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Molecular identification of *Echinococcus granulosus* on the Tibetan Plateau using mitochondrial DNA markers

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ABSTRACT. Cystic echinococcosis (CE) is an important worldwide zoonotic disease that causes large economic losses and human suffering. *Echinococcus granulosus*, the causative agent of CE, exhibits different genotypes in different locations. In order to identify its genotypes and analyze its genetic structure on the Tibetan Plateau, we collected 72 hydatid cysts from different intermediate hosts and amplified and sequenced their mitochondrial cytochrome c oxidase subunit 2 (*cox2*) genes. Seventy isolates were identified as the *E. granulosus* G1 genotype, while two isolates belonged to the G6 genotype. There were 18 haplotypes among the 70 *E. granulosus* isolates, which exhibited a star-like network pattern and shared a common haplotype (H_1). There was little difference between geographical sub-populations. Our results suggest that a recent *E. granulosus* population expansion occurred

on the Tibetan Plateau, suggesting that *E. granulosus* was introduced into China. This study increases the basic molecular data needed for the molecular diagnosis, epidemiology, prevention, and control of *Echinococcus* diseases.

Key words: *Echinococcus granulosus*; Mitochondrial DNA; Tibetan Plateau; Molecular identification

INTRODUCTION

Cystic echinococcosis (CE) is a global zoonotic disease caused by the infection of mammals by the larval (metacestode) stage of *Echinococcus granulosus*. It causes 1 million disability-adjusted life years in humans and an annual loss of 2 billion US dollars in livestock (Budke et al., 2006). Recently, the World Health Organization has included CE as part of a neglected zoonosis subgroup for its strategic plans for the control of neglected tropical diseases (Siracusano et al., 2011).

The metacestode stage of *E. granulosus* inhabits the liver, lungs, and other internal organs of livestock and humans after oral uptake of the eggs, which are produced by adult worms in the canine small intestine (Moro and Schantz, 2009; McManus et al., 2012). Previous studies have identified 10 genotypes of *E. granulosus* (G1 to G10) (Bowles et al., 1992; Bowles et al., 1994; Scott et al., 1997; Lavikainen et al., 2003), but it is currently considered as a complex that consists of at least four species: *E. granulosus sensu strictu* (genotypes G1 to G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6 to G10); however, the species status of *E. canadensis* is ambiguous (Nakao et al., 2010b; McManus, 2013; Nakao et al., 2013).

Mitochondrial DNA has many specific characteristics, such as matrilineal inheritance and no intron structure, which make it an important tool in population evolution and taxonomy studies. Given that different DNA markers have different rates of evolution and conserved sites, the results obtained from analyzing the same samples may be inconsistent. The mitochondrial cytochrome c oxidase subunit 2 (*cox2*) gene has been widely used in studies of evolution and genetic diversity in many species (Frati et al., 1997; Rawson and Burton, 2006), but not in *E. granulosus*. In this study, we investigated whether *cox2* is a suitable marker by sequencing its complete fragments and analyzing the genetic structure and phylogenetic relationships of *E. granulosus* on the Tibetan Plateau. This information will provide basic molecular data for the molecular diagnosis, epidemiology, prevention, and control of CE in this region.

MATERIAL AND METHODS

Samples

A total of 72 hydatid cyst isolates were collected in 2011 and 2013 from humans, sheep, and yaks on the Tibetan Plateau in the Sichuan and Qinghai provinces of southeastern China and in the Tibet Autonomous Region (Table 1). All of the animal isolates were collected from slaughterhouses, while human samples were obtained from the People's Hospital of Ganzi County in Sichuan province. Cysts were washed three times in normal saline and subsequently stored at -80°C until use.

Table 1. *Echinococcus granulosus* samples from the Tibetan Plateau.

	Sichuan	Tibet	Qinghai
Sheep	-	12	35
Human	23	-	-
Yak	1	1	-
Total	24	13	35

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA from each isolate was extracted by the phenol-chloroform method (Jacobs et al., 1997). A complete *cox2* gene fragment was amplified from each sample by PCR using forward (Ps, 5'-TGAGGTAAGTCGTAACAAGG-3') and reverse (Pa, 5'-ATCTACAGCACGAAAAGCC-3') primers. Both primers were designed based on a standard sheep strain (GenBank accession No. AF297617) using Primer Premier software version 5.0 (Premier Biosoft International, CA, USA). The PCR was conducted in a final volume of 20 µL, which contained 10 µL 2X *Taq* PCR Master Mixture (Tiangen, Beijing, China), 1 µL genomic DNA, 1 µL of each primer, and 8 µL ddH_2O. The PCR program consisted of one cycle of primary denaturation (5 min at 95°C) followed by 39 cycles of denaturation (30 s at 94°C), annealing (45 s at 50°C), extension (45 s at 72°C), and a final extension (10 min at 72°C). Positive and negative (no DNA) controls were included with each PCR set. To assess the quality of the PCR amplicons, 8 µL of each PCR product were run on 1.0% (w/v) agarose gels. The PCR products were purified using a TIANgel Midi Purification Kit (Tiangen), and were then sequenced in two directions by the Invitrogen Trading (Shanghai) Co. Ltd. Reference sequences were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Data analysis

Nucleotide sequences were aligned and compared using DNAMAN software (Version 5.2.2.0, Lynnon Biosoft, Quebec, Canada). Amino acid sequences were inferred from the nucleotide sequences using the flatworm mitochondrial genetic code (Nakao et al., 2000) in MEGA version 5.05 (Tamura et al., 2011). Population diversity indices, including the number of haplotypes (H_N), haplotype diversity (H_D), and nucleotide diversity (π), were calculated using DnaSP 5.10 (Librado and Rozas 2009). Maximum likelihood (ML) and Bayesian trees were constructed using MEGA and MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003), respectively, on the default settings. TCS 1.2 software (Clement et al., 2000) was used to build an *E. granulosus* haplotype network by statistical parsimony. The neutrality indices Tajima's *D* (Tajima 1989) and Fu's F_S (Fu 1997) were calculated using the population genetics package Arlequin version 3.5.1.2 (Excoffier and Lischer 2010).

For the geographical sub-population analysis, the nucleotide sequences were grouped into three populations: Qinghai, Sichuan, and Tibet. Population diversity indices, including the number of segregating sites (S), H_N, H_D, and π, were estimated using DnaSP 5.10. Tajima's *D* and Fu's F_S were also calculated for each sub-population. Pairwise genetic differences between the three sub-populations were estimated using Wright's F-statistic (F_{ST}) by an analysis of molecular variance (AMOVA) in Arlequin, and the degree of gene flow (N_M) was also calculated. The average number of pairwise nucleotide differences (K_{XY}), the nucleotide substitution per site (D_{XY}), and the net nucleotide substitution per site (D_A) between the sub-populations were calculated using DnaSP.

Ethics

Human hydatid cysts were kindly donated by the hospital's Center for Genetic Variation Studies and all of the samples were anonymous. According to hospital policy, patients were required to sign informed consent forms for the surgical procedure. The research procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2008.

Animals were handled in accordance with the Animal Protection Law of the People's Republic of China (a draft of the Animal Protection Law was released in China on September 18, 2009). This study was approved by the National Institute of Animal Health, Animal Care, and Use Committee of Sichuan Agricultural University (Approval No. 2011-015).

RESULTS

Nucleotide variability and polymorphisms

All 72 isolates were successfully amplified for the complete *cox2* mitochondrial gene; of these, two isolates from Tibetan sheep (length 576 bp) shared the same sequence (GenBank accession Nos. KC692991 and KC692992) and exhibited a high identity (99.83%) to a previously characterized camel strain (GenBank accession No. AB208063). We identified these two isolates as *E. canadensis* (genotype G6 and haplotype 19 [H_{19}]).

The remaining 70 isolates (GenBank accession Nos. KC692921 to KC692990, length 582 bp, and encoding 193 amino acids) exhibited a high identity (99.48 to 99.83%) to a standard sheep strain (GenBank accession No. AF297617), and were identified as *E. granulosus sensu strictu* (G1 genotype, see **Table S1**). Multiple sequence alignments revealed 18 haplotypes (H_1-H_{18}) and 17 variation sites, in which 10 were single mutation sites and seven were parsimony informative sites. Five non-synonymous mutations (29.4%) were also found in the amino acid sequences (**Table S2**). Only nucleotide substitutions were detected; deletions and insertion mutations were not observed.

Phylogenetic analysis and network

The ML and Bayesian trees of these haplotypes shared the same topological structure. According to these phylogenetic relationships, all of the sequences could be classified into two main branches: the G1 genotype and other genotypes of *E. granulosus*. Haplotypes H_1 to H_{18} were identified as having the G1 genotype, while H_{19} belonged to the G6 genotype (Figure 1). The haplotype network exhibited a star-like expansion, with a common haplotype (H_1) at its center (Figure 2); only one or two mutation steps were detected between the common haplotype and the others. Ten, four, and three geographically unique haplotypes were found in Qinghai, Sichuan, and Tibet, respectively.

Sub-population genetic diversity indices

Population diversity indices were calculated using the nucleotide sequences of the *cox2* gene from the Tibetan Plateau (Table 2). The H_D and π of the sequences were 0.667 ± 0.064 and 0.00148 ± 0.00020, respectively. Among the sub-populations, the highest H_D and π values were obtained in Qinghai. Overall, Tajima's *D* and Fu's F_S were both significant and negative, but

among the sub-populations only the Tajima's *D* value for Qinghai was significant and negative. The AMOVA revealed that 97.21246% of the variation was within the sub-populations and only 2.78754% was between them.

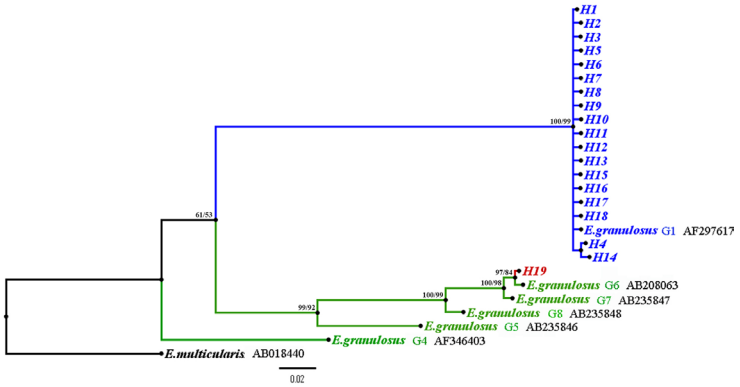

Figure 1. Phylogenetic tree of *Echinococcus granulosus* haplotypes based on maximum likelihood and Bayesian methods. GenBank accession numbers for reference sequences are stated after the species name. Bootstrap values of Bayesian (first value) and maximum likelihood (second value) are stated above each branch.

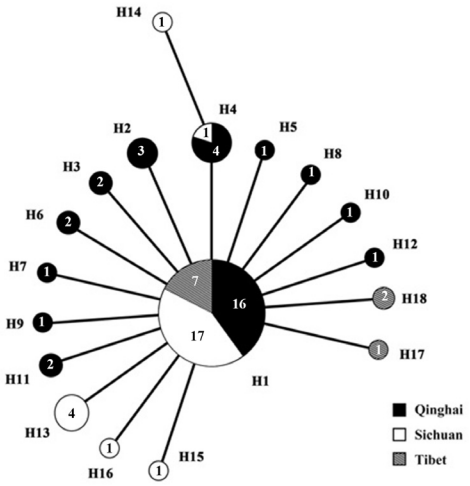

Figure 2. *Echinococcus granulosus* haplotype network on the Tibetan Plateau. Values in circles represent the haplotype distribution frequency.

Table 2. Diversity and neutrality indices of *Echinococcus granulosus* sub-populations.

	No.	*S*	H_N	H_D ± SD	π ± SD	Tajima's *D*	Fu's F_S
Qinghai	35	11	12	0.778 ± 0.068	0.00180 ± 0.00025	-1.896*	-2.070
Sichuan	24	5	5	0.486 ± 0.113	0.00107 ± 0.00033	-1.549	-2.309
Tibet	11	3	4	0.600 ± 0.154	0.00119 ± 0.00038	-1.114	-1.525
Tibetan Plateau	70	17	18	0.667 ± 0.064	0.00148 ± 0.00020	-2.24115***	-19.827**

*P < 0.05; **P < 0.02; ***P < 0.01. No., number of sequences; *S*, number of segregating sites; H_N, number of haplotypes; H_D, haplotype diversity; π, nucleotide diversity.

The K_{XY} values ranged between 0.67636 and 0.00116 (Sichuan and Tibet, respectively), and the D_{XY} values varied between 0.88571 and 0.00152 (Qinghai and Tibet, respectively) (Table 3). F_{ST} values between the sub-populations ranged between 0.00646 (Tibet and Qinghai) and 0.05026 (Tibet and Sichuan), with N_M values of 39.96235 (Tibet and Qinghai) and 4.72440 (Tibet and Sichuan) (Table 4). There was very low genetic differentiation (G_{ST}, 0.01051 to 0.03426; F_{ST}, 0.00646 to 0.05026) and very high gene flow (N_M, 4.72440 to 39.96235) between the three sub-populations.

Table 3. Population genetic indices between different sub-populations of *Echinococcus granulosus*.

Population 1	Population 2	K_{XY}	D_{XY}	D_A	G_{ST}
Sichuan	Qinghai	0.85371	0.00147	0.00005	0.03426
Sichuan	Tibet	0.67636	0.00116	0.00005	0.01051
Qinghai	Tibet	0.88571	0.00152	0.00003	0.01273

K_{XY}, average proportion of nucleotide differences between sub-populations; D_{XY}, average number of nucleotide substitutions per site between sub-populations; D_A, number of net nucleotide substitutions per site between sub-populations; G_{ST}, genetic differentiation index based on haplotype frequency.

Table 4. Pairwise genetic distance (F_{ST} below the diagonal) and gene flow (N_M above the diagonal) between different sub-populations of *Echinococcus granulosus*.

	Qinghai	Sichuan	Tibet
Qinghai		7.39760	39.96235
Sichuan	0.03269***		4.72440
Tibet	0.00646	0.05026	

***P < 0.01.

DISCUSSION

E. granulosus is one of the most important parasites in the world, because it affects human health and causes large economic losses. *E. granulosus* is spread over 27 provinces, autonomous regions, and municipalities in China, and its main areas of endemism are western and northwestern parts of the country. Recent studies that have conducted the molecular identification of *E. granulosus* found that the G1 genotype is the main genotype in this region, and there are a small number of the G3, G6, and G7 genotypes (Cardona and Carmena, 2013; Wang et al., 2014b; Zhang et al., 2014). In this study, 70 isolates were identified as the *E. granulosus* G1 genotype, while two isolates (both derived from sheep in Tibet) belonged to the G6 genotype. The two genotypes on the Tibetan Plateau in southeastern China are consistent with the epidemiological characteristics of human and animal CE in China.

A number of mitochondrial and nuclear fragments have been used as markers to investigate the genotypes, or the genetic structures, of *E. granulosus* in southwestern China, including *cox1*, *nad1*, *nad2*, *cytb*, *atp6*, *12S* rRNA, and *16S* rRNA (Ma et al., 2008; Nakao et al., 2010a; Ma et al., 2012; Wang et al., 2013; Yan et al., 2013; Wang et al., 2014a; Zhong et al., 2014). However, markers of different lengths are not appropriate for the comparison of genetic structures in different regions, and gene fragments may not be representative because every fragment on a gene is unique in terms of its conservation characteristics. Therefore, we amplified the *cox2* gene as a complete sequence, and identified 18 haplotypes of the *E. granulosus* G1 genotype from 70

isolates and found that 40 isolates shared 100% homology with each other, demonstrating that *cox2* is highly conserved. These results suggest that *cox2* could be a candidate marker for the genetic study of *E. granulosus.*

The genetic structures of *E. granulosus* in the Middle East (Iran and Jordan), South America (Peru), and Southeast Asia (China) differ (Casulli et al., 2012; Yanagida et al., 2012). According to previous studies (Nakao et al., 2010a; Ma et al., 2012; Wang et al., 2013; Yan et al., 2013; Wang et al., 2014a), the characterization of The *E. granulosus* genetic network in Southeast Asia is characterized by a star-like pattern, with a predominance of the G1 genotype except in India, where the G3 genotype predominates (Sharma et al., 2013). We also found a radial network based on a common haplotype, but there were few mutation steps between the common haplotype and the others. This may due to the highly conserved nature of *cox2* and the low diversification of *E. granulosus* on the Tibetan Plateau. In addition, *E. granulosus* is a diploid organism that has a mixed sexual and asexual reproduction system. Adult worms are hermaphroditic and exhibit sexual reproduction, while the larval stage is asexual (Haag et al., 1999); this may result in a low level of nucleotide locus variability.

Despite the fact that the environments in Qinghai, Sichuan, and Tibet differ considerably, the inter-population comparison estimates (K_{XY}, D_{XY}, G_{ST}, and F_{ST}) and AMOVA revealed a low level of genetic differentiation. This may have been caused by their common haplotype and the high level of gene flow between these sub-populations, as well as their low level of diversity. All of our results suggest that the three sub-populations have not evolved into distinct populations.

Neutrality tests, such as Tajima's *D* and Fu's F_S, have been developed to test the selective neutrality of nucleotide mutations, and are used to determine population growth. The significant, negative values of Tajima's *D* and Fu's F_S we obtained indicate an excess of rare alleles, which could be a signature of recent population expansion (Tajima, 1989; Fu, 1997). Sheep were first domesticated in the Middle East around 12,000 BC, before spreading into Europe, Africa, the Americas, and Asia. Recent studies have hypothesized that sheep parasites also spread, which caused a parasite population expansion in these regions (Casulli et al., 2012; Yanagida et al., 2012; Sharma et al., 2013). Nakao et al (2010) conjectured that *E. granulosus* spread into Peru from Europe due to livestock importation during the 15th century. We suggest that the "Silk Road" between China and the Middle East accelerated the spread of this parasite by the trading of domestic animals in the 1st century. This hypothesis is consistent with Nakao et al.'s (2010b) timeline; however, archaeoparasitological evidence, molecular epidemiological studies, and comparisons of *E. granulosus* genetic structures in different regions are required to confirm this hypothesis.

In this study, we confirmed that the *E. granulosus* G1 genotype is the predominant genotype in China by using complete *cox2* sequences of 72 samples from the Tibetan Plateau, and a low level of differentiation was found between three geographical sub-populations. In order to fully understand *E. granulosus* transmission, further comparative studies of its genetic structure in different regions should be conducted.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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