*Xinjiang Key Laboratory of Echinococcosis, The First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China*

Received December 11, 2017; Accepted January 7, 2018; Epub February 1, 2018; Published February 15, 2018

Abstract: *Echinococcus multilocularis (E. multilocularis)* is the cause of alveolar echinococcosis (AE) in humans. Differences in gene sequence may exist among strains of E. multilocularis that are isolated from different patients in different areas of Xinjiang Uyghur Autonomous Region of China. Other studies have shown that genetic variation and biomolecular classification of E. multilocularis exists. A total of 47 AE samples were collected from AE patients for sequence analysis of mitochondrial cytochrome c oxidase 1 (cox1) and cytochrome b (cytb) genes using PCR. We compared the obtained sequences with the GenBank database to identify the parasite and 5 haplotypes were detected among the geographical isolates from cox1 and cytb, respectively. Nearly all of the samples originated from Northern Xinjiang. Homology comparison of gene haplotypes in GenBank showed that 3 cox1 haplotypes and one cytb haplotype had 100% homology with sequences in GenBank. Two cox1 haplotypes and 4 cytb haplotypes had no homology with previous deposits in GenBank and thus were considered as newly discovered gene haplotypes. This present study demonstrates that comparatively conservative intraspecific genetic variations of E. multilocularis exist in Xinjiang Uyghur Autonomous Region and the main epidemic haplotypes in Xinjiang are H1 (cox1) and H1 (cytb). Cox1 haplotypes H4, H5, and cytb haplotypes H2, H3, H4, and H5 are considered newly discovered gene haplotype sequences.

Keywords: Alveolar echinococcosis (AE), genotype, cytochrome c oxidase 1 (cox1), cytochrome b (cytb)

Introduction

Alveolar echinococcosis (AE) is one of the globally common pathogenic zoonoses that are caused by *Echinococcus multilocularis*. AE usually occurs in the Northern Hemisphere, especially mountainous regions that are cold or covered by forests. China is one of the most important endemic regions of AE [1, 2]. In AE, infiltrativetumor-like growth of *E. multilocularis* metacestode affects the liver of intermediate hosts, including rodents and humans [3]. The epidemiology, pathogenicity, immunology, and genetics of *E. granulosus* have been extensively studied [4-9] but few studies have been carried out to elucidate the molecular biology of *E. multilocularis,*due to its more limited geographical distribution and fewer affected patients compared with *E. granulosus*. It has been reported that only *E. multilocularis* causes AE and little difference exists among genotypes [10, 11]. Interestingly, 3 subspecies of *E. multilocularis* that have been discovered in the past have been identified to be 3 independent species [12]. *Echinococcus shiquicus*, a species of *Echinococcus* discovered in Sichuan and Qinghai Provinces of China, has special morphologies in larvae and adults as well as different molecular sequences [13]. In the past, AE cases discovered in Xinjiang Uyghur Autonomous Region of China were thought to be caused by infections by European *E. multilocularis*. However, further studies on wild animal and artificial animal hosts have shown that *Echinococcus* spp. in Xinjiang had distinct host body reactions and pathological structure [12]. Moreover, separation of polymorphic microsatellite loci from *E. multilocularis* has confirmed the existence of gene sequence variation among different strains of *E. multilocularis* [14].

Figure 1. Locations of the samples collected in the study.

Of note, our group has also shown more different growth patterns and pathological structures of AE (invasive proliferation, fibrous calcification, and liquefaction necrosis) in past years of clinical practice. AE patients in Xinjiang have wide geographical distribution, large climatic diversity, and a variety of intermediate hosts exist in the province. Therefore, *E. multilocularis* has genetic variation among different isolates [15].

Genetic structures of parasites provide information on their epidemiology and transmission [16]. Mitochondrial genes of *E. granulosus* such as cytochrome c oxidase 1 (cox1), NADH dehydrogenase 1 (nad1), ATP synthase F0 subunit 6 (atp6), cytochrome b (cytb), elongation facto-1 alpha (ef1α), 12 S, and 16S have been investigated [17-22] and the protein-coding genes of cox1 and cytb were chosen as markers for *Echinococcus* mtDNA. In other organisms, mitochondrial control region is generally used to infer genealogical relationships. The characteristics of animal mtDNA usually includes: 1) high number of copies, 2) non-Mendelian maternal inheritance without recombination, 3) homoplasmy in most individuals, and 4) faster evolution than nuclear DNA. Cox1 and cytb genes of *Echinococcus* spp. are promising candidates for classification of intra- and inter-specific variants [23]. In our present study, we investigated the genotypes and genetic diversity of *E. multilocularis* isolates in Xinjiang Uyghur Autonomous Region using cox1 and cytb genes as genetic markers.

Materials and methods

Sample collection, DNA extraction and primer design

A total of 47 patients were obtained from the tissue bank that has been established by the First Affiliated Hospital of Xinjiang Medical University. Surgical resection samples were obtained from the Hepatobiliary Department of the First Affiliated Hospital of Xinjiang Medical University. Geographic origins of isolates are shown in **Figure 1**. All samples were stored at -70°C. The DNA extracted from samples using the DNeasy tissue kit (Qiagen, Hilden, Germany) and used as a template forpolymerase chain reaction (PCR). Based on published *Echinococcus* mitochondrial genome sequences of cox1 and cytb genes in GenBank (http://www.ncbi.nlm.nih.gov/pubmed), DNAman software (http://www.lynnon.com/) was used to design upstream and downstream primers which were synthesized by Genosys (Fargo, North Dakota). Cox1 gene was amplified using the following primer pairs: 5'-TTGAATTTGCACGTTTGAATGC-3' (forward) and 5'-GAACCTAACGACATAACATAATGA-3' (reverse). The cytb gene was amplified using the following primer pairs: 5'-GTCAGATGTCTTATTGGGCTGCC-3' (forward) and 5'-TCTGGGTGACACCCACCTAAATA-3' (reverse).

Amplification and purification of PCR products

To amplify mtDNA fragments of *E. multilocularis*, specific cox1 primers or specific cytb primers were added using conventional meth-

Table 1A. List of mtDNA cox1 gene sequences of *E. multilocularis* retrieved from GenBank, origins, and database accession numbers

Species (Genotype)	GenBank accession numbers	Origins	References
EmRUS12 (cox1 gene)	AB777915	Russia	Konyaev et al. (2013)
EmRUS8 (cox1gene)	AB688132	Russia	Konyaev et al. (2012)
Em haplotype 16 (cox1 gene)	JF906152	Qinghai, China	Ma et al. (2012)
Em haplotype M04 (cox1 gene)	AB491460	China	Nakao et al. (2010)
Em (cox1 gene)	AB461417	Sichuan,China	Nakao et al. (2009)
Em (cox1 gene)	AB385610	USA	Yamasaki et al. (2008)
E. multilocularis	AB018440	Japan	Nakao et al. (2002)
E. granulosus (G1)	AF297617	United Kingdom	Le et al. (2002)
E. oligarthrus	AB208545	Panama	Nakao et al. (2006)
E. vogeli	AB208546	Japan	Nakao et al. (2006)
E. shiquicus	AB208064	Sichuan, China	Nakao et al. (2006)

Table 1B. List of mtDNA cytb gene sequences of *E. multilocularis* retrieved from GenBank, origin, and database accession numbers

Species (Genotype)	GenBank accession numbers	Origins	References
E. multilocularis (cytb gene)	AB461399	Japan	Nakao et al. (2009)
E. multilocularis (cytb gene)	AB461398	Kazakhstan	Nakao et al. (2009)
E. multilocularis A8 (cytb gene)	AB477009	Mongolia	Nakao et al. (2009)
E. multilocularis	AB018440	Japan	Nakao et al. (2002)
E. granulosus (G1)	AF297617	United Kingdom	Le et al. (2002)
E. oligarthrus	AB208545	Panama	Nakao et al. (2006)
E. vogeli	AB208546	Japan	Nakao et al. (2006)
E. shiquicus	AB208064	Sichuan, China	Nakao et al. (2006)

Table 2. Variation sites and genetic distances of cox1 gene of *E. multilocularis*

Haplotype	Locality	N[a]	4	8	9	11	107	115	116	344
H1 (cox1)	Common	42	G	A	G	A	T	A	G	C
H2 (cox1)	Kazakhstan	1	T	T	[b]d	C	-	-	-	-
H3 (cox1)	Aletai, Tacheng	2	-	-	-	-	-	-	-	T
H4 (cox1)	Urumqi	1	-	-	-	-	-	-	T	-
H5 (cox1)	Aletai	1	-	-	-	-	T	G	-	-

Note: [a]Number of isolates; [b]deletion.

ods. The PCR mixture was prepared in a 50 μL final volume containing 5 μL template DNA, 25 μL of Mix (2×PCR Reagent, Tiangen, Beijing), 1 μL of each primer, 18 μL water, and the manufacturer-supplied reaction buffer. Amplification protocol was: initial denaturation step (94°C for 5 min), 40 thermal cycles with a denaturation step (94°C for 30 sec), an annealing step (56°C for 30 sec), an elongation step (72°C for 1 min), and final extension step (72°C for 5 min). Each reaction was set up with negative control without DNA template and positive control with known *E. multilocularis* DNA template. PCR products were run on 1.5% agarose Tris-borate EDTA gel electrophoresis and stained with ethidium bromide to detect PCR amplicons. Target gene bands (cox1-513 bp and cytb-547 bp) were observed under ultraviolet light. Amplification products were purified by using the Nucleospin Extract II kit (Qiagen, Hilden, Germany) and purified PCR products were directly sent for sequencing (Beijing Liuhe BGI Tech Co., Ltd., Beijing, China). ClustalW multiple alignments of nucleotide sequences were then performed using BioEdit 3.03 software and all *E. multilocularis* cox1 and cytb gene sequences were registered in GenBank in January 2016 for phylogenetic analysis (**Table 1A** and **1B**). The genetic distances were calculated using DNAStar 5.0

Table 3. Variation sites and genetic distances of cytb gene of *E. multilocularis*

Haplotype	Locality	N[a]	147	284	414	415	530
H1 (cytb)	Common	42	A	T	-	-	T
H2 (cytb)	Bole	1	G	-	-	-	-
H3 (cytb)	Bayingolin, Urumqi	2	-	-	-	-	G
H4 (cytb)	Tacheng	1	-	A	-	-	-
H5 (cytb)	Yili	1	-	-	[b]G	[b]G	-

Note: [a]Number of isolates; [b]insertion.

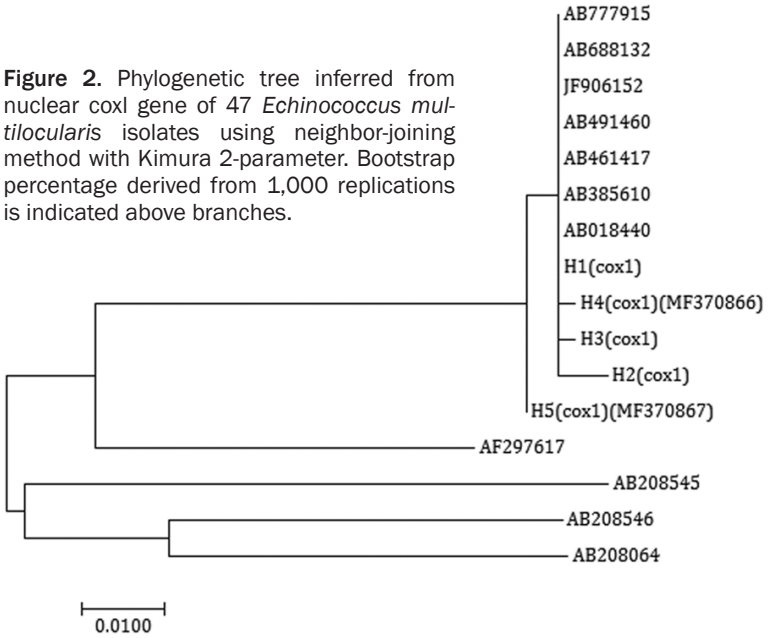

Figure 2. Phylogenetic tree inferred from nuclear coxl gene of 47 *Echinococcus multilocularis* isolates using neighbor-joining method with Kimura 2-parameter. Bootstrap percentage derived from 1,000 replications is indicated above branches.

software (DNASTAR, Inc., Madison, Wisconsin). Phylogenetic trees were constructed from alignments with the neighbor-joining method in MEGA 7.0 software (http://www.megasoftware.net). Confident intervals were obtained via 1,000 bootstrap replications for each branch of the tree.

Results

Liver tissue samples were collected from 47 AE patients by sample library and surgical removal. In total, 94 sequences were obtained from these samples including 47 cox1 gene sequences and 47 cob gene sequences (42 from Xinjiang, 2 from Qinghai, one from Henan, one from Sichuan, one from Kazakhstan) (**Figure 1**).

Sequence analysis of 47 isolates showed that the length of cox1 gene fragment from mtDNA of *E. multilocularis* was 513 bp and showed a higher substitution frequency (1.6%; 8 substitution sites/513 sites) (**Table 2**). Gene mutations included transition, transversion, and deletion but no insertion. In addition, the length of cytb gene fragment was 547 bp and showed 0.9% substitution frequency (5 sites/547 sites) (**Table 3**). Gene mutations included transition, transversion, and insertion but no deletion and high sequences similarity. In addition, all sequences of cytb gene showed more than 98.0% identity with previously published sequences for mtDNA genotypes of *E. multilocularis*.

The phylogenetic analysis based on cox1 gene fragments showed the existence of H1, H2, H3, H4, and H5 haplotypes and their relationship with some previous GenBank deposits (**Figure 2**). After comparing the sequenced gene haplotypes (513 bp) of *E. multilocularis* with the gene haplotypes in GenBank, H1 and H2 haplotypes were found identical to AB777915 (Russia), AB688132 (Russia), JF906152 (China), AB491460 (China), AB461417 (China), AB385610 (USA), and AB018440 (Japan) and H3 was found identical to AB688134 (Russia), AB688133 (Russia), and KX685926 (China) [19, 24-28]. Of note, haplotypes H4 and H5 were not found in the same mutation sequence and submitted sequence data to GenBank and provided GenBank accession numbers was H4 (MF370866) and H5 (MF370867), respectively.

The phylogenetic analysis based on the cytb gene fragments showed the existence of H1, H2, H3, H4, and H5 haplotypes (**Figure 3**). Homology comparison of the sequenced cytb (547) gene haplotypes of *E. multilocularis* with GenBank gene haplotypes showed haplotype H1 identical to AB461399 (Japan), AB461398 (Kazakhstan), AB477009 (Mongolia), and AB018440 (Japan) [24, 25]. However, haplotypes H2, H3, H4, and H5 were not discovered in the same mutation sequence and submitted of sequence data to GenBank and provided

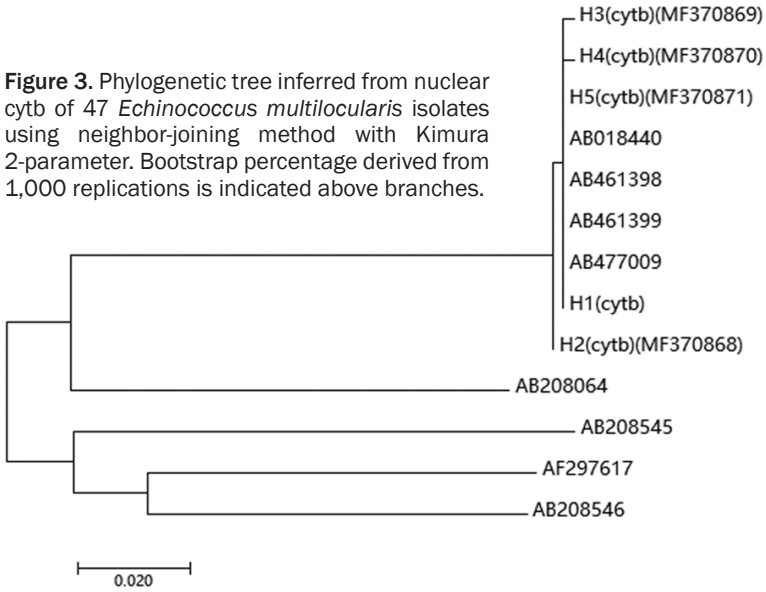

Figure 3. Phylogenetic tree inferred from nuclear cytb of 47 *Echinococcus multilocularis* isolates using neighbor-joining method with Kimura 2-parameter. Bootstrap percentage derived from 1,000 replications is indicated above branches.

GenBank accession numbers was H2 (MF370868), H3 (MF370869), H4 (MF370870) and H5 (MF370871), respectively.

Discussion

A unique reproduction system exists in organisms of the genus *Echinococcus*. Self-fertilization of hermaphroditic adults and clonal amplification of the larvae can cause genetic uniformity in local populations [29, 30]. Mitochondrial genes cox1 and cytb have been used to determine the genotypes of *E. granulosus* [25, 31, 32] and main considered various environments, intermediate hosts, and wide geographical distribution may play an important role in driving the genetic evolution. However, there is no evidence that such a selection is related with the genomic variation of *Echinococcus*.

In our study, we collected mitochondrial sequences for *E. multilocularis* from AE patients, including 47 cox1 and 47 cytb gene sequences, providing basic information for identification of the relationship of host specificity and genetic variation. Alignment and phylogenetic analysis of cox1 sequences from the patients showed five haplotypes whereas the cytb gene sequence analysis showed five haplotypes. The fact is that H1 (cox1) and H1 (cytb) are predominant haplotypes in human AE cases. Our results are consistent with the reports by other researchers who have used different techniques such as mitochondrial DNA sequencing [19, 23, 25] and microsatellite genotyping [14, 33]. Most haplotypes originated from northern Xinjiang which has less solar radiation on the ground than southern Xinjiang, except for H3 (cytb) that is originated from Bayingolin, and H2 (cox1) that is originated from Kazakhstan. Haplotypes MF370866, MF370867, MF370868, MF370869, MF370870, and MF370871 may be considered as newly discovered gene haplotype sequences. However, one genotype (Asian genotype 1) was predominant which may indicate that this genotype of *E. multilocularis* is suitable to the host species and environment and landscape, which impact the transmission of AE [34]. Sequence analysis of *E. multilocularis* cox1 and cytb gene fragments showed relatively low genetic variation among the isolates from AE patients in Xinjiang. Most of the isolates are Asian genotype 1 [15] which may be an indication of its relative high frequency as a source of infection to humans or because of a stronger pathogenicity to humans. These hypotheses need to be further elucidated.

In conclusion, we used a haploid maternally inherited mtDNA marker to examine the population genetic structure of *E. multilocularis* collected from patients infected with alveolar echinococcosis from Xinjiang. The analysis of mitochondrial cox1 and cytb gene fragments show that the parasite exhibits variation at the genomic level with at least five haplotype in China. However, further studies are needed to confirm this conclusion. In addition, it is important to search and track sources of infection, to analyze evolutionary history, and to determine the pathogenicity of echinococcus.

Acknowledgements

This work was financially supported by the National Science Foundation of China (No. 81660108) and Xinjiang Autonomous Regional Science Foundation of China (No. 2016D01C274).

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Tuerganaili Aji and Yingmei Shao, Hepatobiliary & Hydatid Department of Digestive and Vascular Surgery Centre, Xinjiang Key Laboratory of *Echinococcosis*, First Affiliated Hospital, Xinjiang Medical University, 1 Liyushan Road, Urumqi 830011, Xinjiang, China. Tel: +86-09914364556; Fax: +86 -0991-4364556; 13699995676; E-mail: tuergan78@sina.com (TA); syingmei1@163.com (YMS)

References

[1] Torgerson PR, Keller K, Magnotta M and Ragland N. The global burden of alveolar echinococcosis. PLoS Negl Trop Dis 2010; 4: e722.

[2] Nunnari G, Pinzone MR, Gruttadauria S, Celesia BM, Madeddu G, Malaguarnera G, Pavone P, Cappellani A and Cacopardo B. Hepatic echinococcosis: clinical and therapeutic aspects. World J Gastroenterol 2012; 18: 1448-1458.

[3] Brehm K, Wolf M, Beland H, Kroner A and Frosch M. Analysis of differential gene expression in echinococcus multilocularis larval stages by means of spliced leader differential display. Int J Parasitol 2003; 33: 1145-1159.

[4] Ito A, Nakao M, Lavikainen A and Hoberg E. Cystic echinococcosis: future perspectives of molecular epidemiology. Acta Trop 2017; 165: 3-9.

[5] Craig P, Mastin A, van Kesteren F and Boufana B. Echinococcus granulosus: epidemiology and state-of-the-art of diagnostics in animals. Vet Parasitol 2015; 213: 132-148.

[6] Ehsan M, Akhter N, Bhutto B, Arijo A and Ali Gadahi J. Prevalence and genotypic characterization of bovine echinococcus granulosus isolates by using cytochrome oxidase 1 (Co1) gene in Hyderabad, Pakistan. Vet Parasitol 2017; 239: 80-85.

[7] Grosso G, Gruttadauria S, Biondi A, Marventano S and Mistretta A. Worldwide epidemiology of liver hydatidosis including the mediterranean area. World J Gastroenterol 2012; 18: 1425-1437.

[8] Diaz A, Casaravilla C, Barrios AA and Ferreira AM. Parasite molecules and host responses in cystic echinococcosis. Parasite Immunol 2016; 38: 193-205.

[9] Cucher MA, Macchiaroli N, Baldi G, Camicia F, Prada L, Maldonado L, Avila HG, Fox A, Gutierrez A, Negro P, Lopez R, Jensen O, Rosenzvit M and Kamenetzky L. Cystic echinococcosis in South America: systematic review of species and genotypes of echinococcus granulosus sensu lato in humans and natural domestic hosts. Trop Med Int Health 2016; 21: 166-175.

[10] Bowles J and McManus DP. Rapid discrimination of echinococcus species and strains using a polymerase chain reaction-based RFLP method. Mol Biochem Parasitol 1993; 57: 231-239.

[11] Gasser RB, Zhu X and McManus DP. Display of sequence variation in PCR-amplified mitochondrial DNA regions of echinococcus by single-strand conformation polymorphism. Acta Trop 1998; 71: 107-115.

[12] Tang CT, Wang YH, Peng WF, Tang L and Chen D. Alveolar echinococcus species from vulpes corsac in Hulunbeier, Inner Mongolia, China, and differential development of the metacestodes in experimental rodents. J Parasitol 2006; 92: 719-724.

[13] Xiao N, Nakao M, Qiu J, Budke CM, Giraudoux P, Craig PS and Ito A. Dual infection of animal hosts with different echinococcus species in the eastern Qinghai-Tibet plateau region of China. Am J Trop Med Hyg 2006; 75: 292-294.

[14] Nakao M, Sako Y and Ito A. Isolation of polymorphic microsatellite loci from the tapeworm echinococcus multilocularis. Infect Genet Evol 2003; 3: 159-163.

[15] Wu C, Zhang W, Ran B, Fan H, Wang H, Guo B, Zhou C, Shao Y, Giraudoux P, Knapp J, Wen H, Kuang L and Li J. Genetic variation of mitochondrial genes among echinococcus multilocularis isolates collected in western China. Parasit Vectors 2017; 10: 265.

[16] Campbell G, Garcia HH, Nakao M, Ito A and Craig PS. Genetic variation in taenia solium. Parasitol Int 2006; 55 Suppl: S121-126.

[17] Xiao N, Qiu J, Nakao M, Nakaya K, Yamasaki H, Sako Y, Mamuti W, Schantz PM, Craig PS and Ito A. Short report: identification of echinococcus species from a yak in the Qinghai-Tibet plateau region of China. Am J Trop Med Hyg 2003; 69: 445-446.

[18] Ma SM, Maillard S, Zhao HL, Huang X, Wang H, Geng PL, Bart JM and Piarroux R. Assessment of echinococcus granulosus polymorphism in Qinghai province, People's Republic of China. Parasitol Res 2008; 102: 1201-1206.

[19] Nakao M, Li T, Han X, Ma X, Xiao N, Qiu J, Wang H, Yanagida T, Mamuti W, Wen H, Moro PL, Giraudoux P, Craig PS and Ito A. Genetic polymorphisms of echinococcus tapeworms in China as determined by mitochondrial and nuclear DNA sequences. Int J Parasitol 2010; 40: 379-385.

[20] Yan N, Nie HM, Jiang ZR, Yang AG, Deng SJ, Guo L, Yu H, Yan YB, Tsering D, Kong WS, Wang N, Wang JH, Xie Y, Fu Y, Yang DY, Wang SX, Gu XB, Peng XR and Yang GY. Genetic variability of echinococcus granulosus from the tibetan plateau inferred by mitochondrial DNA sequences. Vet Parasitol 2013; 196: 179-183.

[21] Wang N, Wang J, Hu D, Zhong X, Jiang Z, Yang A, Deng S, Guo L, Tsering D, Wang S, Gu X,

Peng X and Yang G. Genetic variability of echinococcus granulosus based on the mitochondrial 16S ribosomal RNA gene. Mitochondrial DNA 2015; 26: 396-401.

[22] Nakao M, Yanagida T, Okamoto M, Knapp J, Nkouawa A, Sako Y and Ito A. State-of-the-art echinococcus and taenia: phylogenetic taxonomy of human-pathogenic tapeworms and its application to molecular diagnosis. Infect Genet Evol 2010; 10: 444-452.

[23] Bowles J, Blair D and McManus DP. Genetic variants within the genus echinococcus identified by mitochondrial DNA sequencing. Mol Biochem Parasitol 1992; 54: 165-173.

[24] Nakao M, Yokoyama N, Sako Y, Fukunaga M and Ito A. The complete mitochondrial DNA sequence of the cestode echinococcus multilocularis (cyclophyllidea: taeniidae). Mitochondrion 2002; 1: 497-509.

[25] Nakao M, Xiao N, Okamoto M, Yanagida T, Sako Y and Ito A. Geographic pattern of genetic variation in the fox tapeworm echinococcus multilocularis. Parasitol Int 2009; 58: 384-389.

[26] Yamasaki H, Nakao M, Nakaya K, Schantz PM and Ito A. Genetic analysis of echinococcus multilocularis originating from a patient with alveolar echinococcosis occurring in Minnesota in 1977. Am J Trop Med Hyg 2008; 79: 245-247.

[27] Konyaev SV, Yanagida T, Ingovatova GM, Shoikhet YN, Nakao M, Sako Y, Bondarev AY and Ito A. Molecular identification of human echinococcosis in the Altai region of Russia. Parasitol Int 2012; 61: 711-714.

[28] Konyaev SV, Yanagida T, Nakao M, Ingovatova GM, Shoykhet YN, Bondarev AY, Odnokurtsev VA, Loskutova KS, Lukmanova GI, Dokuchaev NE, Spiridonov S, Alshinecky MV, Sivkova TN, Andreyanov ON, Abramov SA, Krivopalov AV, Karpenko SV, Lopatina NV, Dupal TA, Sako Y and Ito A. Genetic diversity of echinococcus spp. in Russia. Parasitology 2013; 140: 1637-1647.

[29] Lymbery AJ. Interbreeding, monophyly and the genetic yardstick: species concepts in parasites. Parasitol Today 1992; 8: 208-211.

[30] Haag KL, Araujo AM, Gottstein B, Siles-Lucas M, Thompson RC and Zaha A. Breeding systems in echinococcus granulosus (cestoda; taeniidae): selfing or outcrossing? Parasitology 1999; 118: 63-71.

[31] Jafari R, Sanei B, Baradaran A, Spotin A, Bagherpour B and Darani HY. Genetic characterization of echinococcus granulosus strains isolated from humans based on nad1 and cox1 gene analysis in Isfahan, central Iran. J Helminthol 2017; 1-7.

[32] Gholami S, Jahandar H, Abastabar M, Pagheh A, Mobedi I and Sharbatkhori M. Echinococcus granulosus sensu stricto in dogs and jackals from Caspian Sea Region, Northern Iran. Iran J Parasitol 2016; 11: 186-194.

[33] Knapp J, Staebler S, Bart JM, Stien A, Yoccoz NG, Drogemuller C, Gottstein B and Deplazes P. Echinococcus multilocularis in Svalbard, Norway: microsatellite genotyping to investigate the origin of a highly focal contamination. Infect Genet Evol 2012; 12: 1270-1274.

[34] Cadavid Restrepo AM, Yang YR, McManus DP, Gray DJ, Giraudoux P, Barnes TS, Williams GM, Soares Magalhaes RJ, Hamm NA and Clements AC. The landscape epidemiology of echinococcoses. Infect Dis Poverty 2016; 5: 13.