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Echinococcus species from red foxes, corsac foxes, and wolves in Mongolia

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Running title: *Echinococcus* spp. in Mongolia

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SUMMARY

The small intestines of 420 wild canids (111 corsac foxes, 191 red foxes, and 118 wolves), from Mongolia, were examined for adult worms of the genus *Echinococcus*. The Mongolian genotype of *E. multilocularis* was found in fifteen red foxes and four wolves, whereas two genotypes (G6/7 and G10) of *E. canadensis* were found in two and three wolves, respectively. No adult *Echinococcus* worms were found in the examined corsac foxes. The genotypes of *E. multilocularis* and *E. canadensis* are discussed in terms of host specificity and distribution in Mongolia. The importance of wolves in the completion of the life cycle of *Echinococcus* spp. is also discussed.

KEYWORDS: *Echinococcus multilocularis*, Asian and Mongolian genotypes, *E. canadensis*, red fox, corsac fox, wolf, Mongolia

INTRODUCTION

Echinococcosis, caused by accidental ingestion of the eggs of several species of the genus *Echinococcus*, is now recognized to be a more serious health concern than once believed (Abuladze, 1964; Torgerson and Budke, 2003; Budke *et al*. 2006; Craig *et al*.

2007; Torgerson *et al*. 2006, 2010; Schweiger *et al*. 2007; Fuglei *et al*. 2008; Ito *et al*. 2011a; Jenkins *et al*. 2012; Combes *et al*. 2012; Konyaev *et al*. 2012, 2013; Torgerson, 2013; Schneider *et al*. 2013). Cystic echinococcosis (CE) and alveolar echinococcosis (AE) are the most common echinococcoses worldwide. It has been shown that CE is caused by a variety of genotypes (G1-G10) and that pathogenicity, to humans, differs among these genotypes. The majority of human CE cases are believed to be caused by *E. granulosus* (G1-G3), with fewer cases the result of *E. granulosus* (G5-10) infection (Thompson and McManus, 2002; Thompson, 2008). However, recent molecular re-evaluation of *E. granulosus* sensu lato has revealed that it consists of five independent species: *E. granulosus* sensu stricto (G1-G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6-G10) and *E. felidis* (Nakao *et al*. 2007; Hüttner *et al*. 2008; Knapp *et al*. 2011, Nakao *et al*. 2013b). In contrast, it has been shown that *E. multilocularis* can be differentiated into four (North American, Asian, European, and Mongolian) genotypes (Bretagne *et al*. 1996; Nakao *et al*. 2007; Ito *et al*. 2010; Konyaev *et al*. 2013).

After the collapse of the Soviet Union in 1991, Mongolia's infrastructure to collect data on echinococcosis cases and implement control measures broke down (Torgerson *et al*. 2006). Even though people recognized that CE was common in Mongolia due to the traditional nomadic life style, there are no published nationwide data. Information on very few AE (Spira, 1995; Davaatseren *et al*. 1995; Ebright *et al*. 2003; Ito *et al*. 2010, 2011a; Gurbadam *et al*. 2010) and CE cases (Davaatseren *et al*. 1995; Ebright *et al*. 2003; Ito *et al*. 2010, 2011a; Jabbar *et al*. 2011; Dorjsuren *et al*. in prep.) has ever been published from Mongolia. Therefore, several meetings have recently been conducted to establish a network to improve the diagnosis of echinococcosis in Mongolia as well as

plan regional epidemiological studies on *Echinococcus* spp. (Gurbadam *et al*. 2010; Ito *et al*. 2011b).

In addition to very limited data on human echinococcosis, there are no data on *Echinococcus* spp. infections in animals from Mongolia. This is in contrast to the large number of studies from Russia (Abuladze, 1964). Therefore, in this study, we examined the intestines of foxes and wolves for detection of adult worms of *Echinococcus* spp.

MATERIALS AND METHODS

Wild animals and parasite samples

Small intestines from foxes and wolves captured by hunters, trappers, and wildlife rangers throughout Mongolia during the winters of 2009 to 2011 were sent to the Mongolian Academy of Science in Ulaanbaatar. Geographic locations for the samples were obtained at the province level. The intestines were frozen at -80˚C until they could be examined. A total of 111 corsac foxes (*Vulpes corsac*), 191 red foxes (*Vulpes vulpes*), and 118 wolves (*Canis lupus*) were examined for *Echinococcus* spp. (Table 1, Fig. 1).

Detection of Echinococcus *spp.*

The sedimentation method was used to detect parasites in the provided small intestines. Each small intestine was opened with scissors and all intestinal contents were collected in a 500 ml plastic beaker. Water was added, the beaker contents were mixed, and the supernatant was discarded. This process was then repeated several times. The remaining contents were kept in 50 ml screw cap tubes. *Echinococcus* specimens were confirmed by microscopic examination (Matoba et al. 2006).

Mitochondrial DNA analysis

Genomic DNA was extracted from ethanol-fixed adult worms using the DNeasy blood and tissue kit (Qiagen). Obtained DNA was used as templates for polymerase chain reaction (PCR). One to twelve worms from each host animal were used for the analysis. The entire mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene was amplified by PCR as reported previously (Hüttner *et al*. 2008). PCR amplicons were treated with illustra ExoStar (GE Healthcare) to remove excess primers and dNTPs, and directly sequenced with a BigDye™ Terminator v3.1 and a 3500 DNA sequencer (Life Technologies). Obtained sequences of each *cox1* haplotype were aligned by Clustal W 2.0 (Larkin *et al*. 2007) with representative *cox1* sequences of *Echinococcus* spp. available in the GenBank database. A phylogenetic tree was constructed using the neighbour-joining method and Kimura's two-parameter model (Kimura, 1980) in Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b (Swofford, 2002). The robustness of the phylogenetic tree was tested by bootstrapping with 1,000 replicates. For tree construction, *Versteria mustelae* was used as an out-group because the species is a sister to members of the genus *Echinococcus* (Nakao *et al*. 2013a).

RESULTS

Table 1 summarizes the results from the intestinal analyses of the provided wild canids, with samples collected from 10 of the 17 provinces in Mongolia (Fig. 1). A total of fifteen red foxes and nine wolves were confirmed to be harbouring *Echinococcus* adult worms. Nucleotide sequences of the *cox1* gene (1608 bp) were determined for a total of 99 isolates of *Echinococcus* spp. and, consequently, 7 haplotypes were obtained. The

nucleotide sequences of these haplotypes were deposited into DDBJ/EMBL/GenBank databases under the accession numbers AB813182-AB813188. Based on the phylogenetic analysis, these isolates were identified as *E. canadensis* (G6/7 and G10) and *E. multilocularis* (Fig. 2). Three *cox1* haplotypes of *E. multilocularis* (EmMGL1-3) were further categorized as belonging to the Mongolian genotype.

Echinococcus multilocularis was confirmed in red foxes from Arkhangai, Bulgan, Dornod, and Zavkhan provinces (Table 1 and Fig. 1). In Dornod, *E. multilocularis* was found during 2011 (6/16) and 2013 (1/5), but not in 2012 (0/20). Two of the three *cox1* haplotypes (EmMGL1 and 2) were found in red foxes, with three foxes in Dornod harbouring both haplotypes. While EmMGL1 was confirmed in red foxes from all four provinces, EmMGL2 was confirmed only in Arkhangai and Bulgan (Fig. 2). *Echinococcus multilocularis* adult worms were found in four wolves in Khentii, Sukhbaatar, Tuv (Central) and Zavkhan (Table 1). EmMGL1 was found in Sukhbaatar and Tuv, EmMGL2 was found in Khentii and Zavkhan, and EmMGL3 was found in Sukhbaatar. *Echinococcus canadensis* G6/7 was confirmed in 1/7 wolves from Gobi-Altai and in 1/56 wolves from Zavkhan. The wolf from Gobi-Altai harboured both *cox1* haplotypes (EcMGL1 and 2), and the wolf from Zavkhan harboured the EcMGL2 haplotype. *Echinococcus canadensis* G10 was confirmed in 3/5 wolves from Zavkhan. EcMGL3 and EcMGL4 were obtained from two and one wolves, respectively.

DISCUSSION

This is the first report of *Echinococcus* spp. from animals in Mongolia. Although we believe that both red foxes and corsac foxes are important in the transmission of *E.*

multilocularis in Mongolia, in this study, we only found red foxes infected with *E. multilocularis*. By contrast, wolves were found to be definitive hosts to both *E. canadensis* and *E. multilocularis* in Mongolia (Table 1).

The Mongolian genotype is, thus far, the only *E. multilocularis* genotype confirmed from wild canids in Mongolia (Ito *et al*. 2010). This genotype was originally described by Tang *et al*. (2007) as a new species, *E. russicensis*, found in corsac foxes in Inner Mongolia, China. Subsequently, it was confirmed to be an intraspecies variation of *E. multilocularis* and described as the Inner Mongolian genotype (Nakao *et al*. 2009). Recently, genetic variations have been described in two human AE cases, infected with this genotype, originating in Mongolia (Ito *et al*. 2010). This has resulted in the Inner Mongolian genotype being re-described as the Mongolian genotype (Ito *et al*. 2010; Konyaev *et al*. 2013). Our results do not exclude the possibility that corsac foxes are definitive hosts of the Mongolian genotype, since corsac foxes are well known as definitive hosts of *E. multilocularis* and *E. granulosus* s.l. in Russia (Abuladze, 1964). A more interesting finding is that red foxes, as well as wolves, can harbour the Mongolian genotype. Therefore, the Mongolian genotype may be the major genotype in Mongolia. However, this study is only the first to show direct evidence of *Echinococcus* spp. infection in animals from Mongolia. Additional studies, including evaluation of seasonal differences in infection rates and confirmation of the intermediate host animals are needed to understand the local life cycle of *Echinococcus* spp.

Three human AE cases were recently identified from Mongolia. One of the cases was confirmed to be infected with the Asian genotype and the other two cases were confirmed to be infected with the Mongolian genotype (Ito *et al*. 2010). This was the

first report describing AE cases, in Mongolia, using a molecular approach. We expect that the primary definitive host for both the Mongolian and Asian genotypes in Mongolia, is the red fox since the red fox is known as the definitive host for the Asian genotype in other countries (Nakao *et al*. 2009). It is also expected that *E. multilocularis* is widely distributed in wild animals in Mongolia (Fig. 1). Additional studies are needed to confirm which genotypes predominate among the various wild canid definitive hosts as well as to better understand the role played by the corsac fox in the transmission of *E. multilocularis* in Mongolia. There are no published studies on the parasitic fauna in any animals in Mongolia. We are, therefore, in the process of analyzing intestinal helminthic fauna from a variety of species for future publication.

The importance of wolves in the transmission of both *E. multilocularis* and *E. granulosus* has been reported previously. *E. multilocularis* has been reported from wolves in Russia (Abuladze, 1964; Dzhabarova *et al*. 1993; Konyaev *et al*. 2013), China (Wang *et al*. 1989), Bulgaria (Breyer *et al*. 2004), Latvia (Bagrade *et al*. 2009), Iran (Beiromvand *et al*. 2011), and Kazakhstan (Abdybekova and Torgerson, 2012), whereas *E. canadensis* and *E. granulosus* s.l. have been reported from wolves in Eurasia and North America (Rausch and Schiller, 1951; Abuladze, 1964; Dzhabarova *et al*. 1993; Rausch, 2003; Hirvelä-Koski *et al*. 2003; Guberti *et al*. 2004; Breyer *et al*. 2004; Tang *et al*. 2004; Moks *et al*. 2006; Schantz, 2006; Sobrino *et al*. 2006; Bagrade *et al*. 2009; Foreyt *et al*. 2009; Abdybekova and Torgerson, 2012; Bryan *et al*. 2012; Guerra *et al*. 2013; Konyaev *et al*. 2013). Among these reports, *E. granulosus* genotypes G1 and G10 have been confirmed from Bulgaria (Breyer et al. 2004) and Estonia (Morks *et al*. 2006), respectively, genotypes G8 and G10 have been confirmed from Canada (Bryan *et al*. 2012), and genotypes G6, G8, and G10 have been confirmed from Russia (Konyaev *et*

al. 2013). In addition, genotype G7 has been confirmed from Portugal (Guerra *et al*. 2013). However, the present study is the first report on the sympatric occurrence of the G6/7 and G10 genotypes in wolves. Both *E. multilocularis* and *E. granulosus* s.l. have also been confirmed from wolves (Abuladze, 1964; Dzhabarova *et al*. 1993; Bagrade *et al*. 2009; Beiromvand *et al*. 2011; Abdybekova and Torgerson, 2012; Konyaev *et al*. 2013) in the same geographic locations, with dual infections also reported (Abuladze, 1964; Beiromvand *et al*. 2011). Recent increases in wolf populations in Europe and North America, and especially in Arctic and Sub-Arctic areas, should be noted with caution in terms of transmission of *E. multilocularis* (Martínek *et al*. 2001; Bagrade *et al*. 2009), *E. canadensis*, and *E. granulosus* s.s. (Rausch, 2003; Schantz, 2006; Sobrino *et al*. 2006; Moks *et al*. 2006; Foreyt *et al*. 2009; Bagrade *et al*. 2009; Abdybekova and Torgerson, 2012).

Additional studies are needed to evaluate infection prevalence in wolves located in the three main eco-regions of Mongolia (the Gobi Desert in the south, the mountainous or forest region in the north and west, and the steppes). As shown in Fig. 1 and Table 1, *E. multilocularis* worms have been found in red foxes living in the mountainous and steppe regions of Mongolia where nomadic or semi-nomadic herdsmen live. Therefore, monitoring *E. multilocularis* infections in both red and corsac fox populations is also necessary for future control programs.

Echinococcus canadensis (G6/7 and G10) was found in wolves living in the western part of Mongolia. Recent studies, using 50 human CE samples from Mongolia, revealed that *E. granulosus* s.s. (n = 30) was confirmed mainly from the eastern regions of the country, whereas patients infected with *E. canadensis* (G/7 and G10) (n = 20) were from

the western part of the country, including Gobi-Altai and Zavkhan provinces (Jabbar *et al*. 2011). The fact that *E. granulosus* s.s. was not detected in wild canids, in the present study, may support the belief that *E. granulosus* s.s. tends to be associated primarily with domestic dogs. Therefore, we would anticipate detecting adult worms of *E. granulosus* s.s. from dogs located in the eastern part of the country where Jabbar *et al*. (2011) confirmed human infection with *E. granlulosus* s.s.

Our results suggest that *E. canadensis* is strongly linked with wolves in Mongolia. Possible animal definitive hosts should be evaluated for *Echinococcus* spp. in all provinces. Special attention should be given to the Northern provinces that border Russia and to the northwestern provinces that border China or China and Russia since these regions are mountainous and provide suitable habitat for wolves and foxes. As shown in Fig. 1 and Table 1, Zavkhan Province is an important area for further studies. Zavkhan Province is unique in that an ethnic minority, in this province, is primarily composed of reindeer (*Rangifer tarandus*) herders, with reindeer expected be a suitable intermediate host for *E. canadensis* (Konyaev *et al*. 2013). Neighbouring Khubsugul Province is also believed to be important, since human CE cases infected with genotype G6/7 have been identified from this province (Jabbar *et al*. 2011). Although only four red foxes were examined from Khubsugul, additional samples from red foxes, corsac foxes, wolves, and dogs are needed, from this province, since this region contains a national park and is considered the largest resort area in Mongolia.

Prior to the current study, only human infections with *Echinococcus* spp. have been reported from Mongolia. As an initial evaluation of infections in animals, we focused on wild canids due to availability of these animals from local hunters, trappers, and rangers.

However, infection in domestic dogs needs to be studied since the nomadic life style of the Mongolian people brings them in close contact with dogs. It is expected that dogs in nomadic households might show similar levels of infection as the red foxes and wolves in this study. Furthermore, each household typically has at least one dog, which is usually not tied and is often left to hunt for its own food during the summer season. In these situations, dogs will hunt small mammals and scavenge offal from domestic livestock. It is also common for herdsmen to train dogs to hunt marmots (*Marmota sibirica*), which could potentially be an intermediate host for *Echinococcus* spp.

Recent serological studies of dog owners in Ulaanbaatar carried out at the National Center of Communicable Diseases (NCCD) (Davaasuren *et al*. in prep.) have revealed that a higher proportion of dog owners are positive for CE by both ELISA and immunoblot using the recombinant Antigen B8/1 compared to individuals that do not own dogs (Mamuti *et al*. 2004; Mohammadzadeh *et al*. 2012). Therefore, it is imperative to evaluate *Echinococcus* spp. infection in dogs.

The number of echinococcosis patients treated surgically at the First Hospital of Ulaanbaatar in 1993 was approximately two times higher than the number treated in 1950. The proportions of surgical cases due to CE and AE, at the same hospital, in 1950 were 7.8% and 1.9%, respectively, with AE cases numbering approximately a quarter of CE cases (Spira, 1995; Ebright *et al*. 2003). In the last decade, there have only been three reports on human echinococcosis in Mongolia, one describing three cases of AE (Ito *et al*. 2010) and the other describing fifty cases of CE (Jabbar *et al*. 2011). Only three AE cases, with samples kept at the Pathology Center, Ulaanbaatar, were available for molecular analysis (Ito *et al*. 2010). We expect that many AE cases in rural areas have been misdiagnosed as hepatic cancers and that greater numbers of AE

cases would be identified if a national notification system for echinococcoses was established. It is important that the NCCD and the Mongolian Center of Infectious Diseases with Natural Foci (CIDNF) form collaborations with other academic institutions and hospitals. Therefore, we strongly recommend that the Mongolian government establish a network for the control of echinococcoses in humans, which includes a monitoring system for infection in dogs, wild animals, and livestock (Gurbadam *et al*. 2010).

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Table 1. Occurrences of *Echinococcus* spp. from corsac foxes, red foxes and wolves from 10 Provinces, Mongolia

Province	Corsac fox	Red fox	Wolf	Total
Arkhangai	0/8	1^{Emul}/3	NS	1/11
Bulgan	0/26	3^{Emul}/60	NS	3/86
Tuv (Central)	NS	NS	1^{Emul}/1	1/1
Dornod	NS	7^{Emul}/41	0/4	7/45
Gobi-Altai	NS	NS	$1^{G6/7}$/7	1/7
Khovd	NS	NS	0/5	0/5
Khentii	NS	0/1	1^{Emul}/2	1/3
Khubsugul	0/45	0/6	NS	0/51
Sukhbaatar	NS	0/1	1^{Emul}/29	1/30
Zavkhan	0/32	4^{Emul}/79	1^{Emul}, $1^{G6/7}$, 3^{G10}/70	9/181
Total	0/111	15^{Emul}/191	4^{Emul}, $2^{G6/7}$, 3^{G10}/118	24/420

NS: no sample.

^{Emul}: *E. multilocularis*

$^{G6/7}$: *E. canadensis* (G6/7)

^{G10}: *E. canadensis* (G10)

FIGURE LEGENDS

Fig. 1. A map of Mongolia showing the number of *Echinococcus* positive animals.

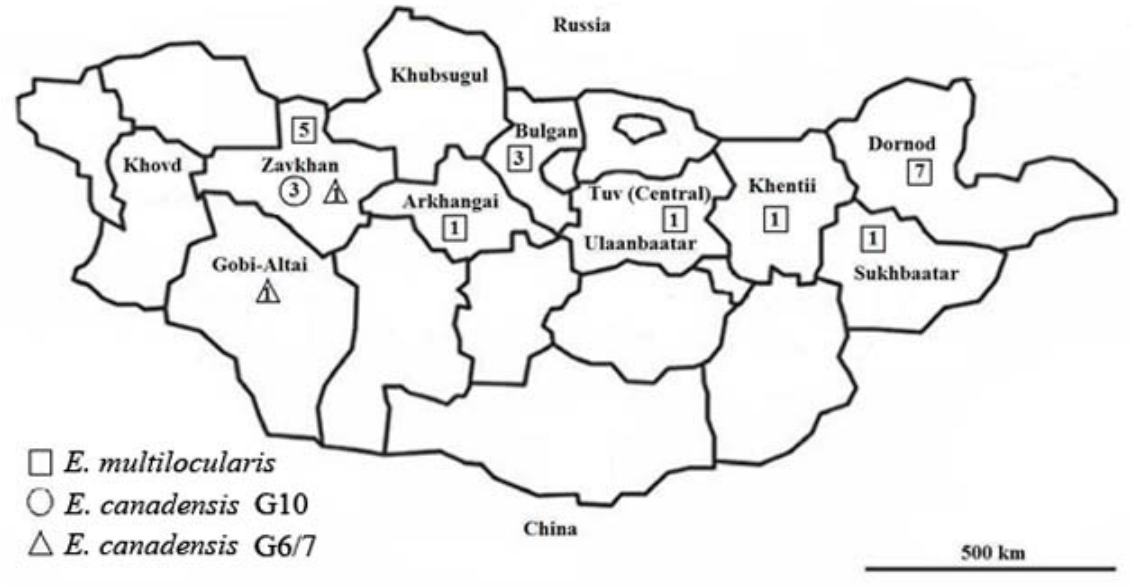
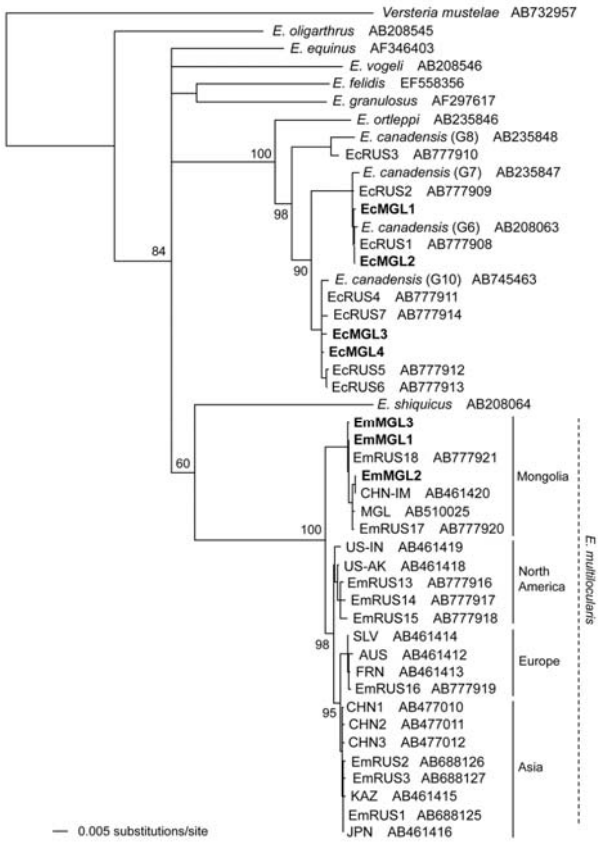


Fig. 2. A neighbour-joining tree of *Echinococcus* spp. constructed from the nucleotide sequences of mitochondrial *cox1* gene. Numbers on the nodes are bootstrap values. The names of the haplotypes obtained in the present study are shown in bold. CHN-IM, Chinese Inner Mongolia; MGL, Mongolia; US-AK, Alaska (St Lawrence Island); US-IN, Indiana; US-SD, South Dakota; SLV, Slovakia; AUS, Austria; FRN, France; KAZ, Kazakhstan; CHN, China (Sichuan); JPN, Japan (Hokkaido).


Versteria mustelae AB732957
E. oligarthrus AB208545
E. equinus AF346403
E. vogeli AB208546
E. felidis EF558356
E. granulosus AF297617
E. ortleppi AB235846
E. canadensis (G8) AB235848
EcRUS3 AB777910
E. canadensis (G7) AB235847
EcRUS2 AB777909
EcMGL1
E. canadensis (G6) AB208063
EcRUS1 AB777908
EcMGL2
E. canadensis (G10) AB745463
EcRUS4 AB777911
EcRUS7 AB777914
EcMGL3
EcMGL4
EcRUS5 AB777912
EcRUS6 AB777913
E. shiquicus AB208064
EmMGL3
EmMGL1
EmRUS18 AB777921
EmMGL2
CHN-IM AB461420
MGL AB510025
EmRUS17 AB777920
US-IN AB461419
US-AK AB461418
EmRUS13 AB777916
EmRUS14 AB777917
EmRUS15 AB777918
SLV AB461414
AUS AB461412
FRN AB461413
EmRUS16 AB777919
CHN1 AB477010
CHN2 AB477011
CHN3 AB477012
EmRUS2 AB688126
EmRUS3 AB688127
KAZ AB461415
EmRUS1 AB688125
JPN AB461416
Mongolia
North America
Europe
Asia
E. multilocularis
100
98
84
90
60
100
98
95
0.005 substitutions/site