International Journal for Parasitology (2005. May) 35(6):693-701.

Echinococcus shiquicus n. sp., a taeniid cestode from Tibetan fox and plateau pika in China

Xiao, Ning ; Qiu, Jiamin ; Nakao, Minoru ; Li, Tiaoying ; Yang, Wen ; Chen, Xingwang ; Schantz, Peter M ; Craig, Philip S ; Ito, Akira

Manuscript number: 04/351
Revised

Echinococcus shiquicus n. sp., a taeniid cestode from Tibetan fox and plateau pika in China ☆

Ning Xiao[a,b], Jiamin Qiu[b], Minoru Nakao[a], Tiaoying Li[b], Wen Yang[b], Xingwang Chen[b], Peter M. Schantz[c], Philip S. Craig[d], Akira Ito[a,]*

[a] *Department of Parasitology, Asahikawa Medical College, Asahikawa 078-8510, Japan*
[b] *Institute of Parasitic Diseases, Sichuan Center for Disease Control and Prevention, Chengdu, Sichuan 610041, China*
[c] *Division of Parasitic Diseases, National Centers for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30341, USA*
[d] *Cestode Zoonoses Research Group, Bioscience Research Institute & School of Environment and Life Sciences, University of Salford, Great Manchester, M5 4WT, UK*

Note

☆ Nucleotide sequence data reported in this paper are available in DDBJ/EMBL/GenBank databases under the accession numbers AB159136-43.

* Corresponding author. Tel.: +81-166-68-2420; fax: +81-166-68-2429.
E-mail address: akiraito@asahikawa-med.ac.jp (A. Ito).

Abstract

The taeniid cestode *Echinococcus shiquicus* n. sp. was found from the Tibetan fox *Vulpes ferrilata* and the plateau pika *Ochotona curzoniae* in the Qinghai-Tibet plateau region of China. In the adult stage, *E. shiquicus* from the foxes is morphologically similar to *Echinococcus multilocularis*. However, the new species is differentiated by its smaller rosteller hooks, fewer segments, distinct position of genital pore in the mature segment and fewer eggs in the gravid segment. Hydatid cysts of *E. shiquicus* found in the livers from the pikas were essentially unilocular but an oligovesicular cyst was also found. The data of mitochondrial and nuclear DNA sequences proved *E. shiquicus* to be a valid taxon.

Keywords: Qinghai-Tibet plateau; Tibetan fox; Plateau pika; *Echinococcus shiquicus* n. sp.

1. Introduction

Species of *Echinococcus* Rudolphi, 1801 (Cestoda: Taeniidae) are minute tapeworms of carnivores. Their larvae are known as hydatids which proliferate asexually in various mammals including humans. The taxonomy of this genus has been controversial owing to inadequate descriptions and sympatric occurrences of subspecies. A total of 16 species and 13 subspecies have been described but only four species (i.e. *Echinococcus granulosus*, *Echinococcus multilocularis*, *Echinococcus oligarthrus* and *Echinococcus vogeli*) are generally accepted as valid taxa (Rausch and Bernstein, 1972; Kumaratilake and Thompson, 1982). The former two species are widely distributed, whereas the latter two species are restricted to Central and South America. These species are distinguishable by a number of morphological characteristics of both adult and larval stages. However, several strains of *E. granulosus,* which show substantial genetic diversity, have been classified into 10 genotypes (G1-G10) (Bowles et al., 1992, 1995; Bowles and McManus, 1993, Scott et al., 1997; Lavikainen et al., 2003). Recently, Thompson and McManus (2003) proposed the following taxonomic revision; the G1 (sheep strain) genotype is the prototypical species of *E. granulosus* but the G4 (horse strain) and G5 (cattle strain) genotypes are distinct species of *Echinococcus equinus* and *Echinococcus ortleppi*, respectively. Thus, the biological entity of sibling or cryptic species should be considered in the taxonomy of *Echinococcus*.

Our research group is currently collecting specimens of *E. multilocularis* throughout the Holarctic region for a large-scale genetic study of the species.

During the course of mitochondrial DNA (mtDNA) sequencing, we noticed that a larval specimen from the plateau pika, *Ochotona curzoniae,* in the Qinghai-Tibet plateau region of China showed a characteristic sequence, which was dissimilar to any published sequences of *Echinococcus* spp. The same sequence was subsequently found in adult specimens from the Tibetan fox, *Vulpes ferrilata*. This unknown species is distributed sympatrically with *E. multilocularis* and the *E. granulosus* G1 genotype. Qiu et al. (1995) have already observed its morphological characteristics but considered it to be a variant of *E. multilocularis*. Taxonomic criteria including morphology, host preference, molecular genetics and geographical distribution have led us to describe a new species. In this article, we present the morphological features of both adult and larval stages and provide molecular evidence to support the validity of the new species.

2. Materials and methods

2.1. Parasite samples and morphological observations

From July 2001 to November 2003, larval and adult specimens of *Echinococcus* spp. were collected from foxes, dogs, pikas, voles and sheep in Shiqu County, the Qinghai-Tibet plateau region of western Sichuan, China (Table 1). All samples were collected following the local laws for the preservation of domestic animals and wildlife. Since the Tibetan foxes were strictly protected from hunting, parasites were taken from the carcasses killed by attacks of stray dogs. Tapeworms from canine intestines were relaxed in tap water and then fixed in 4% formalin. Hydatid tissues from intermediate hosts were also fixed in 4% formalin. Parts of both larval and adult samples were stored in 70-99%

ethanol for DNA preservation. The formalin-fixed samples were subjected to morphological observations. The tapeworms were stained overnight with Delafield's haematoxylin, destained with 70% ethanol containing 1% hydrochloric acid, dehydrated in ethanol, cleared with xylene and mounted in Canada balsam. Eggs were obtained from broken gravid segments. To examine rostellar hooks, tapeworms placed on a glass slide were crushed with pressure on a coverslip. The hydatid tissues were embedded in paraffin-wax. Sections (3-5 µm thick) were stained with haematoxylin and eosin.

2.2. Sequence analysis

DNA was purified from hydatid tissues by using a spin column kit (DNeasy tissue kit; Qiagen, Germany). As reported previously (Nakao et al., 2003a), tapeworms were individually lysed in 10 µl of 0.02 N NaOH at 95°C for 10 min. The larval DNA or the adult lysate was used as a template for polymerase chain reaction (PCR). A DNA polymerase with 3'-5' exonuclease proofreading activity (Ex-*Taq*; Takara Biomedicals, Japan) was used for PCR amplification. PCR was carried out in a 50 µl reaction mixture containing 1 µl template, 200 µM of each dNTP, 0.2 µM of each primer, 1U of Ex-*Taq* polymerase and the manufacturer-supplied reaction buffer. Thermal reactions were performed for 35 cycles of denaturation (94 °C for 30 s), annealing (54-56 °C for 30 s) and extension (72 °C for 60-90 s). Primer pairs used for the amplification of mitochondrial or nuclear DNA regions are shown in Table 2. The PCR products were directly sequenced by using a dye terminator cycle sequencing kit (DYEnamic ET terminator; Amersham Biosciences, UK) and an automated sequencer (ABI PRISM 377; Applied Biosystems, USA).

The mitochondrial genomes of *E. multilocularis* (database accession no. **AB018440**), *E. granulosus* (**AF297617** and **AF346403**) and *Taenia solium* (**AB086256**) served as reference sequences (Nakao et al., 2002, 2003b; Le et al., 2002). Published mtDNA sequences of *cox1* (**M84661-71** and **AF525457**), *nad1* (**AJ237632-43**) and *atp6* (**AY056611-5**) were used for comparison (Bowles et al., 1992, 1993, 1994; McManus et al., 2002; Lavikainen et al., 2003). The *elp* locus of an ezrin-radixin-moesin (ERM)-like protein (**AJ012663**) was used to compare nuclear DNA (Brehm et al., 1999). Multiple alignments of sequences were achieved by the Clustal W program (http://www.ddbj.nig.ac.jp). Gaps and missing data were deleted from the alignments. Percentage divergences of nucleotide sequences were corrected by Kimura's 2 parameter model (Kimura, 1980). Phylogenetic trees were constructed from the alignments by using the neighbor-joining method in the MEGA2 software (Saitou and Nei, 1987; Kumar et al., 2001). All three codon positions were used to analyze nucleotide sequences. Confidence values for each branch of the trees were determined by 1000 bootstrap replications.

3. Results

3.1. Description of the adult worm

Adults of *Echinococcus shiquicus* n. sp. were found only in Tibetan foxes. During the survey period, 6 (37.5%) of 16 Tibetan foxes were confirmed to be infected with *E. shiquicus* by DNA sequencing. Adult specimens from 2 foxes, whose morphological conditions remained better, were used for observation.

As shown in Fig. 1, the adults containing a gravid segment were divided into two types. The first type consisted of only pre-mature and gravid segments (Fig. 1A). Early ovary and testes were formed in the pre-mature segment but its genital pore was closed. This unique type constituted the majority of the specimens. The second type consisted of immature, mature and gravid segments (Fig. 1B). The number of segments in fully developed adults did not exceed three. The adults of the second type (*n*=20) were used for the following description. All measurements are in micrometers, except where indicated.

Length of whole body 1.3-1.7 (mean 1.5) mm. Strobila extremely small, with only three segments (Fig. 2A). Genital pores irregularly alternating. Lateral osmoregulatory canals running through scolex to gravid segment. Scolex with four suckers. Suckers oval, 63-73 (mean 69) in maximum diameter. Rostellum armed with tiny hooks. The number is 18-34 (Qiu et al., 1995). To measure the length of hooks, six worms retaining both large and small hooks were selected from several hundred worms. Large hooks 20-23 (mean 21, *n*=19) long, small hooks 16-17 (mean 17, *n*=6) long (Fig. 2B). Neck absent. Immature segment 80-150 (mean 115) long by 160-230 (mean 192) wide, genital primordium present. Mature segment 300-475 (mean 386) long by 250-350 (mean 285) wide. Genital pore lateral, opened at 1/4 anterior portion of mature segment. Cirrus pouch pyriform, enclosing minute cirrus and coiled vas deferens, 120-138 (mean 131) long by 45-63 (mean 56) wide, located in anterior portion of segment extending beyond osmoregulatory canals to midline. Ovary bilobed, lobes subcircular in dorso-ventral view, 53-75 (mean 63) in maximum diameter. Vitelline gland subspherical, 68-83 (mean 77) in maximum diameter, postovarian. Ovary and vitelline gland located in centre of mature segment. Testes spherical, 25-45 (mean 36) in diameter, 12-20 (mean 16) in number, mainly distributed posterior to vitelline gland. Few testes anterior to genital pore, 0 to 2 in number. Gravid segment 625-800 (mean 708) long by 275-350 (mean 325) wide. Genital pore located at 1/3 anterior portion of gravid segment. Cirrus pouch, vagina and seminal receptacle still remaining in gravid segment. Gravid uterus branchless, sac-like, extending to posterior 1/3 of segment. Number of eggs in gravid uterus 37-94 (mean 76). Mature eggs 34-40 (mean 38) in diameter (*n*=24, from 3 worms), containing hexacanth embryo (Fig. 2C).

3.2. Morphological features of larva

The metacestode of *E. shiquicus* was found only in plateau pikas. DNA sequencing revealed that 5 (5%) of 101 pikas harbored hydatid cysts of *E. shiquicus* in their livers. A pulmonary hydatid cyst was also found in one pika. Most of the larval forms were unilocular cysts of 10 mm in diameter but one showed an oligovesicular form (Fig. 3A). The cysts included no daughter cysts. Fully developed brood capsules containing many protoscoleces were attached firmly to germinal layers. The protoscoleces were 125-140 µm (mean 128) long by 105-125 µm (mean 117 µm) wide (*n*=20, from 1 cyst). Numbers of hooks in the protoscolex were 19-24 (mean 21, *n*=12), and their length ranged from 16 to 21 µm (mean 18 µm, *n*=30, from 5 protoscoleces). Host inflammatory reactions to cysts appeared minimal. The adventitial layer around cysts was thin, but the laminated layer of cysts was relatively broad, being 5-38 µm in thickness. A protrusion of cyst was found, suggesting that exogenous budding may occur (Fig.

3B).

3.3. Molecular analyses

Since three species of *Echinococcus* are distributed sympatrically in the survey area, partial fragments of mitochondrial *cob* and nuclear *elp* were amplified and sequenced to confirm species identity (Table 1). The length of *cob* sequenced was 549 base pairs (bp). Intraspecific variation of the *cob* sequence was observed in *E. shiquicus* and *E. granulosus* but not in *E. multilocularis* (Table 3). Numbers of variable nucleotide sites were 15 (2.7% of total length) in *E. shiquicus* and 1 (0.2%) in *E. granulosus*. Transitional substitutions occurred at all variable sites. The maximum percentage of divergence reached 1.3 when 23 sequences of *E. shiquicus* were compared with each other. The intron VII sequences of nuclear *elp* locus were determined in 16 samples of *E. shiquicus*, 18 samples of *E. multilocularis* and 7 samples of *E. granulosus*. As shown in Table 3, the intron sequences of *E. shiquicus* were different from those of *E. multilocularis* and *E. granulosus*. Similarly, both *E. multilocularis* and *E. granulosus* had their unique sequences.

The DNA fragments containing complete mitochondrial genes were amplified from the hydatid tissue of *E. shiquicus* and sequenced. The full lengths of *E. shiquicus* mitochondrial genes determined in this study were 1608 bp in *cox1*, 897 bp in *nad1*, 513 bp in *atp6*, 1068 bp in *cob* and 985 bp in *rrnL*. The lengths were similar to those of *E. multilocularis* (Nakao et al., 2002) and *E. granulosus* G1 and G4 (Le et al., 2002). Table 4 shows the pairwise divergence values of nucleotide sequences between *E. shiquicus* and other *Echinococcus* species.

The values indicated that *E. shiquicus* is almost equidistant from other *Echinococcus* species regardless of the genes examined. Moreover, the values were at interspecific level when compared with those between *E. multilocularis* and *E. granulosus* G1 genotype. Among the genes examined, *cox1* showed the minimum divergence values (7.8-10.6%). In contrast, the maximum values (18.4-22.1%) were observed in *atp6*. Of the *Echinococcus* spp. and genotypes examined, only 79 (21.6%) of 366 nucleotide sites were variable in *cox1*, whereas 196 (38.2%) of 513 sites were variable in *atp6*. The extreme bias toward thymine base was observed in the coding strand of all protein genes examined. The thymine contents were 46.2-48.7% in *cox1*, 46.1-49.9% in *nad1*, 51.9-53.4% in *atp6* and 47.7-48.8% in *cob*.

The phylogenetic trees of *Echinococcus* were obtained from the neighbor-joining analysis using nucleotide sequences of partial *cox1,* partial *nad1* and complete *atp6*. As shown in Fig. 3, the resultant trees depicted that *E. shiquicus*, *E. multilocularis*, *E. vogeli, E. oligarthrus*, *E. granulosus* G1 (= *E. granulosus*), *E. granulosus* G4 (= *E. equinus*) and *E. granulosus* G5 (= *E. ortleppi*) are distantly related to each other. However, the branching patterns of the trees were different from each other. The phylogenetic positions of these 7 species were unclear because of low bootstrap values in each tree. On the other hand, the genotypes G6-G10 of *E. granulosus* (camel, pig and cervid strains) formed a single cluster in the *nad1*-tree, suggesting that these genotypes may belong to a single species. Phylogenies were reconstructed using deduced amino acid sequences; however, the interspecific relationships were also ambiguous (data not shown). Although mitochondrial rRNA gene is regarded as a good candidate for the study of deep phylogeny (von Nickisch-Rosenegk et al., 1999), the usefulness of *rrnL* gene was not examined because the sequences of *rrnL* have been determined only in *E. shiquicus*, *E. multilocularis* and the *E. granulosus* G1 and G4 (Table 4).

4. Discussion

Apart from the neotropical species *E. oligarthrus* and *E. vogeli*, *E. shiquicus* n. sp. must be differentiated from *E. granulosus* and *E. multilocularis*. In the adult stage, *E. shiquicus* is easily distinguishable from *E. granulosus* by its shorter length, branchless gravid uterus and anterior position of genital pore in the gravid segment. As shown in Table 5, *E. shiquicus* overlapped in most morphological features with *E. multilocularis* reported in China (Zhu et al., 1983; Li et al., 1985; Tang et al., 1988; Wang et al., 1989). However, undersized rostellar hooks and the upper position of genital pore in mature segment are characteristic of *E. shiquicus*. The strobila of *Echinococcus* consists of several segments, whose reproductive organs gradually develop toward the posterior end. In most species of *Echinococcus*, the gravid segment is connected to the mature segment. However, a strobila consisting of only two segments (a gravid segment directly attaches to a pre-mature segment) is unique to *E. shiquicus* (Fig. 1A). Fewer eggs in the gravid segment of *E. shiquicus* (less than 100) is also useful for differentiation because *E. multilocularis* in China shows higher fecundity (200-400 eggs per gravid segment) as reported by Zhu et al. (1983). In the larval stage, *E. shiquicus* is quite different from *E. multilocularis*. A unilocular minicyst containing fully developed brood capsules is typical of *E. shiquicus*. Unlike *E. granulosus*, no daughter cysts appear within the fertile cyst of *E. shiquicus*. The larval development of *E. shiquicus* in hosts other than plateau pika is unknown. In morphologically questionable cases of both adult and larva, the sequencing of mitochondrial DNA is recommended for the identification of species.

The mammalian fauna of the Qinghai-Tibet plateau consists of elements of the Palaearctic and Oriental realms (Feng et al., 1980). In this region approximately 4000 meters above sea level, many wild and domestic mammals including foxes, dogs, voles, pikas, hares, sheep and yaks are involved in the transmission cycles of *Echinococcus* (Qiu et al., 1995; Xiao et al., 2003, 2004). In this study, we found that the Tibetan fox *V. ferrilata* and the plateau pika *O. curzoniae*, which are endemic to the plateau, serve as natural hosts for *E. shiquicus*. A high density of the pika (Lai and Smith, 1996) is probably important to maintain the life cycle of *E. shiquicus*. Both the pika and the fox are adapted to the high altitude steppe but do not survive in lowlands. Accordingly, we predict that the distribution of *E. shiquicus* is restricted within the plateau and adjacent highlands. In contrast, it seems likely that *E. granulosus* was recently introduced into the plateau by human activities associated with livestock farming. We also speculate that *E. multilocularis* recently invaded the plateau together with the red fox *V. vulpes* which has expanded its own niche into the high altitude steppe. The high level of intraspecific variation in *cob* sequences of *E. shiquicus* supports its ancient endemism; however, further phylogenetical and ecological studies are required to verify our speculation.

Little is known about interspecific mating in parasitic flatworms under natural conditions. Molecular genetic evidence for interspecific hybridization has been reported in the members of the Schistosomatidae (Morgan et al., 2003), but similar cases have not been found in taeniid cestodes. Nuclear DNA sequence can serve as a genetic marker to evaluate the consequence of interspecific hybridization. The nuclear *elp* gene in *E. multilocularis* represents a single locus, and various species of *Echinococcus* contain its homologues in their genomes (Hemmings and McManus, 1991; Brehm et al., 1999). Therefore, its intron VII sequences were compared among the sympatric species of *E. shiquicus*, *E. multilocularis* and *E. granulosus*. In examining specimens available to us, there was no evidence of interspecific hybridization, suggesting that the three species are reproductively isolated.

The segregating mechanism, which maintains the genetic identity of these parasites, is unclear. To explain this mechanism, we present the following two hypotheses. The first is an ecological isolation, which is associated with the predator–prey relationship of host mammals and their susceptibility to the parasites. In the Qinghai-Tibet plateau, domestic dogs and sheep are involved in the life cycle of *E. granulosus*. On the other hand, wild animals are natural hosts for *E. multilocularis* and *E. shiquicus*. Rodents of the Arvicolidae most commonly serve as intermediate hosts for *E. multilocularis*. If red foxes mainly hunt rodents whereas Tibetan foxes show a particular preference for pikas, *E. multilocularis* and *E. shiquicus* might acquire their own niches. A dietary analysis of canines in the plateau is necessary to understand the transmission dynamics of *Echinococcus* spp. However, the segregating mechanism can not be explained from the ecological aspect alone. The second hypothesis is a physiological isolation concerning the reproduction of parasites. On the plateau, there are no documented records of canines concurrently infected with different *Echinococcus* species. However, we assume that mixed infections might sometimes occur. Both male and female reproductive organs share a common genital pore in the mature segment of *Echinococcus*. Therefore, the parasite has the potential for both cross- and self-insemination. Based on morphological observations, Kumaratilake et al. (1986) suggested that the self-insemination by inserting cirrus into the adjacent vagina is common in *E. granulosus* but is rare in *E. multilocularis*. The shorter cirrus and lack of vaginal sphincter in *E. multilocularis* are probable causes of the rarity. A recent population genetic study supported the hypothesis that cross-insemination occurs in *E. multilocularis* (Nakao et al., 2003a). The frequency of self-insemination in *E. shiquicus* is unknown. If self-insemination predominates, *E. shiquicus* could retain its genetic identity even though mixed infections occur in a fox. We also assume that gamete incompatibility and hybrid inviability may be responsible for preventing the crossing between different *Echinococcus* species.

Shiqu County, located in the Qinghai-Tibet plateau region, is a highly endemic area of human echinococcosis. An epidemiological survey using ultrasonography, X-ray and serological tests estimated that 97 (7.8%) of 1249 residents in three townships were infected with *Echinococcus* (Qiu et al., 2000). Among them, 60 were diagnosed as cystic echinococcosis and 37 as alveolar echinococcosis. However, the diagnoses were not confirmed by inspecting surgically removed lesions or biopsy samples (Li et al., 2004). The Tibetan people of Shiqu who live in high altitude steppe are in close contact with canines. Further studies are required to examine the possibility of human infections with *E. shiquicus*.

In this study, the sequence data of mitochondrial DNA were especially useful in demonstrating the validity of *E. shiquicus*. However, the phylogenetic trees deduced from sequences of *cox1*, *nad1* and *atp6* were insufficient to resolve comprehensive relationships among various species of *Echinococcus*. The ambiguity of the trees is probably due to several factors, such as the short length of sequences examined (366 bp for *cox1*, 442 bp for *nad1* and 513 bp for *atp6*), the strong mutational bias toward thymine and the saturation of nucleotide substitutions. To infer an exact phylogeny of *Echinococcus*, the DNA sequencing of mitochondrial genomes and nuclear rRNA genes is required in various species.

Recently, Tang et al. (2004) reported that a variant of *E. multilocularis* in Inner Mongolia of China should be regarded as a new species. In their report, the subspecies name of *E. multilocularis sibiricensis* was used for the variant. The lengths of its rostellar hooks were 26-27 µm (large) and 20-22 µm (small) and its hydatid cysts in voles and mice showed an alveolar form. Considering these morphological features, the variant may be unrelated to *E. shiquicus*.

5. Taxonomic summary

5.1. Echinococcus shiquicus n. sp.

Type host: Tibetan fox, *Vulpes ferrilata*.

Site of infection: The lower part of small intestine (ileum). The number of worms ranges from hundreds to ten thousands.

Type locality: Shiqu County, the Qinghai-Tibet plateau region of western Sichuan, China.

Type specimens: The type series consists of fully developed adult specimens. Holotype (slide no. ScCDCPTE001) and 9 paratypes (ScCDCPTE002-010) are kept in Institute of Parasitic Diseases, Sichuan Center for Disease Control and Prevention, Chengdu, Sichuan, China.

Intermediate host: Plateau pika, *Ochotona curzoniae.* The metacestode develops into unilocular cyst mainly in liver.

Etymology: The new species is named after its locality of occurrence.

Acknowledgements

The authors thank Patrick Giraudoux, Francis Raoul (Université de Franche-Comte, France), Xiaoming Wang (East China Teaching University, China) and Kenichi Takahashi (The Institute of Health of Hokkaido, Japan) for their help in sample collection. This investigation was supported by the National Institute of Health (1R01 TW01565-01; Principal Investigator, Philip S. Craig), the Thrasher Fund to Peter M. Schantz and a Grant-in-Aid from the Japan Society of Promotion of Science to Akira Ito (14256001).

References

Bowles, J., Blair, D., McManus, D.P., 1992. Genetic variants within the genus *Echinococcus* identified mitochondrial DNA sequencing. Mol. Biochem. Parasitol. 54, 165-174.

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

Bowles, J., Blair, D., McManus, D.P., 1994. Molecular genetic characterization of the cervid strain (‘northern form’) of *Echinococcus granulosus*. Parasitology 109, 215-221.

Bowles, J., McManus, D.P., 1993. NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*. Int. J. Parasitol. 23, 969-972.

Brehm, K., Jensen, K., Frosch, P., Frosch, M., 1999. Characterization of the genomic locus expressing the ERM-like protein of *Echinococcus multilocularis*. Mol. Biochem. Parasitol. 100, 147-152.

Feng, Z., Zheng, C., Cai, G., 1980. Mammals from southeastern Xizang (in Chinese). Acta Zool. Sinica 26, 91-97.

Hemmings, L., McManus, D.P., 1991. The diagnostic value and molecular characterisation of an *Echinococcus multilocularis* antigen gene clone. Mol. Biochem. Parasitol. 44, 53-62.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol.

Evol. 16, 111-120.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17, 1244-1245.

Kumaratilake, L.M., Thompson, R.C.A., 1982. A review of the taxonomy and speciation of the genus *Echinococcus* Rudolphi 1801. Z. Parasitenkd. 68, 121-146.

Kumaratilake, L.M., Thompson, R.C.A., Eckert, J., D'Alessandro, A., 1986. Sperm transfer in *Echinococcus* (Cestoda: Taeniidae). Z. Parasitenkd. 72, 265-269.

Lai, C.H., Smith, A.T., 1996. Keystone status of plateau pikas (*Ochotona curzoniae*): effect of control on biodiversity of native birds. In: Peter, J.S., Wang, S., Xie, Y. (Eds.), Conserving China's Biodiversity (II). China Environmental Science Press, Beijing, pp. 222-230.

Lavikainen, A., Lehtinen, M.J., Meri, T., Hirvelä-Koski, V., Meri, S., 2003. Molecular genetic characterization of the Fennoscandian cervid strain, a new genotypic group (G10) of *Echinococcus granulosus*. Parasitology 127, 207-215.

Le, T.H., Pearson, M.S., Blair, D., Dai, N., Zhang, L.H., McManus, D.P., 2002. Complete mitochondrial genomes confirm the distinctiveness of the horse-dog and sheep-dog strains of *Echinococcus granulosus*. Parasitology 124, 97-112.

Li, T.Y., Qiu, J.M., Craig, P.S., Ito, A., Yang, W., Vuitton, D.A., Xiao, N., Chen, X.W., Yu, W., Schantz, P.M., 2004. Review of 311 cases of alveolar echinococcosis and criteria for classification of hepatic ultrasound images.

S.E. Asian J. Trop. Med. Publ. Health 35, 213-217.

Li, W.X., Zhang, G.C., Lin, Y.G., Hong, L.X., 1985. The occurrence of *Echinococcus multilocularis* Leuckart, 1863 the natural animal host in China and its morphological study (in Chinese). Acta Zool. Sinica 31, 365-371.

McManus, D.P., Zhang, L., Castrodale, L.J., Le, T.H., Pearson, M., Blair, D., 2002. Molecular genetic characterization of an unusually severe case of hydatid disease in Alaska caused by the cervid strain of *Echinococcus granulosus*. Am. J. Trop. Med. Hyg. 67, 296-298.

Morgan, J.A., DeJong, R.J., Lwambo, N.J., Mungai, B.N., Mkoji, G.M., Loker, E.S., 2003. First report of a natural hybrid between *Schistosoma mansoni* and *S. rodhaini*. J. Parasitol. 89, 416-418.

Nakao, M., Sako, Y., Ito, A., 2003a. Isolation of polymorphic microsatellite loci from the tapeworm *Echinococcus multilocularis*. Infect. Genet. Evol. 3, 159-163.

Nakao, M., Sako, Y., Ito, A., 2003b. The mitochondrial genome of the tapeworm *Taenia solium*: a finding of the abbreviated stop codon U. J. Parasitol. 89, 633-635.

Nakao, M., Yokoyama, N., Sako, Y. Fukunaga, M., Ito, A., 2002. The complete mitochondrial DNA sequence of the cestode *Echinococcus multilocularis* (Cyclophyllidea: Taeniidae). Mitochondrion, 1, 497-509.

Qiu, J.M., Chen, X.W., Ren, M., Luo, C.X., Liu, D.L., Liu, X.T., He, D.L., 1995. Epidemiological study on alveolar hydatid disease in Qinghai-Xizang plateau (in Chinese). J. Pract. Parasit. Dis. 3, 106-109.

Qiu, J.M., Liu, F.J., Schantz, M.P., Ito, A., Delker, C., He, J.G., Zhang, Y., Chen, X.W., 2000. Epidemiological study on human hydatidosis in Tibetan region of western Sichuan (in Chinese). Chin. J. Zoonoses 16, 77-80.

Rausch, R.L., Bernstein, J.J., 1972. *Echinococcus vogeli* sp. n. (Cestoda: Taeniidae) from the bush dog, *Speothos venaticus* (Lund). Z. Tropenmed. Parasitol. 23, 25-34.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Scott, J.C., Stefaniak, J., Pawlowski, Z.S., McManus, D.P., 1997. Molecular genetic analysis of human cystic hydatid cases from Poland: identification of a new genotypic group (G9) of *Echinococcus granulosus*. Parasitology 114, 37-43.

Tang, C.T., Cui, G.W., Qian, Y.C., Lu, S.M., Lu, H.C., 1988. On the occurrence of *Echinococcus multilocularis* in Hulunbeier Pasture, Nei Mongolian Autonomous Region (in Chinese). Acta Zool. Sinica 34, 172-179.

Tang, C.T., Quian, Y.C., Kang, Y.M., Cui, G.W., Lu, H.C., Shu, L.M., Wang, Y.H., Tang, L., 2004. Study on the ecological distribution of alveolar *Echinococcus* in Hulunbeier Pasture of Inner Mongolia, China. Parasitology 128, 187-194.

Thompson, R.C.A., McManus, D.P., 2003. Towards a taxonomic revision of the genus *Echinococcus*. Trends Parasitol. 18, 452-457.

von Nickisch-Rosenegk, M., Lucius, R., Loos-Frank, B., 1999. Contributions to the phylogeny of the Cyclophyllidea (Cestoda) inferred from mitochondrial 12S rDNA. J. Mol. Evol. 48, 586-596.

Wang, W., Ding, Z.X., Wu, Y., Wu, J.G., 1989. The occurrence of *Echinococcus* *multilocularis* Leuckart, 1863 in fox and wolf in Tacheng, Xinjiang (in Chinese). Endemic Dis. Bull. 4, 8-11.

Xiao, N., Qiu, J.M., Nakao, M., Nakaya, K., Yamasaki, H., Sako, Y., Mamuti, W., Schantz, P.M., Craig, P.S., Ito, A., 2003. Identification of *Echinococcus* species from a yak in the Qinghai-Tibet plateau region of China. Am. J. Trop. Med. Hyg. 69, 445-446.

Xiao, N., Li, T.Y., Qiu, J.M., Nakao, M., Chen, X.W., Nakaya, K., Yamasaki, H., Schantz, P.M., Craig, P.S., Ito, A., 2004. The Tibetan hare *Lepus oiostolus*: a novel intermediate host for *Echinococcus multilocularis*. Parasitol. Res. 92, 352-353.

Zhu, Y.B., Qiu, J.M., Qiu, D.C., Yi, D.Y., 1983. The discovery of *Echinococcus multilocularis* in China (in Chinese). Sichuan J. Zool. 2, 44.

Figure legends

Fig. 1. Adults of *Echinococcus shiquicus* n. sp. in a naturally infected Tibetan fox. The adults containing a gravid segment were classified into two types (A and B). gs, gravid segment; is, immature segment; ms, mature segment; pms, pre-mature segment; sc, scolex.

Fig. 2. Morphological features of adult *Echinococcus shiquicus* n. sp. (A) Fully developed adult. cp, cirrus pouch; e, eggs; gpo, genital pore; gpr, genital primordium; gu, gravid uterus; o, ovary; oc, osmoregulatory canals; r, rostellum; s, sucker; t, testes; u, uterus; vg, vitelline gland. (B) Adult hooks. lh, large hook; sh, small hook. (C) Eggs in gravid uterus.

Fig. 3. Larval *Echinococcus shiquicus* n. sp. developed in a plateau pika. (A) Hepatic hydatid. bc, brood capsule. (B) Cross section of the hydatid. p, protrusion; ps, protoscolex.

Fig. 4. The neighbor-joining phylogenetic trees of *Echinococcus*. The trees were constructed from mitochondrial nucleotide sequences of partial *cox1* (A), partial *nad1* (B) and complete *atp6* (C). *Egra*G1-10, *Echinococcus granulosus* genotypes; *Emul*, *Echinococcus multilocularis*; *Eoli*, *Echinococcus oligarthrus*; *Eshi*, *Echinococcus shiquicus* n. sp.; *Evog*, *Echinococcus vogeli*; *Tsol*, *Taenia solium* (an outgroup). Numbers at individual nodes are the bootstrap confidence values (%). The scale bars represent the estimated number of nucleotide substitutions per nucleotide site.

Table 1

Origins of *Echinococcus* samples collected in Shiqu County, the Qinghai-Tibet plateau region of China

Species (developmental stage)	Final or intermediate hosts (no. infected)	No. samples used for sequencing (no. hosts) [a]	
		cob	*elp*
E. shiquicus n. sp. (adult)	Fox, *Vulpes ferrilata* (6)	18 (6)	12 (4)
E. shiquicus n. sp. (larva)	Pika, *Ochotona curzoniae* (5)	5 (5)	4 (4)
E. multilocularis (adult)	Fox, *Vulpes ferrilata* (1)	4 (1)	4 (1)
E. multilocularis (adult)	Fox, *Vulpes vulpes* (1)	4 (1)	4 (1)
E. multilocularis (adult)	Dog, *Canis familiaris* (3)	6 (3)	6 (3)
E. multilocularis (larva)	Vole, *Microtus fuscus* (4)	4 (4)	4 (4)
E. multilocularis (larva)	Vole, *Pitymys irene* (1)	1 (1)	0 (0)
E. granulosus G1 (adult)	Dog, *Canis familiaris* (5)	8 (5)	6 (3)
E. granulosus G1 (larva)	Sheep, *Ovis aries* (1)	1 (1)	1 (1)

[a] *cob*, cytochrome *b*; *elp*, ezrin-radixin-moesin (ERM)-like protein. The partial nucleotide sequences of mitochondrial *cob* gene and the intron VII sequences of nuclear *elp* locus were determined to confirm the identification of species. In the adult stage, 1-4 worms per host were used for sequencing.

Table 2

Primer pairs used for PCR amplification

Target genes [a]	Sequences (5’-3’) of primer pairs [b]
cox1 (mtDNA)	F: AGAGAAAATTGTGGAGTTACTGCT
	R: ATTACTAATCAACTTAGACTTACA
nad1 (mtDNA)	F: TAGTTTAATTAGAATGTCGGTTTG
	R: TCTTGAAGTTAACAGCATCACGA
atp6 (mtDNA)	F: GCATCAATTTGAAGAGTTGGGGATAAC
	R: CCAAATAATCTATCAACTACACAACAC
cob (mtDNA)	F1: GTTTAAACTGGTAGATTGTGGTTC
	R1: CTCCACAGTAGAAATCACCATCA
	F2: GTCAGATGTCTTATTGGGCTGC
	R2: TCTGGGTGACACCCACCTAAATA
rrnL (mtDNA)	F: ATGCGTTGGATTGATGATTGTAAT
	R: AAACAAACTTCATGCAGCCAATG
elp (nuclear DNA)	F: ATGCGCGTGAGAGTCTTCAGAAGA
	R: ATTCTGCGAAGCTCAGCTTCA

[a] *cox1*, cytochrome *c* oxidase subunit 1; *nad1*, NADH dehydrogenase subunit 1; *atp6*, ATPase subunit 6; *cob*, cytochrome *b*; *rrnL*, large-subunit rRNA; *elp*, ezrin-radixin-moesin (ERM)-like protein. Primers were designed from the mtDNA genome (Nakao et al., 2002) and the *elp* exons (Brehm et al., 1999) of *E. multilocularis*.

[b] Forward (F) and reverse (R) primers. Partial fragments of *cob* were amplified and sequenced by using primers F2 and R2 to confirm the identification of species.

Table 3

Intraspecific variation of mitochondrial *cob* sequences and pairwise comparison of the intron VII sequences of nuclear *elp* locus among three species of *Echinococcus* collected in Shiqu County

Species	Maximum percent divergence of *cob* within species [a]	Pairwise divergence (%) of *elp* intron [b]	
		E. shiquicus	*E. multilocularis*
E. shiquicus n. sp.	1.3 (23)	-	
E. multilocularis	0 (19)	5.2	-
E. granulosus G1	0.2 (9)	4.7	5.3

[a] The partial nucleotide sequences (549 bp) were determined and the maximum values of percentage divergence were compared within the species. The number of samples examined was shown in parentheses.

[b] Lengths of the intron sequences were 866 bp in *E. shiquicus*, 864 bp in *E. multilocularis* and 872 bp in *E. granulosus* G1. There were no intraspecific variations in 18 samples of *E. multilocularis* and 7 samples of *E. granulosus* G1. In *E. shiquicus*, 1 out of 16 samples showed a variation (1 base substitution).

Table 4
Percentage divergences of mitochondrial nucleotide sequences between *Echinococcus shiquicus* n. sp. and other *Echinococcus* species

E. shiquicus compared to:	Mitochondrial genes [a]				
	cox1	*nad1*	*atp6*	*cob*	*rrnL*
E. multilocularis	9.0	16.9	21.5	13.1	14.2
E. oligarthrus	9.0	20.0	21.1	- [b]	-
E. vogeli	7.8	18.5	19.4	-	-
E. granulosus G1	9.4	21.0	22.1	14.2	14.2
E. granulosus G2	8.8	20.1	-	-	-
E. granulosus G3	9.1	20.1	-	-	-
E. granulosus G4	8.1	17.6	18.4	11.1	13.4
E. granulosus G5	9.0	18.1	-	-	-
E. granulosus G6	10.2	18.1	21.1	-	-
E. granulosus G7	10.6	17.5	20.8	-	-
E. granulosus G8	-	18.2	20.5	-	-
E. granulosus G9	-	20.3[c]	-	-	-
E. granulosus G10	9.7	17.5	-	-	-
	(9.9)[d]	(19.3)	(22.8)	(13.6)	(11.3)

[a] The alignments of *cox1* and *nad1* were made by using partial sequences, whereas complete sequences were aligned in *atp6*, *cob* and *rrnL*. The numbers of nucleotide sites examined were 366 in *cox1*, 442 in *nad1*, 513 in *atp6*, 1068 in *cob* and 970 in *rrnL*.

[b] Sequence data were unavailable in databases.

[c] The sequence of *E. granulosus* G9 (human isolate GS) was taken from published data (Scott et al., 1997).

[d] Percentage divergences between *E. multilocularis* and *E. granulosus* G1 were shown in parentheses.

Table 5

Morphological comparison between adult worms of *Echinococcus shiquicus* n. sp. and *Echinococcus multilocularis* in China

			E. multilocularis in four localities of China (host)			
		E. shiquicus	Sichuan (Dog)	Ningxia (Red fox)	Xinjiang (Wolf)	Nei Mongolia (Corsac fox)
Body length (mm)		1.3-1.7	1.3-3.0	1.1-2.4	1.3-1.7	1.8-3.3
No. of segments		2-3	4-5	2-5	3-7	3-4
No. of hooks		18-34 [a]	29-40	30-32	24-30	28-30
Length of hooks (μm) [b]	L	20-23	29-31	28-32	28	26-27
	S	16-17	16-26	20-26	23	20-22
No. of testes		12-20	15-29	16-22	12-16	14-20
Position of testes		Majority posterior to genital pore. In Nei Mongolia samples, none were located anterior to genital pore.				
Position of genital pore		Anterior to the middle of lateral margin. The pore of *E. shiquicus* was located more anterior than that of *E. multilocularis*, particularly in the mature segment.				
Gravid uterus		Branchless and sac-like shape in all samples.				
Data cited		This study	Zhu et al. (1983)	Li et al. (1985)	Wang et al. (1989)	Tang et al. (1988)

[a] Data from Qiu et al. (1995).
[b] L, large hook; S, small hook.

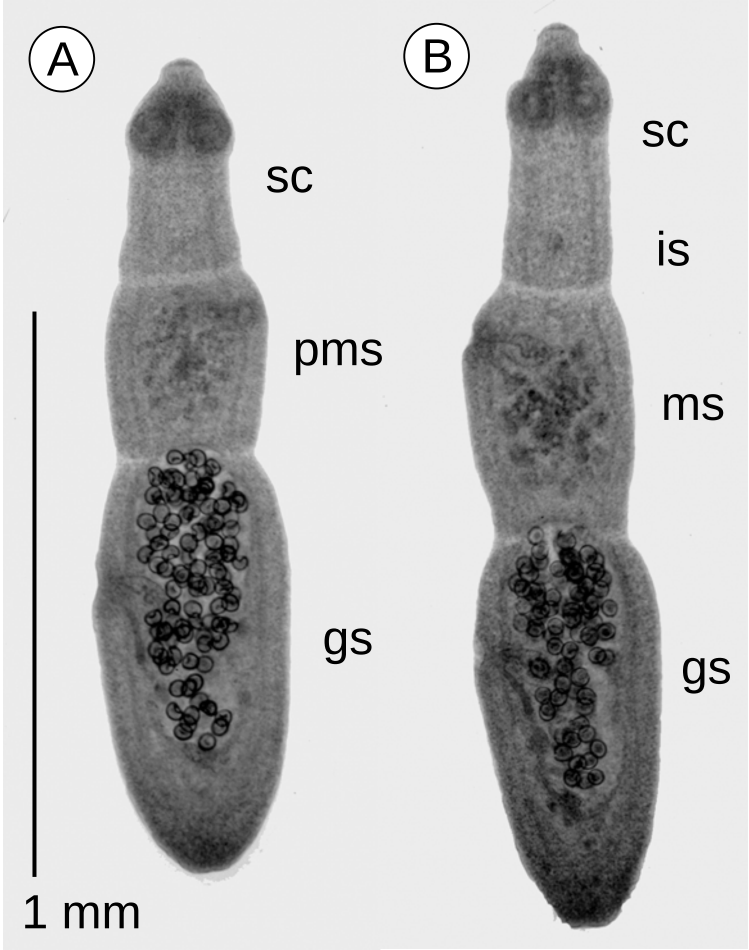

Fig. 1 Xiao et al

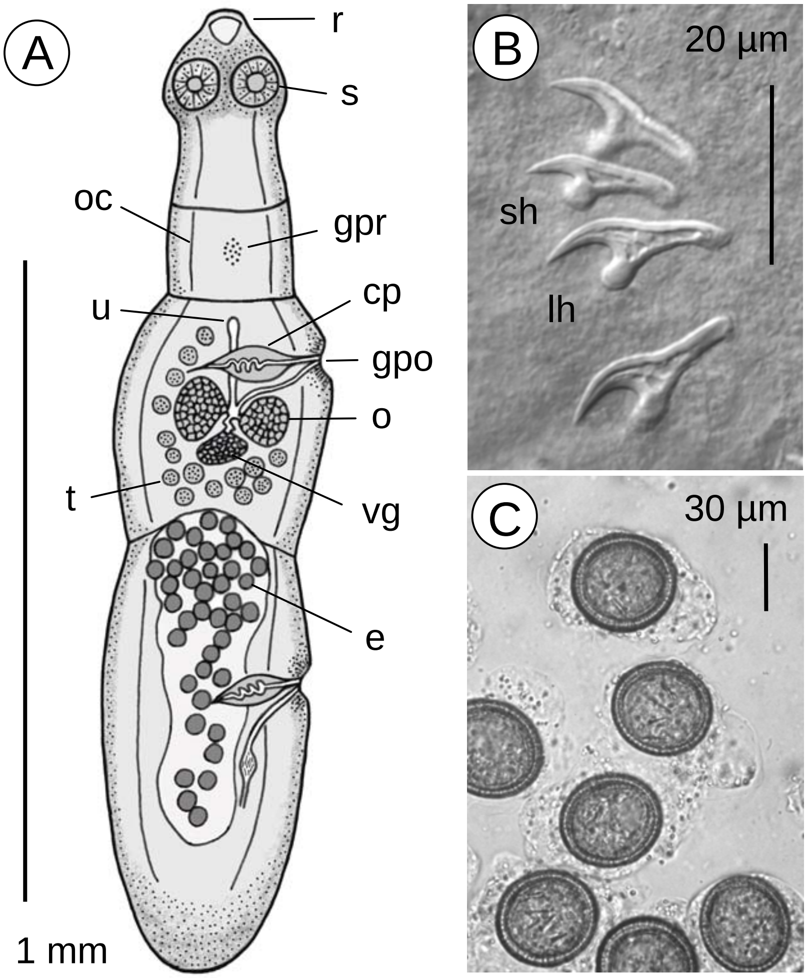

Fig. 2 Xiao et al

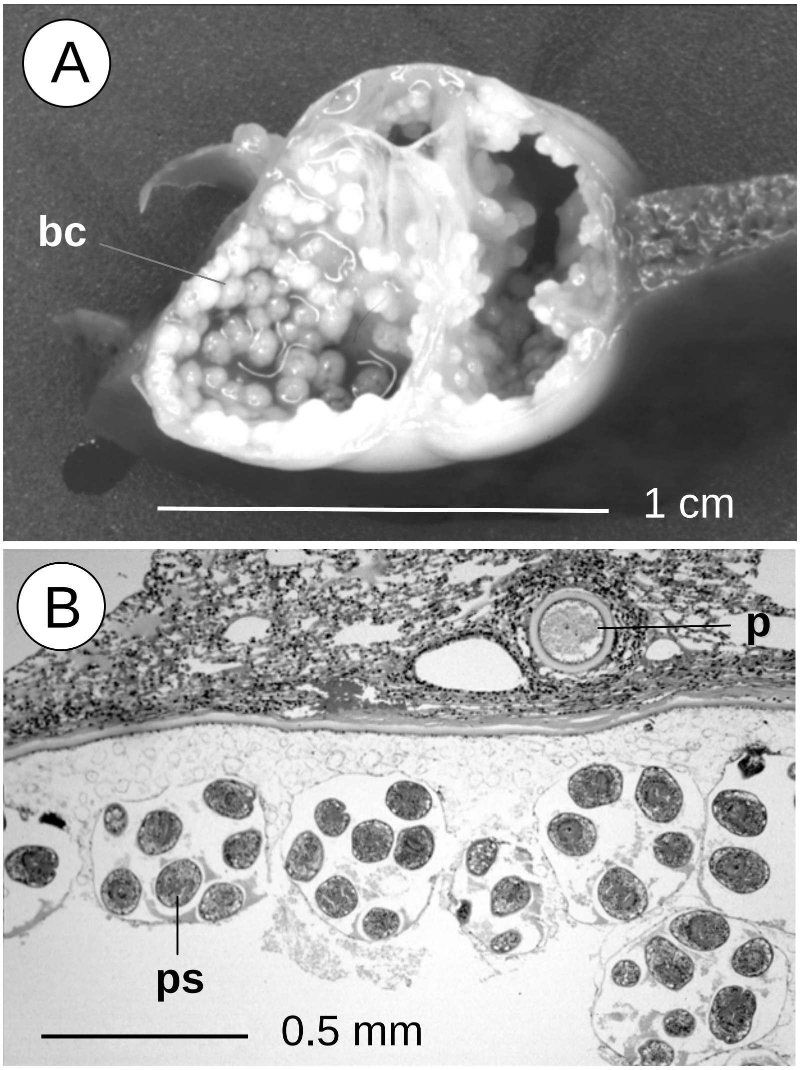

Fig. 3 Xiao et al

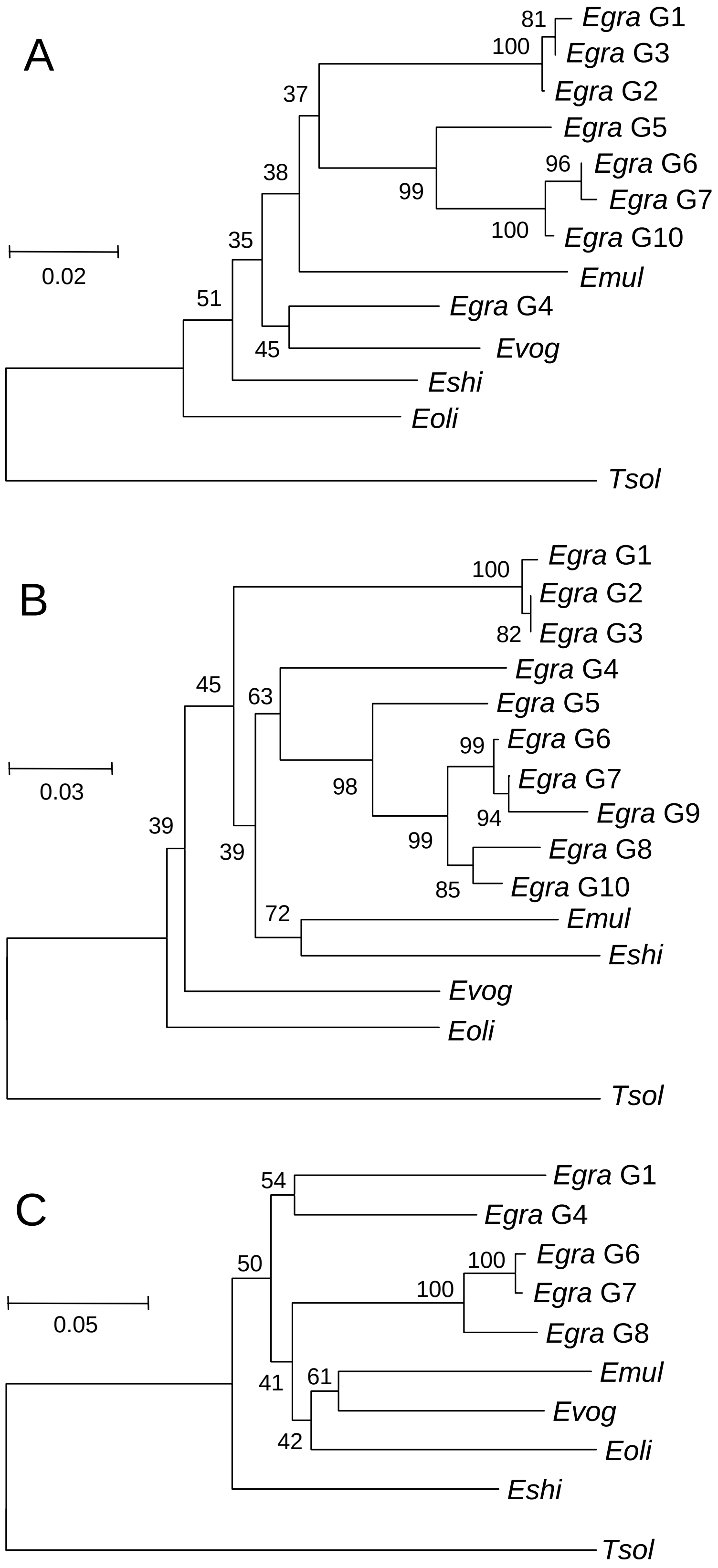

Fig. 4 Xiao et al