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Genetic polymorphisms of *Echinococcus* tapeworms in China as determined by mitochondrial and nuclear DNA sequences ☆

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Abstract

The genetic polymorphisms of *Echinococcus* spp. in the eastern Tibetan Plateau and the Xinjiang Uyghur Autonomous Region were evaluated by DNA sequencing analyses of genes for mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear elongation factor-1 alpha (*ef1a*). We collected 68 isolates of *Echinococcus granulosus* sensu stricto (s.s.) from Xinjiang and 113 isolates of *E. granulosus* s. s., 49 isolates of *Echinococcus multilocularis* and 34 isolates of *Echinococcus shiquicus* from the Tibetan Plateau. The results of molecular identification by mitochondrial and nuclear markers were identical, suggesting the infrequency of introgressive hybridization. A considerable intraspecific variation was detected in mitochondrial *cox1* sequences. The parsimonious network of *cox1* haplotypes showed star-like features in *E. granulosus* s. s. and *E. multilocularis*, but a divergent feature in *E. shiquicus*. The *cox1* neutrality indexes computed by Tajima's D and Fu's Fs tests showed high negative values in *E. granulosus* s. s. and *E. multilocularis*, indicating significant deviations from neutrality. In contrast, the low positive values of both tests were obtained in *E. shiquicus*. These results suggest the following hypotheses: (i) recent

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founder effects arose in *E. granulosus* and *E. multilocularis* after introducing particular individuals into the endemic areas by anthropogenic movement or natural migration of host mammals, and (ii) the ancestor of *E. shiquicus* was segregated into the Tibetan Plateau by colonizing alpine mammals and its mitochondrial locus has evolved without bottleneck effects.

Keywords

Echinococcus; Mitochondrial DNA; Genetic diversity; Population genetic structure; China

1. Introduction

Metacestodes of the dog tapeworm *Echinococcus granulosus* sensu stricto (s.s.) and the fox tapeworm *Echinococcus multilocularis* are highly pathogenic to humans, and cause cystic and alveolar echinococcoses, respectively. Humans become infected through oral ingestion of eggs derived from faeces of canine definitive hosts. Sheep are a main intermediate host for *E. granulosus* s. s., whereas arvicoline rodents serve as intermediate hosts for *E. multilocularis* (Eckert and Deplazes, 2004). Besides the main two species, Echinococcus equinus, Echinococcus ortleppi, Echinococcus canadensis, Echinococcus felidis, Echinococcus shiquicus, Echinococcus vogeli and *Echinococcus oligarthrus* have been regarded as valid by recent phylogenetic studies (Xiao et al., 2005; Nakao et al., 2007; Hüttner et al., 2008). However, the species status of *E. canadensis* is still debatable (Thompson, 2008).

In China, *E. granulosus* s. s. and *E. multilocularis* are widespread in western, northern and central parts of the country (Wang et al., 2008), and hyperendemic foci exist within pastoral areas of the eastern Tibetan Plateau (Schantz et al., 2003; Li et al., 2008) and the Xinjiang Uyghur Autonomous Region (Wang et al., 2001). Both endemic regions are geographically separated by the Kunlum mountains and the Taklamakan Desert. The alpine steppe of the Tibetan Plateau supports human pastoral activity for the raising of yak and sheep. In Xinjiang, nomads and semi-nomads keep livestock on low-altitude grassland. This type of sheep husbandry system, including the use of pastoral dogs, is essential to maintain the synanthropic cycle of *E. granulosus* s. s. In contrast, the rodent fauna of grassland and the migration of foxes are key factors in establishing the endemic foci of *E. multilocularis* (Giraudoux et al., 2006). Human infections with *E. multilocularis* are extremely frequent in the Tibetan communities of Sichuan province (Craig, 2006), and the role of dogs in the communities is important to infections (Wang et al., 2006). Thus, the life cycle of *E. multilocularis* is altered to be synanthropic in hyperendemic areas.

Species of *Echinococcus* prevailing in China have been clarified by molecular taxonomic studies using mtDNA markers (McManus et al., 1994; Zhang et al., 1998; Yang et al., 2005; Bart et al., 2006; Xiao et al., 2004, 2005, 2006; Ma et al., 2008; Li et al., 2008). The molecular identification of *Echinococcus* isolates from various origins showed the following host-parasite relationships in China: (i) domestic mammals (sheep, cattle, goats, yaks and dogs) for *E. granulosus* s. s., (ii) domestic mammals (camels, cattle and dogs) for *E. canadensis* (G6 genotype), (iii) wildlife (voles, hares, pikas, red foxes and Tibetan foxes) and domestic mammals (dogs) for *E. multilocularis* and (iv) wildlife (pikas and Tibetan foxes) for *E. shiquicus*. Human infections have been confirmed for all species except *E. shiquicus*. Interestingly, *E. canadensis* G6 has been found only in Xinjiang (Zhang et al., 1998) and *E. shiquicus* seems to be restricted in the Tibetan Plateau (Xiao et al., 2005). All of the epidemiological information provides a basis to consider the natural history of *Echinococcus* in China. Previous studies demonstrated that intraspecific mtDNA variations occurred in *E. granulosus* s. s. (Yang et al., 2005; Bart et al., 2006; Ma et al., 2008), *E. multilocularis* (Yang

et al., 2005) and *E. shiquicus* (Xiao et al., 2005). However, the genetic populations of *Echinococcus* spp. in China have never been characterized in an evolutionary context.

In this study, the genetic diversities of *E. granulosus* s. s., *E. multilocularis* and *E. shiquicus* were explored by using mtDNA and nDNA markers. We sampled the isolates of *E. granulosus* s. s. from Xinjiang and the isolates of all three species from the eastern Tibetan Plateau. The main purpose of this study was to evaluate the population genetic structures of the three species. The resultant data and epidemiological information enabled us to suggest evolutionary hypotheses on how the parasites have spread in China.

2. Materials and methods

2.1. Isolates collected

An *Echinococcus* isolate was defined as a unilocular cyst or a separated alveolar cyst from an intermediate host. During the period from 2002 to 2007, the larval isolates of *Echinococcus* spp. from various hosts were collected in the eastern Tibetan Plateau (Qinghai and Sichuan provinces) and the Xinjiang Uyghur Autonomous Region. Table 1 summarizes the number and origin of isolates examined in the two localities. For *E. granulosus* s. s., 113 isolates in the highlands and 68 isolates in the lowlands were treated as two separate populations. On the other hand, 49 isolates of *E. multilocularis* and 34 isolates of *E. shiquicus* were obtained only from the highlands. One isolate in the lowlands was identified as *E. canadensis* G6 genotype. In addition, 57 isolates of *E. granulosus* s. s. in Peru (Moro et al., 2009) served as a foreign control for population genetic analyses. The species identification of those isolates was validated by DNA sequencing described below.

2.2. PCR amplification and sequencing

The genomic DNA of each isolate was prepared from ethanol-preserved larval cysts by using a DNeasy blood and tissue kit (Qiagen), and used as a template for PCR. Partial fragments of a mitochondrial gene for cytochrome *c* oxidase subunit 1 (*cox1*) and a nuclear gene for elongation factor-1 alpha (*ef1a*) were amplified by PCR using specific primers reported previously (Nakao et al., 2000; Moro et al., 2009). The PCR mixture was prepared in a 25 μl final volume containing 1 μl template DNA, 200 μM of each dNTP, 0.2 μM of each primer, 0.5 U of Ex-*Taq* polymerase (Takara) and the manufacturer-supplied reaction buffer. Thermal reactions were performed for 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s. Amplified DNA fragments were purified with QIAquick spin columns (Qiagen), and sequenced directly with a BigDye terminator cycle sequencing kit (Applied Biosystems). The resultant sequence ladders were read by an ABI PRISM 377 genetic analyzer (Applied Biosystems).

2.3. Data analysis

In each species of *Echinococcus*, multiple alignments in NEXUS format were made manually by editing the plain text files of nucleotide sequences. Amino acid sequences were inferred from the nucleotide sequences by echinoderm mitochondrial genetic code (Nakao et al., 2000; Telford et al., 2000) or standard genetic code. Percentage divergence values of nucleotide sequences were determined using the MEGA4 package (Tamura et al., 2007) using Kimura's two parameter model (Kimura, 1980) with a γ-shaped parameter (a=0.5). The identification of haplotypes and the drawing of their networks were executed by TCS 1.2 software (Clement et al., 2000) using statistical parsimony (Templeton et al. 1992). The network estimation was run at a 95% connection limit.

Population diversity indexes (number of haplotypes, haplotype diversity and nucleotide diversity) were calculated using DnaSP 4.5 software (Rozas et al., 2003). The population

genetics package Arlequin 3.1 (Excoffier et al., 2005) was employed to calculate the neutrality indexes of Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997). The degree of gene flow between two populations was estimated using a pairwise fixation index (Fst) as determined by the Arlequin package. The three geographic populations of *E. granulosus* s. s. from Xinjiang, the eastern Tibetan Plateau and Peru (out of China) were used to compute the Fst values.

3. Results

3.1. Variations in nucleotide sequences

In our targeted regions of *Echinococcus* DNA, deletion or insertion mutations were not observed, even in different species. The total numbers of nucleotides examined were therefore stable in mitochondrial *cox1* (789 sites) and nuclear *ef1a* (656 sites). The *cox1* sequences could be amplified in all of the isolates of *E. granulosus* s. s. (n = 181), *E. multilocularis* (n = 49), *E. shiquicus* (n = 34) and *E. canadensis* G6 (n = 1). However, the PCR positive rate of *ef1a* was relatively lower than that of *cox1*, probably due to the low copy number of the nuclear gene. Each *Echinococcus* sp. retained the species-specific nucleotide sequences of *cox1* and *ef1a*.

Considerable intraspecific variations were detected only in the mtDNA sequences of *cox1* (Table 1), indicating its primary use for population genetic analyses. Synonymous substitutions exceeded non-synonymous substitutions in the *cox1* sequences of *E. granulosus* s. s. and *E. shiquicus*. Out of all of the point mutations, 24 sites (49.0%) of *E. granulosus* s. s., one site (25.0%) of *E.multilocularis* and 11 sites (64.7%) of *E. shiquicus* were parsimony informative. Relatively to *E. shiquicus*, singleton substitutions were abundant in *E.granulosus* s. s. and *E. multilocularis*. The pairwise divergence of the *cox1* sequences was computed among individual isolates at an intraspecific level. The maximum values of the divergence were 0.9% in *E. granulosus* s. s., 0.3% in *E. multilocularis* and 1.5% in *E. shiquicus*. The autochthonous species *E.shiquicus* appeared to have the most variable mtDNA.

3.2. Haplotype networks

In *E. granulosus* s. s., 43 mtDNA haplotypes were found in 181 isolates from Xinjiang and the eastern Tibetan Plateau (Qinghai and Sichuan). To discern a genealogical relationship among the haplotypes, we constructed a statistical parsimony network. As shown in Fig. 1, each of the two regions possessed geographically specific haplotypes. The network, however, showed a star-like expansion, and one common haplotype (G01) occupied the centre of the network. The numbers of mutational steps between the common haplotype and the others ranged from one to five, and the frequency of the common haplotype was 53.6% in the population. A similar star-like network was observed in the Peruvian population of *E. granulosus* s. s. (Fig. 2A). Five mtDNA haplotypes were detected in the 57 Peruvian isolates (Moro et al., 2009), but the majority of the isolates (93.0%) belonged to the haplotype G01, which was the most common in the Chinese populations. A single-nucleotide variation was identified between members of the five haplotypes, three of which were geographically specific to Peru.

We detected five mtDNA haplotypes in 49 isolates of *E. multilocularis* from the eastern Tibetan Plateau. These were illustrated as a star-like network with one major haplotype (M01), which comprised 89.8% of the isolates examined (Fig. 2B). All variations found in the five haplotypes were single-nucleotide polymorphisms. As opposed to the convergent networks of *E. granulosus* s. s. and *E. multilocularis*, a divergent network was found in *E. shiquicus* (Fig. 2C). Although 10 mtDNA haplotypes were detected in 34 isolates of *E. shiquicus*, major haplotypes were absent in the population. The maximum number of mutational steps was 13 in the network of *E. shiquicus*.

3.3. Diversity and neutrality indexes

Diversity indexes for *Echinococcus* populations in each locality were calculated using the data set of *cox1* (Table 3). In the Chinese populations of *Echinococcus* spp., both values of haplotype and nucleotide diversities were the highest in *E. shiquicus* but the lowest in *E. multilocularis*. In the case of *E. granulosus* s. s., the Peruvian population showed the lowest values compared with the Chinese populations. The high levels of haplotype diversity were kept in the Chinese populations of *E. granulosus* s. s., but their nucleotide diversity was relatively low because of the richness of single nucleotide substitutions.

Neutrality indexes calculated by Tajima's D and Fu's Fs tests are also shown in Table 3. The highly negative values were recorded in the Chinese populations of *E. granulosus* s. s. and *E. multilocularis*, indicating a significant deviation from neutrality. The Peruvian population of *E. granulosus* s. s. also showed significant negative values. By contrast, relatively low positive values were obtained in the population of *E. shiquicus*, which kept highly polymorphic mtDNA.

3.4. Fixation index for the populations of E. granulosus s. s

Using the data set of mtDNA, the values of the pairwise fixation index (Fst) were computed to estimate the degree of gene flow among three geographic populations of *E. granulosus* s. s in China and Peru (Table 4). Since one common haplotype existed predominantly in the three localities, the Fst values between the populations were very small, ranging from 0.036 to 0.009. These low values implied that the populations were not genetically differentiated from one another.

4. Discussion

Echinococcoses caused by *E. granulosus* s. s. and *E. multilocularis* lead to considerable social and economic losses in the endemic communities of the eastern Tibetan Plateau (Budke et al., 2005). Furthermore, *E. shiquicus* has been recently discovered in the plateau (Xiao et al., 2005). Information on the population genetic structures of these sympatric species is necessary to better understand the process of intra- and interspecific gene flows, and may provide a foundation for future epidemiological studies on the transmission dynamics of the parasites. In the present study, mtDNA revealed the basic structures of *Echinococcus* populations in the eastern Tibetan Plateau.

We chose the protein-coding gene *cox1* as a marker for *Echinococcus* mtDNA. In other organisms, the mitochondrial control region has been generally used to infer genealogical relationships. However, the corresponding mtDNA regions of *Echinococcus* spp. contain highly repetitive sequences, which are unsuitable for phylogenetic studies (Nakao et al., 2007). The *cox1* gene of *Echinococcus* spp. has already been shown to be a promising candidate for the classification of intra- and interspecific variants even in the short sequence (366 nucleotide sites) (Bowles et al., 1992). In this study, we determined the relatively long sequence of *cox1* (789 nucleotide sites), and detected a sufficient number of haplotypes to analyze the population genetic structures of each species. The haplotypes are only loosely correlated with the intraspecific genotypes of *E. granulosus s. s.* (G1, G2 and G3) and *E. multilocularis* (M1 and M2) defined by Bowles et al. (1992). The negative selection of the *cox1* for the purging of deleterious mutations has been demonstrated by the codon-based Z-test (Tamura et al., 2007) using the corresponding sequences of *E. granulosus* s. s., *E. multilocularis*, *E. equinus*, *E. ortleppi*, *E. canadensis*, *E. felidis*, *E. vogeli* and *E. oligarthrus* (M. Nakao, unpublished data). The fragments of the nuclear gene *ef1a* were also sequenced in this study, but their species-specific sequences were not polymorphic at an intraspecific level. The results of species identification by the mitochondrial and nuclear markers were identical, suggesting the infrequency of introgressive hybridization among the sympatric species.

The *cox1* haplotypes of *E. granulosus* s. s. found in this study did not show an apparent phylogeographic structuring in China. The parsimony network analysis revealed that the haplotypes exhibit a star-like expansion from a main founder haplotype, suggesting that the populations of eastern Tibet and Xinjiang are not fully differentiated from each other. It is noteworthy that the same founder was predominant in the Peruvian population. It seems unlikely that mutations of the founder haplotype are advantageous because the amino acid sequence deduced from the founder is the same as those from other minor haplotypes. The common genetic structure between geographically unrelated populations enables us to speculate that one particular lineage of *E. granulosus* s. s. is widespread globally. The genetic non-differentiation between the local populations is also demonstrated by extreme low values of the fixation index Fst. Furthermore, the significant negative values of the neutrality indexes Tajima's D and Fu's Fs suggest that bottleneck events might occur in the recent past. It is most likely that demographic expansions of the parasite occurred after introducing particular individuals into the endemic areas by anthropogenic movements of host mammals (sheep and dogs).

It is assumed that *E. granulosus* s. s. was introduced into South America from Europe through livestock importation after the colonial period. The higher values of haplotype and nucleotide diversities in the Chinese populations of *E. granulosus* s. s. suggest that China historically preceded Peru in the time of initial founder introduction. One could speculate that bottleneck events might also occur in the ancestral population of *E. granulosus* s. s. during the colonization of domestic sheep as an intermediate host. Archaeological and genetic evidence suggest that sheep were domesticated in the Ancient Near East (Pedrosa et al., 2005), but the genealogical survey of Chinese domestic sheep showed the possibility that additional domestication events occurred independently in other regions (Chen et al., 2006). The lack of archaeoparasitological data does not permit us to infer how and when the parasite invaded China. However, the Ancient Near East is one possible candidate for the cradle of *E. granulosus* s. s. The population genetic structures of *E. granulosus* s. s. should be compared in various endemic areas to clarify its ancestral origin and the process of its worldwide dispersal.

Our previous study has already indicated the rarity of mtDNA polymorphism in the Tibetan population of *E. multilocularis* (Xiao et al., 2005). The present study furthermore revealed that a particular *cox1* sequence was positioned as a basal haplotype, suggesting that a founder effect arose in the population. Our recent phylogeographic study on *E. multilocularis* showed that the worldwide isolates were classified into European, Asian and North American clades except the Inner Mongolia isolates from the corsac fox *Vulpes corsac* (Nakao et al., 2009). The geographic clustering indicates a possibility that genetic changes occurred in *E. multilocularis* after the fragmentation of the population during the Pleistocene ice ages. The red fox *Vulpes vulpes*, which has a flexible ability to adapt to various environments, extended its distributional range in the Holarctic region, and might play an essential role in introducing *E. multilocularis* into new areas. It seems likely that an epidemic of the parasite in the eastern Tibetan Plateau was initiated by natural migration of red foxes in the recent past.

The Tibetan indigenous species *E. shiquicus* showed a quite different pattern of population genetic structure when compared with *E. granulosus* s. s. and *E. multilocularis*. Statistical neutrality tests and haplotype network analyses suggest a possibility that the mitochondrial locus of *E. shiquicus* has evolved without bottleneck effects. Our previous report clarified that *E. shiquicus* utilizes the Tibetan fox *Vulpes ferrilata* as a definitive host and the plateau pika *Ochotona curzoniae* as an intermediate host (Xiao et al., 2005). Both the autochthonous mammals are adapted to the high altitude steppe but do not survive in lowlands. We can therefore consider that *E. shiquicus* has been segregated in the plateau since the parasite's ancestor colonized the alpine mammals. The lasting geographic segregation seems to be a cause for extraordinary richness of polymorphism in Tibetan *E. shiquicus*.

Diploid organisms having a mixed sexual and asexual reproduction system show different patterns from theoretical population genetic models (Prugnolle et al., 2005). The adult tapeworms of *Echinococcus* are hermaphroditic, and self-fertilization mainly occurs in the small intestine of canine definitive hosts (Haag et al., 1999). The larvae furthermore proliferate asexually in the viscera of intermediate hosts, and the clonal offspring develop into adults in a definitive host. The biphasic reproduction of *Echinococcus* spp. may strongly affect their population genetic structures and promote a very low genetic variability of nuclear loci. In this study we used a haploid maternally inherited mtDNA marker to examine the population genetic structure of *Echinococcus* because of the lack of appropriate nuclear markers. A panel of single-locus nuclear markers is required for further population genetic studies to elucidate the evolutionary backgrounds of *Echinococcus* worldwide.

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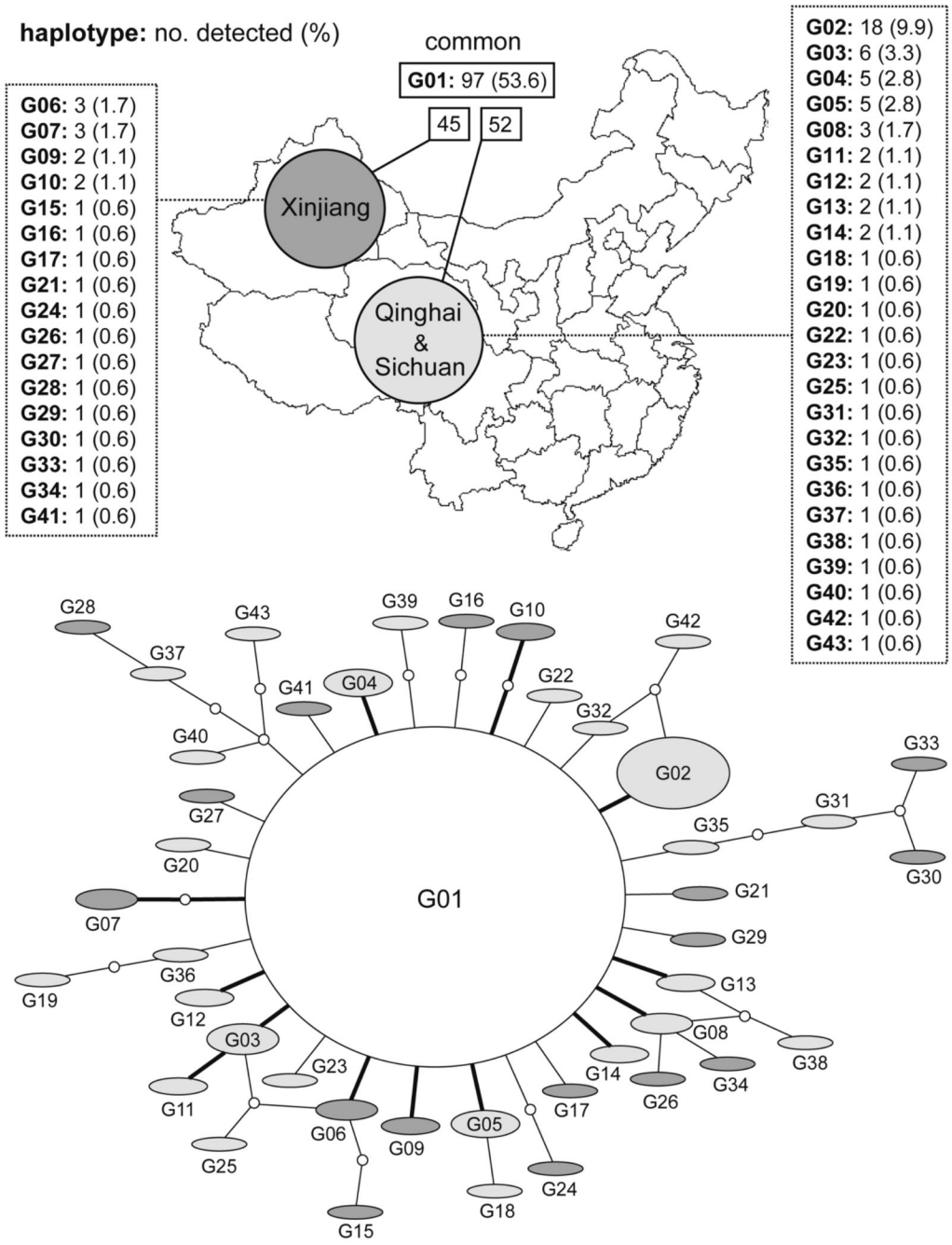

Fig. 1.

Frequencies of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) haplotypes in Chinese *Echinococcus granulosus* and their network based on statistical parsimony. In the network, the size of ovals indicates the frequency of the haplotypes. Small circles show hypothetical haplotypes. The haplotypes whose frequencies are more than 1% are connected with bold lines. Dark ovals represent the haplotypes found in Xinjiang, whereas the localities of gray ovals are Qinghai and Sichuan. A white oval shows the common haplotype.

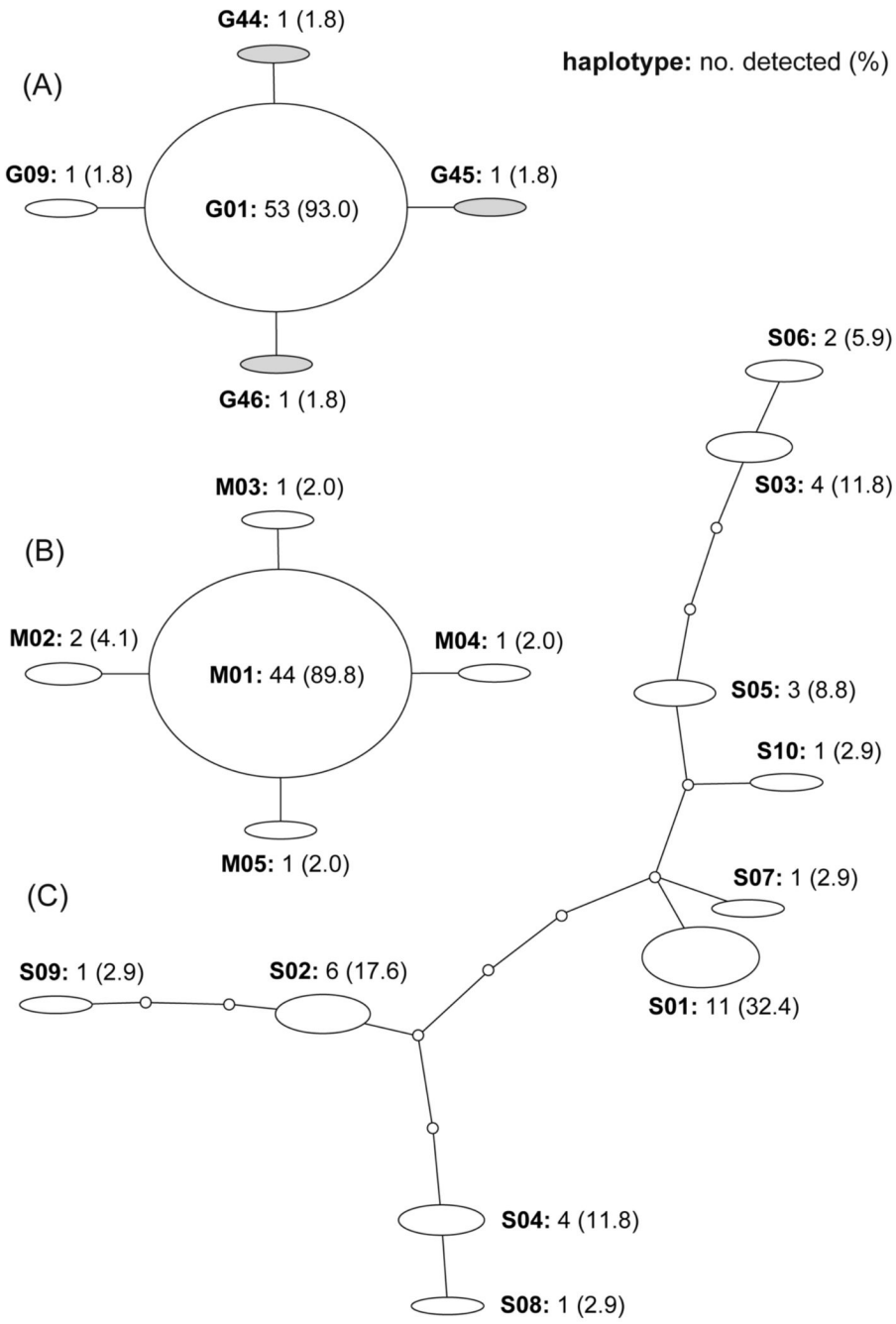

Fig. 2.

The statistical parsimony networks of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) haplotypes in *Echinococcus* spp. The size of ovals indicates the frequency of the haplotypes. Small circles show hypothetical haplotypes. (A) The Peruvian population of *Echinococcous granulosus* sensu stricto. Gray ovals represent the haplotypes specific to Peru. (B) The Tibetan population of *Echinococcus multilocularis*. (C) The Tibetan population of *Echinococcus shiquicus*.

Table 1

Number of *Echinococcus* isolates used for this study.

Species and localities	Origins of larval isolates						Total
	Human	Sheep	Yak	Rodent [a]	Hare [b]	Pika [c]	
Echinococcus granulosus							
Qinghai & Sichuan	37	57	19	0	0	0	113
Xinjiang	54	14	0	0	0	0	68
Total	91	71	19	0	0	0	181
Echinococcus multilocularis							
Qinghai & Sichuan	20	0	0	26	1	2	49
Echinococcus shiquicus							
Qinghai & Sichuan	0	0	0	0	0	34	34
Echinococcus canadensis (G6)							
Xinjiang	1	0	0	0	0	0	1

[a] *Microtus fuscus*, *Microtus limnophilus* and *Cricetulus kamensis*.

[b] Lepus oiostolus.

[c] Ochotona curzoniae.

Table 2

Number of nucleotide substitutions in mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear elongation factor-1 alpha (*ef1a*) genes amplified from *Echinococcus* spp. in China.

	***cox1* (789)**			***ef1a* (656)**		
Species	***n***	**S**	**NS**	***n***	**S**	**NS**
Echinococcus granulosus	181	30	19	122	0	0
Echinococcus multilocularis	49	1	3	38	0	0
Echinococcus shiquicus	34	16	1	30	3	0

Number of the total nucleotide sites examined is shown in parentheses. Abbreviations are number of isolates examined (*n*), synonymous substitutions (S) and non-synonymous substitutions (NS).

Table 3

Diversity and neutrality indexes for *Echinococcus* populations calculated from the nucleotide data set of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene.

Species and localities	*n*	Diversity		Neutrality		
		Hn	Hd ± S.D.	π ± S.D.	D	Fs
Echinococcus granulosus						
Qinghai & Sichuan	113	26	0.760 ± 0.038	0.0017 ± 0.0002	−2.323[a]	−26.023[a]
Xinjiang	68	18	0.562 ± 0.073	0.0015 ± 0.0003	−2.456[a]	−15.762[a]
Total (China)	181	43	0.702 ± 0.038	0.0017 ± 0.0002	−2.536[a]	−61.569[a]
Out of China (Peru)	57	5	0.137 ± 0.062	0.0002 ± 0.0001	−1.849[a]	−5.889[a]
Echinococcus multilocularis						
Qinghai & Sichuan	49	5	0.195 ± 0.075	0.0003 ± 0.0001	−1.765[a]	−4.788[a]
Echinococcus shiquicus						
Qinghai & Sichuan	34	10	0.847± 0.040	0.0055 ± 0.0004	0.164	0.258

Abbreviations are number of isolates examined (*n*), number of haplotypes (Hn), haplotype diversity (Hd), nucleotide diversity (π), Tajima's D (D) and Fu's Fs (Fs).

[a]Significant *P* values ($P < 0.01$).

Table 4

Pairwise fixation index (Fst values) between *Echinococcus granulosus* sub-populations calculated from the nucleotide data set of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene.

	1	2
1. Qinghai & Sichuan		
2. Xinjiang	0.031[a]	
3. Out of China (Peru)	0.036[a]	0.009

[a]Significant *P* values ($P < 0.01$).