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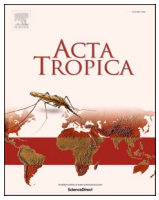

The polymorphic analysis of *cox1* and *cob* genes of *Echinococcus granulosus* in the Ngari region of Tibet in China

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ABSTRACT

Cystic echinococcosis (CE) is an important zoonotic parasitic disease caused by *Echinococcus granulosus* (*E. granulosus*). CE seriously threatens human health and the development of animal husbandry. The Ngari region is one of the world's highest endemic regions for CE, while genetic polymorphisms of *E. granulosus* were unclear. Paraffin slices of liver Cyst were collected from seventy-nine surgical patients with echinococcosis in the Ngari region. DNA was extracted from samples. The *cox1* and *cob* genes of mitochondrial DNA of *E. granulosus* were simultaneously amplified and sequenced. The sequencing results were compared with the standard sequence (KU925397.1and HF947574.1). Phylogenetic trees and the haplotype network of *cob* and *cox*1 genes were constructed and analyzed genotypes of *E. granulosus* isolated from humans in the Ngari Region of Tibet. Out of 79 hydatid cyst samples collected from surgery patients, 60 isolates were identified as G1/ G3, and two isolates were identified as G6/ G7. Analysis of the *cob*/ *cox*1 genes revealed 9/7 mutations resulting in 8/6 haplotypes, respectively. The *cob* and *cox*1 neutrality indices computed by Tajima's D and Fu's Fs tests showed high negative values in *Echinococcus granulosus sensu stricto* (*E. granulosus s. s.*). The result suggested that *E. granulosus* in the Ngari region experienced population expansion or a negative selection. We found that G1/ G3 was still the main genotype, and G6/ G7 was found occasionally in humans of the Ngari region. Therefore, we recommend future surveys and control efforts to investigate G1/ G3 and G6/ G7 transmission in the Ngari region.

1. Introduction

Cystic echinococcosis (CE), a globally distributed parasitic zoonosis, is caused by the larval stage of *Echinococcus granulosus* (*E. granulosus*) and is considered a problem of global public health (Torgerson et al., 2020; Godazande et al., 2020). The disease burden of human CE has reached 1009,662 (95% CI, 862,119–1175,654) Disability Adjusted Life Years (Budke et al., 2006). The livestock-associated production losses due to CE were $1.2 billion annually (Wu et al., 2018a). The prevalence of echinococcus in the Tibet Autonomous Region (TAR) was 1.71% (1371/80,384), and CE cases accounted for 87.67%, which is the highest prevalence in China (Wu et al., 2018b).

Ngari region is located southwest of the Qinghai-Tibet Plateau, bordering India, Nepal, and Kashmir. The average altitude is above 4 500 m, with low oxygen content. The climate is cold, and the land is wide and sparsely populated. The population is mainly Tibetan, with a backward education level. In the Ngari region, health education is little, and the prevention and control basis is weak. The infection rate of hydatid cysts in livestock was 28.82% (66/229), and the prevalence of CE among children was 1.12% (13/1158), which is the highest in Tibet (Suolang et al., 2018; Xue et al., 2018). Due to high altitude, insufficient staff, vast territory, and inconvenient transportation, identifying genetic polymorphisms of *E. granulosus* was not easy.

Many studies have revealed that different genotypes of *E. granulosus*

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are different in their life cycle, morphology, host species, pathogenicity, and vaccine sensitivity (Romig et al., 2015; Khan et al., 2020). It provided references for diagnosis, therapy, prevention, and control strategy to the CE (Bonelli et al., 2020). There is a high level of genetic variation within *E. granulosus*. It was recently characterized into five independent species, including *E. granulosus sensu stricto* (s.s.) (genotype G1 and G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6-G10), and *E. felidis* (Barazesh et al., 2020). It has been reported that G2 and G9 were not valid genotypes, where G2 was a microvariant of G3 and G9 belonged to G7 (Kinkar et al., 2017; Cucher et al., 2016). Mitochondrial DNA sequence evolved rapidly and had low recombination rates, reflecting population genetic structure, differentiation, and species relationships (Alvi et al., 2020). The *cox*1 and *cob* genes were widely applied in the analysis of polymorphism in *E. granulosus* (Li et al., 2008; Pestechian et al., 2014).

In our study, *cob* and *cox*1 genes were used as genetic markers to describe the genetic variation of *E. granulosus* in the Ngari region. It will provide the dominant genotypes, and genetic variation characteristics of *E granulosus* isolates from humans.

2. Materials and methods

2.1. Ethical statement

The hydatid cysts from patients were collected with the consent of the patient and families. The ethical standards (IPD No. 2019-002) were reviewed and approved by the LAWEC Committee of the National Institute of Parasitic Diseases, Chinese centre for Disease Control and Prevention.

2.2. Study area

Ngari region is located in the northern part of the Tibet plateau, with an average altitude of more than 4500 m. The Ngari region has an area of 345 000 sq. km, 87% grassland. The climate is cold and dry. The average temperature is 0 °C(full range −25.03 °C to 10.30 °C) (Lei et al., 2020). The daily average temperature varies greatly. Most livestock is suitable hosts for the survival of *E. granulosus*. This research was carried out in seven counties in Ngari region including Gar, Pland, Zada, Ritu, Geji, Gaze, and Cuoqin.

2.3. Samples collection and DNA extraction

Paraffin slices of liver cyst was collected from seventy-nine surgical patients with echinococcosis in the Ngari region. The age ranged from six to 76years, including 30 males and 49 females. Nine cases were younger than 14 years old, twenty-eight were between fifteen and 29 years old, nineteen were between thirty and 44 years old, seventeen were between forty-five and fifty-nine years old, and six were over sixty years old.

Paraffin slices were trimmed using a disposable razor blade and placed into a 1.5 mL microcentrifuge tube. The total gDNA of *E. granulosus* was extracted according to the DNA isolation kit protocol (TRANS EasyPure FFPE Tissue Genomic DNA Extraction Kit, Code: EE191–01). In brief, Dimethylbenzene (1 mL) was added to the tubes in the fume hood, vortex vigorously for 10 s, centrifuge at 12,000 g for 2 min, and discarded the supernatant. Absolute ethanol, 90% ethanol, and 80% ethanol were added and vortexed violently for 15 s, 12 000 g for 2 min, and discarded the supernatant, respectively. Samples were completely lysed by adding LB32H and Proteinase K, vortexing vigorously for 15 s, and incubating at 56 °c for 1 h. RNAse A was added to remove RNA. Add 10 mL isopropyl alcohol and 600μL BB32 in turn and swirl. Magnetic FFPE beads are used to isolate DNA. Finally, the DNA samples were stored at −20 °C.

2.4. DNA amplification and sequencing

The *cox1* and *cob* genes of *E. granulosus* were amplified by polymerase chain reaction (PCR). The *cob* gene (600 bp) was amplified using the forward (5′- GTCAGATGTCTTATTGGGCTGC-3′) and reverse (5′-TCTGGGTGACACCCACCTAAATA-3′) primers (Li et al., 2008). The *cox1* gene (450 bp) was amplified using the forward (5′-TTTTTTGGGCATCCTGAG GTTTAT −3′) and reverse (5′-TAAAGAAAGAACATAATG AAAATG-3′) primers (Pestechian et al., 2014). The primers were synthesized by Sain biological (Shanghai, China). The PCR amplification system was as follows, 1.0 μL (10 μmol/L) of upstream and downstream primers, 25 μL of 2 × Ex Taq DNA polymerase, 1.0 μL of DNA template, and 22 μL of distilled water. At the same time, the Genomic DNA extracted from cysts in the infected sheep was used as a positive control, and distilled water was the blank. The PCR program was performed as follows, 95 °C for 5 min, 35 cycles of 95 °C for 15 s, 54 °C (*cox1*) or 56 l°C (*cob*) for the 30 s, 72 °C for 1 min, and 72 °C for 10 min. Each PCR reaction yielded were detected in a 1.5% (w/v) agarose gel stained with GelRed® (Biotium, Fremont, USA). Five microliters of the amplicon were used for visualization. The rest were used for sequencing by Sain biological (Shanghai, China).

2.5. Molecular and phylogenetic analysis

The PCR amplification products were sent to Sain biological (Shanghai, China) for bidirectional sequencing, and the sequencing results were spliced and manually corrected using Contig software. On the NCBI website (HTTP: //blast. ncbi. nlm. nih. Gov/ Blast. cgi), the genotype of *E. granulosus* in the Ngari region of Tibet was compared with the reference sequences which were downloaded from GenBank. The sequences were AF297617.1 (*E. granulosus* G1), MG682544.1 (*E. granulosus* G3), AB786665.1 (*E. equinus* G4), AB745463.1 (*E. canadensis* G10), AB732958.1 (*E.felidis*), AB235846.1 (*E.ortleppi* G5), AB208063.1 (*E. canadensis* G6), AB235847.1 (*E. canadensis* G7), AB235848.1 (*E. canadensis* G8), AB208064.1 (*E.shiquicus*), AB018440.2 (*E. multilocularis*), KU925397.1 (*E. granulosus* G1), and HF947574.1 (*E. granulosus* G1). Sequence alignment of *cob* and *cox1* genes was performed using MEGA 7.0, and mutation sites were found. Phylogenetic trees of *cob* and *cox*1 genes were constructed and tested using the Neighbor-Joining method by MEGA 7.0. We selected the Kimura two-parameter model and repeated the bootstrapping test 1000 times. DnaSP (v5.10.01) was used to analyze the haplotype diversity (Hd), nucleotide diversity, and Tajima's D. Diagrams of the haplotype network were made using Network software.

3. Results

3.1. PCR and sequencing analysis

In our study, a total of 79 from Ngarin-fixed paraffin-embedded (FFPE) tissues were used as DNA source, and samples were collected from Geji (29/79; 36.71%), Gaize (14/79; 17.72%), Ritou (9/79; 11.39%), Pulan (10/79; 12.66%), Cuoqin (7/79; 8.86%), Zhada (6/79; 7.59%), Geer (4/79; 5.06%) in the Ngari region of Tibet.

PCR was carried out with CE patients' samples using *cob* and *cox*1 genes primers. The *cob* and *cox1* gene fragments were amplified on 79 samples in the Ngari region, and 62 samples were successfully amplified (Table 1). The lengths of *cob* and *cox1* gene fragments were 600 bp and 450 bp, respectively (Fig. 1). Out of 62 samples, 59 (95.16%) isolates were identified as G1, two (3.23%) isolates were identified as G6/G7, one isolate (1.61%) was identified as G3 (Table S1-S2).

MEGA7.0 was used to compare the *cob* gene with the standard sequence (KU925397.1), and nine mutations were found. Missense mutations were observed at three positions: 99, A to G, leading to isoleucine to valine; 294, G to T, leading valine to phenylalanine; and 301, G to T, leading tryptophan to isoleucine. Compared with the

Table 1. The
The results of PCR amplification and sequence alignment.

Cob	*cox*1	Number of samples	genotype
+	+	19	G1
	+	34	G1
+		6	G1
+		1	G3
+		1	G6
	+	1	G7
		17	

+, positive amplification; -, negative amplification.

reference sequence HF947574.1, seven mutations were based on the *cox*1 gene. Missense mutations were observed at two positions: 80, C to T, leading alanine to valine; 303, G to A, leading valine to isoleucine (Table 2).

3.2. Phylogenetic tree and haplotype network analysis

The *cob*-65 and AB208063.1 sequence (G6) were on the same branch, the *cob*-40 and MG682544.1 (G3) were on the same branch, and the other *cob* sequences were identified as G1 genotype (Fig. 2A, Table S1). Based on the *cox*1 gene, the genetic distance from *cox*1–66 to the branches between genotypes G6 (AB208063.1; MH301019) and G7 (AB235847.1) was the same, and the other *cox*1 sequences were on a giant branch and belonged to G1 (AF297617.1) (Fig. 2B, Table S2). The genotypes of G1 and G3 belong to *E. granulosus s. s.* The genotypes of G6, G7, G8, and G10 belong to the Canadian strain of *E. granulosus.*

Eight haplotypes were based on the *cob* gene, and the dominant haplotype was H-4 (Fig. 3A). Haplotype diversity (Hd), nucleotide diversity (Pi), Tajima's D, and Fu's Fs statistics were 0.640, 0.00154, −1.88629, and −5.101($P<0.005$), respectively. There were six haplotypes based on the *cox1* gene, and the dominant haplotype was H1 (Fig. 3B). Haplotype diversity (Hd), Nucleotide diversity, Tajima's D, and Fu's Fs statistics were 0.3636, 0.00116, −1.89592, and −3.526 ($P<0.005$).

4. Discussion

CE has been listed as a neglected tropical disease (NTD) by World Health Organization (Maldonado et al., 2017). The disease harms human health and results in enormous financial losses to livestock farming (Metwally et al., 2018). CE is mainly prevalent in western China and the annual incidence of human CE in TAR (32/100,000) was the highest in China (Wen and Yang, 1997).

Ngari Prefecture is one of the areas with the most serious *E. granulosus* infection in the Tibet Autonomous Region. The positive rate of echinococcosis in livestock in the Ngari region (28.82%) is the first, the prevalence rate of echinococcosis in children (1.84%) and the positive rate of eggs detection of *E. granulosus* in dog feces (8.04%) are the second in Tibet Autonomous Region (Xue et al., 2018; SuoLang et al., 2018; KangZhu et al., 2018). And it is difficult to control the final host dog. The viscera of livestock are discarded everywhere after slaughter, which are easy to be swallowed by the definitive host, such as dogs and wolves, causing infection (Yang et al., 2015). High altitude and low temperature in the Ngari district were beneficial to the long-term

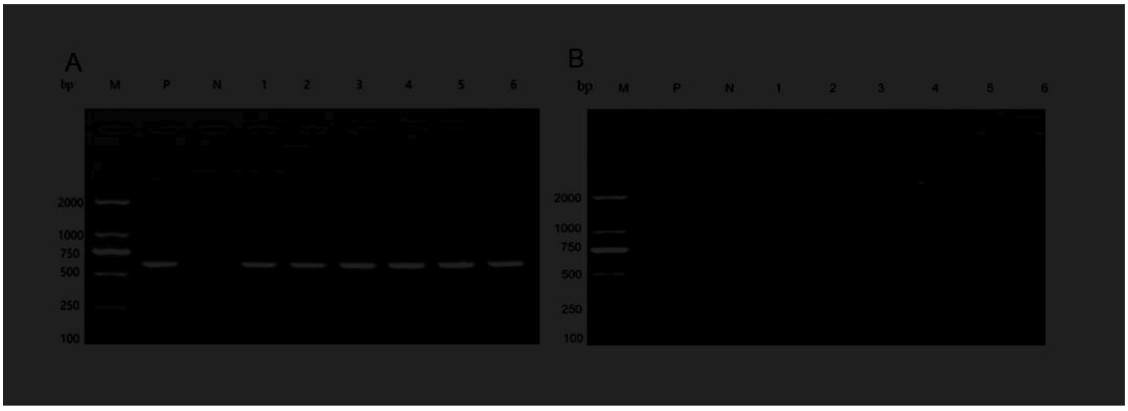

Fig. 1. PCR. products amplified from *E. granulosus* isolates based on *cob* and *cox1* genes from patients in Ngari region A PCR products amplified based on *cob* gene; B PCR products amplified based on *cox*1 gene; M: DNA marker; P: Positive control; N: Negative control; 1–6:PCR products of Samples.

Table 2
Base mutation compared with HF947574.1 and KU925397.

No. of isolates Position	Mutation sites in the *cob* nucleotide sequences								
	99	212	215	218	294	301	321	324	368
KU925397.1	A	C	G	T	G	G	T	C	T
cob-39	G								
cob-69		T							
cob-56			T		T				
cob-40				C			C		
cob-32						T			
cob-73								T	
cob-63									C
No. of isolates Position	Mutation sites in the *cox*1 nucleotide sequences								
	80	90	135	270	281	303	411		
HF947574.1	C	C	C	G	T	G	T		
*cox*1–39	T								
*cox*1–48		T			C				
*cox*1–67				T					
*cox*1–62			T			A			
*cox*1–24							C		

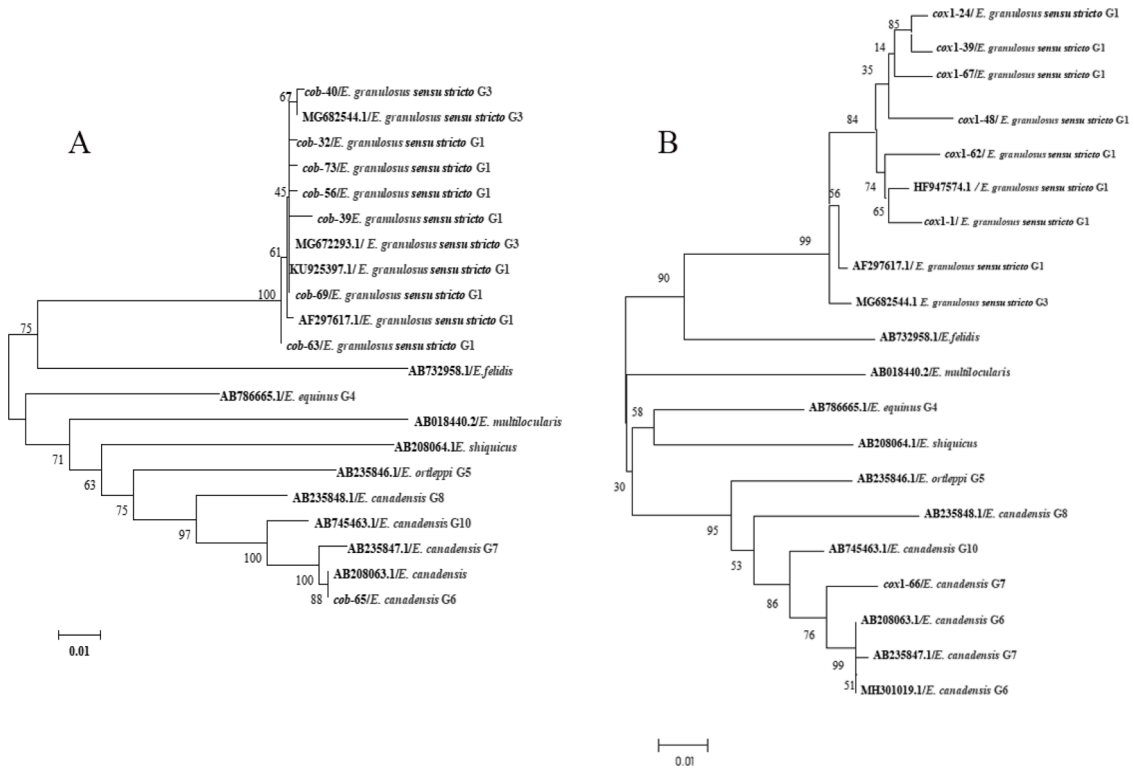

Fig. 2. Neighbor-Joining phylogenetic tree based on mitochondrial DNA sequences of *cob* and *cox*1. A. Phylogenetic relationships of *cob* in the Ngari region. B. Phylogenetic relationships of *cox1* in the Ngari region.

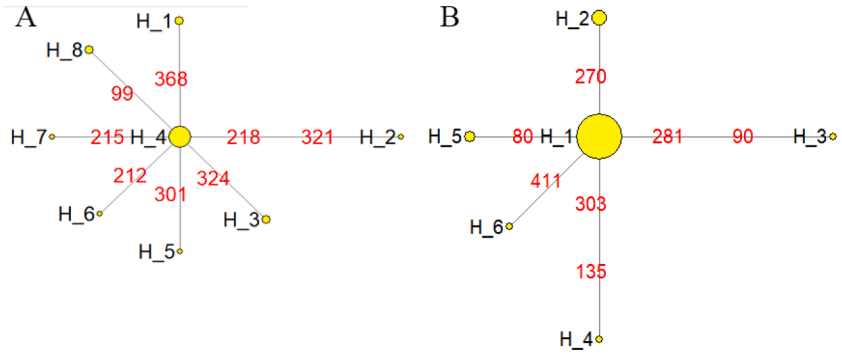

Fig. 3. Haplotypes network analysis of *E. granulosus* isolates based on *cox*1 and *cob* in the Ngari region. A. There were eight haplotypes based on the *cob* gene. The dominant haplotype of *cob* was H-4 in the Ngari region. B. There were six haplotypes based on *cox*1 gene. The dominant haplotype of *cox*1 were H-1 in the Ngari region. The size of the circle indicates the frequency of the haplotypes. .

survival of *E. granulosus* eggs and increased the risk of intermediate host infection. Non-standard slaughtering of livestock, low temperature, and lack of medical conditions have resulted in the local epidemic of echinococcosis, which seriously threatens people's health and life safety (Ma et al., 2021).

While different genotypes may result in a different immune response, it is important to identify the genetic diversity of *E. granulosus*. For example, EG95 was the most effective vaccine, which has been observed differences in the amino acid between *E. granulosus* (G1) and *E. Canadensis* (G6) (Chow et al., 2008). However, there was little data about the genetic diversity of *E. granulosus* in the Ngari region. In our research, 79 patient samples were collected from seven counties of the Ngari region, and the gene polymorphism of *E. granulosus* was investigated.

PCR amplification was employed based on *cob* and *cox*1 gene sequences and the amplification efficiency of different genes is different. 27 and 54 samples were successfully amplified based on *cob* and *cox*1 gene fragments. 17 samples were negative results, which might be DNA degradation during the fixation procedure or PCR inhibition caused by high polysaccharide content in the cyst tissues (Schneider et al., 2008; Khan et al., 2020). The length of the amplified product from FFPE tissue was not more than 450 bp (Zada et al., 2018). In this study, we used a DNA isolation kit protocol (TRANS EasyPure FFPE Tissue Genomic DNA Extraction Kit, Code: EE191-01) to extract DNA from FFPE tissue. In the rehydration, using absolute ethanol, 90%, and 80% ethanol may reduce DNA degradation.

In our study, *E. granulosus s. s.* (G1/G3) and *E. canadensis* (G6/G7) were detected in the Ngari region. The similarity between the *cob*-40 and MG682544.1 (G3) was 100%. The *cob*-40 and G3 genotypes were found in the same branch. But short *cob* or *cox*1 sequences could not differentiate G3 from G1, so *E. granulosus s. s.* (G1/G3) were detected in this study (Romig et al., 2015). *E. granulosus s. s.* (G1) is the most common genotype causing human CE in the world and China (Alvarez Rojas et al., 2014; Hua et al., 2022; Zhao et al., 2022). Transmission of *E. granulosus*

in the Qinghai-Tibet Plateau is almost certainly related to the widespread distribution of definitive hosts (dogs) and intermediate hosts (sheep, goats, yaks, pigs) (Wang et al., 2021; Craig et al., 2019; Wu et al., 2018c; Li et al., 2017; Liu and Yin, 2022). Sheep have been considered a suitable intermediate host for *E. granulosus,* the main domestic animal in the Ngari region (Ma et al., 2021). The evolution distance from *cox*1-66 to genotypes G6 (AB208063.1; MH301019) and G7 (AB235847.1) were the same. Too many intermediate variants make it difficult to distinguish between G6 and G7. It's better to treat cluster *E.canadensis* (G6/7) as a common haplotype (Addy et al., 2017). Only with complete mtDNA could we differentiate G6/G7, while it was impossible with key genes of *cox1, nad1, nad2*, and *atp6* (Zhao et al., 2022).

Except for *E. granulosus s. s.* (G1), *E. canadensis* (G6) is the second most cause leading human CE in most parts of the country (Shang et al., 2019). The *E. canadensis* G6/G7 genotype in China has been reported in sheep, bigs, cattle, and yaks (Hua et al., 2022). Our research focused on genotypes of *E. granulosus* in humans. The result indicated that control transmission of *E. granulosus s. s.* (G1/G3) and *E. canadensis* (G6/G7) in humans is important in the Ngari region.

Haplotype diversity is often used to measure inter-population and intra-population diversity. As depicted by the median-joining network, the parsimony network of *E. granulosus* in the Ngari region was exhibited by a star-like configuration. It was in line with some previous studies from China (Ohiolei et al., 2019; Nakao et al., 2010; Yan et al., 2013). Other haplotypes expanded from the main haplotype, suggesting that the populations of seven counties are not fully differentiated (Nakao et al., 2010). It is similar to previous observations in China and differs from the Middle East. Still, this observation may be a geographical limitation or a result of an analyzed short DNA fragment (Ohiolei et al., 2019). In the study, the low nucleotide diversity (Pi<0.005) based on the *cox1* and *cob* gene has been observed in the Ngari region. The low nucleotide has also been observed in other parts of China, which supported the demographical expansion of *E. granulosus* (Ohiolei et al., 2019). The negative Tajima's D values and Fu's Fs statistic of the *cox*1 and *cob* genes indicated that it experienced population expansion or a negative selection, a common feature of *E. granulosus* in the Tibetan plateau (Ohiolei et al., 2019; Han et al., 2019; Wang et al., 2014).

This study is an important molecular analysis of *E. granulosus in* humans in the Ngari region. Results indicated that *E. granulosus s. s.* (G1/G3) and *E. canadensis* (G6/G7) were prevalent in humans. There were eight/ six haplotypes based on the *cob/ cox*1gene, and the dominant haplotype was H-4/ H-1. Our results suggested *E. granulosus* in the Ngari region experienced population expansion or a negative selection. It filled the genetic patterns and provided evidence for future control measures in the Ngari region. Therefore, we recommend it is necessary to investigate *E. granulosus s. s.* (G1/G3) and *E. canadensis* (G6/G7) transmission further in the Ngari region.

Author statement

Yuhuan Wei: complemented the experiments, wrote the draft.
Liwu Jun, Chunhai Shao, Hai Zhao: collected the samples.
Yuan Hu, Hua Liu: designed experiments and revised the manuscript.
Jianping Cao: revised the manuscript.

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Supplementary materials

Table S1. The blast result of cob in the Ngari region
Table S2. The blast result of cox1 in the Ngari region

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships to influence the work in this paper.

Data availability

The data that has been used is confidential.

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