Taeniid cestodes in Tibetan foxes (*Vulpes Ferrilata*) detected by copro-PCR: Applications and challenges

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ABSTRACT

Tibetan foxes (*Vulpes ferrilata*) have been confirmed as the main wild definitive hosts in echinococcosis transmission in the eastern Tibetan Plateau. However, little information is available about the epidemiology in wildlife from the perspective of the Taeniidae family, which is essential knowledge in understanding the epidemiology and phylogeography of cestode species in the Tibetan plateau. Therefore, in this study, we used copro-PCR techniques, by amplifying *nad1* and *cox1* gene fragments, to detect the taeniid species from Tibetan fox feces collected in Shiqu County, (Sichuan Province, China), eastern Tibetan Plateau. Phylogenetic relationships between amplified sequences and existed *Taenia* species genotypes were evaluated. Then, the maximum prevalence (positive PCR results from at least one primer pair) and the conservative prevalence (positive PCR results from at least two primer pairs) were calculated. Thirty-six Tibetan fox feces were analyzed. *Echinococcus multilocularis* (conservative prevalence ± 95% CI: 22.2% ± 13.6%; maximum prevalence ± 95% CI: 33.3% ± 15.4%) and *E. shiquicus* (2.8 ± 5.4%; 8.3 ± 9.0%) was detected. Meanwhile, DNA fragments of *T. polyacantha* were detected with high similarity to NCBI sequences (*cox1*, 94.0%) and to the larva sample DNA sequenced in this study (93.4%), and were supported by phylogenetic analysis. Thus, *T. polyacantha* might infect Tibetan foxes (5.6% ± 7.5%, 11.1% ± 10.3%). Our limited findings in the epidemiology of parasitic *Taenia* species suggest that sylvatic transmission cycles for a more species-rich Taeniid community must be established between wild canids and small mammals than just for the two *Echinococcus* species. Besides, discrepancies in different primer pairs in detecting the taeniid species were evaluated. The sensitivity of some widely used universal primer pairs was poor in detecting *Taenia* species from canid copro-DNA samples. It is still challenging to the development of effective taeniid species-specific molecular markers especially for non-zoonotic species.

1. Introduction

The family Taeniidae is composed of more than 150 recognized species in genus *Taenia, Echinococcus, Hydatigera,* and *Versteria* (Nakao et al., 2013a), in which many species can cause serious parasitic human and animal diseases (see review Hoberg, 2002, 2006). The intestinal infections (taeniasis) caused by the adult stages of Taeniidae cestodes, systemic infections (cysticercosis, coenurosis, echinococcosis) agented by their larvae phase have been uncovered within a range of humans, domestic animals, and wildlife (Eckert et al., 2004; Nakao et al., 2013b; Lavikainen et al., 2014). Understanding the epidemiology and phylogeography of the Taeniidae species is not only vital for understanding the evolutionary mechanisms of cestode species, but also essential in the surveillance and prevention of infectious pathogenic organisms.

The eastern Tibetan Plateau is recognized as a serious, endemic area of human parasitic diseases caused by the *Echinococcus* and *Taenia* species (Wang, 2016; Zhou, 2018). The transmission of echinococcosis has been shown to involve a complex wildlife reservoir in which Tibetan

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foxes (*Vulpes ferrilata*) are the main wild definitive hosts (Xiao et al., 2008; Jiang et al., 2012; Boufana et al., 2013). Although *Taenia* species infectious to humans have mainly been reported between humans and domestic animals (Li et al., 2006), fox species have been found to be infected by many *Taenia* species worldwide. For example, the red fox (*Vulpes vulpes*) was confirmed to be the host species of *T. crassiceps* (Rietschel, 1981), *T. serialis*, *T. hydatigena*, and *T. polycantha* (Ziadinov et al., 2010) in various areas of the Eurasian continent, and Karamon et al. (2018) further determined that the infection rate of the *Taenia* species in red foxes could be 42.5% in central Poland. Regarding the Tibetan plateau, limited information about the epidemiology of taeniid cestodes in wildlife has been reported. For example, Li et al. (2013) and Cui et al. (2015) reported that Tibetan foxes were infected with *T. pisiformis*, *T. crassiceps*, *T. multiceps,* and *T. taeniaeformis* (*Hydatigera taeniaeformis* suggested by Nakao et al., 2013a) based on adult worm morphological and molecular identification by hunted individuals post-mortem in Qinghai Province. As an endangered and widely distributed canid species in the Tibetan plateau (Wang and Xie, 2009), general studies addressing the parasitology of Tibetan foxes are usually hampered due to there being limited specimens for analysis.

Non-invading sampling techniques are more proper and effective methods to detect and surveille the prevalence of tapeworms in Tibetan fox populations. For example, molecular identification technologies based on copro-DNA have been used in studies of the *Echinococcus* spp. prevalence in a Tibetan fox population in the eastern Tibetan plateau (Jiang et al., 2011, 2012; Yang et al., 2014). Therefore, we tried to establish a general copro-DNA analytic protocol to detect infection of taeniid species in Tibetan foxes based on fecal samples collected in this field study. This approach may be helpful in understanding the under-researched epidemiology and the phylogeography of Taeniidae in wildlife in this region.

2. Materials and methods

2.1. Sample collection

We collected Tibetan fox fecal samples from July to August 2012 in Yongbo Valley (32°19′- 34°20′N, 97°20′-99°15′E; elevation, 4300 m), Shiqu County, Ganzi Autonomous Prefecture, Sichuan Province, China. According to the protocol by Jiang et al. (2012), fox feces were collected along line transects. Line transects were designed to start from the bank of the river running through the center of the valley and end at the top of hills on both sides of the valley. The distance between any two lines was more than 500 m to control repeated sampling feces from a same individual (Zuo et al., in composing). Thus, a total of >40 km long line transects were set to cover a 20 km^2 sampling area. Tibetan fox feces were firstly identified by their dark, small, slender shape, and full of small mammal hairs. Feces were stored in 50-ml centrifuge tubes with 75% ethanol separately, and for safety reasons were kept for at least 3 weeks at −80 °C. Fecal samples needed further tests for copro-DNA quality by checking their origins using Tibetan fox *mt*DNA analyses before further processing (see 2.2. for details). However, because of the dry and sunny climate in the plateau, fox feces were dried and decomposed quickly in the field. Thus, we classified the freshness of Tibetan fox feces into four levels: fresh, medium, dry, and old (decomposed) (Supplemental Fig. S1). Although *Echinococcus* DNA could be detected even in a level three (e.g., dry) fecal sample (Wang et al., unpublished data), Tibetan fox *mt*DNA can only be detected in fresh or medium level fecal samples. Therefore, only fresh and medium fox fecal samples were used in this study.

Adult worm samples of *E. multilocularis*, *E. shiquicus*, *E. granulosus*, *T. solium*, and *T. asiatica* were acquired from the Sichuan Center for Disease Control and Prevention, among which *E. multilocularis*, *E. shiquicus*, *E. granulosus* were collected from Shiqu County, and *T. solium* and *T. asiatica* were collected from Yajiang County, Ganzi Autonomous Prefecture, Sichuan Province. Larva samples of

T. polyacantha were acquired from the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, which were collected from a common vole (*Microtus arvalis*) in Xinyuan County, Yili Autonomous Prefecture, Xinjiang Uygur Autonomous Region, China.

2.2. Copro-PCR, cloning, and sequencing

2.2.1. Sample pretreatment and copro-DNA extraction

The pretreatment and copro-DNA extraction followed the protocol in Jiang et al. (2012). Briefly, dissolved and emulsified raw materials from three to four g of each fecal sample were divided evenly into sample A and sample B. Sample A was not treated with a procedure of mechanical disruption (PMD) of taeniid eggshells, while Sample B was. Then, copro-DNA in both Sample A and B would be extracted by QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany). Sample A would be used for host species (i.e., the Tibetan fox) *mt*DNA test (see 2.2.2. for details) and negative control tests for the presence of *Taenia* eggs (see 2.2.4. for details), while Sample B would be used to test taeniid species DNA directly (see 2.2.2.). Tissue DNA extraction from adult worm and larva samples was performed using the TIANamp Genomic DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions.

2.2.2. Copro-PCR

Before taeniid species detection, Sample A of each fecal sample was checked for the Tibetan fox origin using a corpo-PCR protocol developed by Jiang et al. (2011) to detect the Tibetan fox mitochondrial cytochrome *b* (*cytb*) gene. Briefly, copro-PCR products using a canid universal primer pair (L14724/H15149; Wayne et al., 1997) were further digested by restriction enzymes *Bam*HI and *Ssp*I for Tibetan fox DNA confirmation. This copro-PCR test also worked as a corpo-DNA quality control to exclude fecal samples with poor DNA quality or strong fecal negative conditions inhibiting copro-PCR amplification, both of which can strongly influence the evaluation of the molecular prevalence of taeniid species using Tibetan fox feces (Jiang et al., 2012). Therefore, only fecal samples confirmed with the existence of the Tibetan fox *cytb* gene will be used for taeniid species detection.

When detecting taeniid species, Sample B of each fecal sample was used. A total of three pairs of universal primers were used in this study. The first pair of Taeniidae universal primers CO1 JP (JP3/4.5) was selected according to Bowles et al. (1992) to amplify *c.*440 bp fragments of the mitochondrial cytochrome oxidase subunit 1 (*cox1*) gene in taeniid species. However, this primer pair is in fact able to amplify DNA from a wide scope of helminth worm species including the Taeniidae family. To restrict the process to Taeniidae species, we designed a new primer pair CO1 ZQ based on CO1 JP. In addition to *cox1* gene fragments, ND1 JP (JP11/12) (Gasser et al., 1999) were used to amplify a *c.* 500 bp region of the mitochondrial NADH dehydrogenase subunit 1 (*nad1*) gene. Moreover, four pairs of *Echinococcus* species-specific primers were used to double-check *Echinococcus* species detecting results by universal primer tests. Because *T. asiatica* has been detected in fecal samples by the universal primer pair COI ZQ (see Table 2), the species-specific primer pair PHDP2TSAF3 (González et al., 2004) was used to reconfirm the attendance of *T. asiatica*. All primer pairs information is listed in Table 1.

All amplifications were generated in a 50 μl reaction volume consisted of 25 μl of Premix Ex Taq (Takara Biotechnology, Dalian, China), 1 μl of bovine serum albumin (Takara), 1 μl of each primer pair, 20 μl of ddH$_2$O and 2 μl of sample DNA and were performed using a Bio-Rad DNA Engine PTC-200 instrument (Bio-Rad, Hercules, CA, USA). Amplification conditions were as follows: Initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s; annealing at 52°C–60 °C for 30 s (Table 1); extension at 72 °C for 45 s; and a final extension step of 72 °C for 10 min. Negative controls were included in each PCR run. All PCR products underwent electrophoresis in 1.5% agarose gels that stained with ethidium bromide (Biotium, Hayward,

Table 1
Primer information of target gene.

	Family/Species	Primer pair name	Gene	Original Primer code	Primer	Amplicon lengths	Annealing Temperature	Reference
Universal primers	Taeniidae	CO1 JP	*cox1*	JP3	TTTTTTGGGCATCCTGAGGTTTAT	444 bp	55 °C	Bowles et al. (1992)
				JP4.5	TAAAGAAAGAACATAATGAAAATG			
	Taeniidae	CO1 ZQ	*cox1*	ZQ1	TGATTAGTCATATATGTTTAAGAATAAG	339 bp	58 °C	Developed in this study
				ZQ2	AAACTTTATCCAATACACAAGC			
	Taeniidae	ND1 JP	*nad1*	JP11	AGATTCGTAAGGGGCCTAATA	500 bp	55 °C	Gasser et al. (1999)
				JP12	ACCACTAACTAATTCACTTTC			
Species specific primers	*E. multilocularis*	CO1 Em	*cox1*	Em1	GTCATATTTGTTTAAGTATAAGTGG	243 bp	52 °C	Nonaka et al. (2008)
				Em2	CACTCTTATTTACACTAGAATTAA			
	E. multilocularis	ND1 Em	*nad1*	EmF19/3	TAGTTGTTGATGAAGCTTGTTG	207 bp	53 °C	Boufana et al. (2013)
				EmR6/1	ATCAACCATGAAAACACATATACAAC			
	E.shiqucus	CO1 Es	*cox1*	Es1	GTTGGTTACGTTACCGGTT	420 bp	52 °C	Jiang et al. (2012)
				Es2	TCTTATTAACATTTGAATTCAAC			
	E.shiqucus	ND1 Es	*nad1*	EsF50	TTATTCTCAGTCTCGTAAGGGTCCG	442 bp	60 °C	Boufana et al. (2013)
				EsR73	CAATAACCAACTACATCAATAATT			
	T. asiatica	HDP2F1R1	*HDP2*	PTs7S35F1	CAGTGGCATAGCAGAGGA-GGAA	599bp	63 °C	Gonzaĺez et al. (2004)
				PTs7S35R1	GGACGAAGAATGGAGTTGAAGGT			
Universal primer	Canidae	Canidae	*cytb*	L14724	GATATG AAAAACCATCGTTG	478bp	55 °C	Wayne et al. (1997)
				H15149	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA			

CA, USA). Gels were developed in UV illumination using a GelDoc-IT™ imaging system (Alpha Innotech Ltd., Staffordshire, U.K.) and the successful amplifications were cut and purified using a TIANgel midi purification kit (Tiangen).

2.2.3. Cloning and sequencing

Target fragments contained in purified products were ligated into a pMD^{Tm}19-T vector using a pMD^{Tm}19-T vector cloning kit (Takara), introduced into *E.coli* DH5α competent cells (Takara) and then cultivated using A-X-I LB medium plates in 37 °C for 8−12h. The procedure followed the manufacturer's instructions. At least 20 positive colonies were chosen and sequenced for each plate. The sequencing products accomplished by Majorbio (Shanghai) were analyzed using Bio-edit v7.0.9.0 (Hall, 1999) and MEGA7 (Kumar, 2015), and compared with NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.4. Definitive host species confirmation

Other than the *Echinococcus* genus, little data about *Taenia* species in Tibetan foxes were available. Therefore, when copro-PCR detected *Taenia* DNA, to confirm if the Tibetan fox is a newly discovered viable definitive host species of this taeniid species, *Taenia* eggs in the fecal sample were collected according to a modified protocol from Ito (1980) and Mathis et al. (1996). To each target fecal sample, two to five g of feces were dissolved in a 50-ml centrifuge tube with dH2O, passed through a 100 μm sieve to remove large particles, and then centrifuged at 3600 g for 30 min. Sediments were kept and re-suspended by saturated sucrose solution. After centrifuged at 1000 g for 30 min, the supernatant was passed through a 20 μm mesh size sieve. If *Taenia* eggs (diameter approximately 31–43 μm) existed in the supernatant, they should be retained in the 20 μm sieve, and could be washed out with dH2O and checked out under a microscope. To determine which species these collected eggs belonging to, PMD of taeniid eggshells to release the oncosphere was used (Jiang et al., 2012). Oncosphere DNA was then extracted using the TIANamp Genomic DNA kit (Tiangen) according to the manufacturer's instructions. Then copro-PCR and molecular species identification were carried out using the method explained in section 2.2.1. and 2.2.2.

However, empirical studies suggested that the detecting efficiency of taeniid eggs in field collected fecal samples might be low. For instance, Nonaka et al. (2008) detected 30% of *T. taeniaeformis* eggs added in dog feces, and Jiang (2012) reported a 40%. Our previous tests suggested that the *Echinococcus* DNA detecting rate was only 13.3% (16/120 feces) without PMD, and 62% (74/120) with PMD (Jiang, 2012). Taeniid DNA in eggs would be kept longer than tissue materials in Tibetan fox feces by

the protection of eggshells. Therefore, disrupting eggshells and releasing egg DNA in fox feces can facilitate a significantly higher taeniid DNA detecting rate. Therefore, to each *Taenia* DNA detected fecal sample (i.e., Sample B with PMD), we used its Sample A (i.e., without PMD) to detect taeniid DNA again following the taeniid species detection procedure for the Sample B as explained in section 2.2.2. above. We supposed that if the fecal sample did contain taeniid eggs, the copro-PCR result of Sample A should be negative while positive in Sample B.

2.3. Phylogenetic analyses

Little data has been reported on *Taenia* species that infect wildlife in the Tibetan plateau (but see Li et al., 2013; Cut et al., 2015). Thus, if *Taenia* species *cox1* and *nad1* gene fragments were detected, maximum likelihood phylogenetic trees (ML trees) were constructed to evaluate the phylogenetic relationships with existing *Taenia* species. The *cox1* and *nad1* sequences downloaded from NCBI were aligned and edited with our sequences detected in fecal samples by MEGA 7 (Kumar, 2015). Then, *cox1* and *nad1* genotypes of taeniid species were calculated by DnaSP 6 (Librado and Rozas, 2009) to build ML trees using all the genotypes from the nine recognized *Echinococcus* species as outgroups. Modeltest v3.7 (Posada and Crandall, 1998) was used to test for the best-fit models of nucleotide substitution, and ML trees were built using MEGA 7. The robustness of all ML trees was calculated by bootstrapping with 1000 replicates. Detailed information about the sequences and genotypes used in the phylogenetic analyses is provided in Supplemental Table S1.

2.4. Statistical analysis

PCR amplification results using different primer pairs can be quite inconsistent because of different amplification efficiency, complexity of copro-DNA background conditions, and other unknown stochastic factors (Jiang et al., 2011, 2012). To evaluate the prevalence of a specific taeniid species based on the 36 fecal samples, we established a maximum prevalence by defining a positive sample as at least one positive PCR result from one pair of primers, and the conservative prevalence by at least two positive results from two pairs of primers (Wang et al., 2018). A 95% confidence interval (CI) was used for each prevalence. The $\chi2$ goodness of fit test was used to test whether the detection efficiency was statistically significantly different between primer pairs, especially between universal primer pairs and species-specific primer pairs. All statistics were computed using R 3.5.2 (http://www.r-project.org).

3. Results

A total of 63 fox feces samples were collected and 36 of them were detected with Tibetan fox *cytb* DNA thus used for further analyses.

3.1. Echinococcus species detection

Both *E. multilocularis* and *E. shiquicus* were detected in fecal samples by ND1 JP or CO1 ZQ universal primer pairs and further confirmed by species-specific primer pairs (Table 2). *Echinococcus multilocularis* DNA sequences were detected from 12 fecal samples, in which *cox1* and *nad1* sequences were 98.3%–98.8% and 98.1%–100% identical to the two referential *E. multilocularis* sequences (accession number: NH259774 and MH259778), respectively, from Qinghai, China (Table 2, Supplemental Table S2). The conservative prevalence was 22.2% ± 13.6% (±95% CI) and the maximum prevalence was 33.3% ± 15.4% (Supplemental Table S3). Regarding *E. shiquicus*, DNA in only three fecal samples was detected by the *cox1* primer pairs as 98.3%–99.5% identical to the referential sequence detected in the Tibetan Plateau of China. The conservative prevalence of *E. shiquicus* was 2.8 ± 5.4%, and the maximum prevalence was 8.3 ± 9.0% (Table S3). Mix infection of the two *Echinococcus* species was only detected in one fecal sample (F12056, Table 2).

3.2. Teania species detection

DNA sequences of a *Taenia* species were amplified in four fecal samples (Sample B) by CO1 ZQ and ND1 JP universal primer pairs. The *cox1* sequences amplified using CO1 ZQ showed 93.4% identical to the *T. polyacantha* larva sample and 94.0% identical to *T. polyacantha* from Cape Bathurst, Canada (accession number: EU544595), while the *nad1* sequences showed 89.0% identical to the larva sample and 89.3% to *T. polyacantha* from Lappeenranta, Finland (accession number: EU544637) (Table 2, Table S2). The conservative (5.6% ± 7.5%) and maximum (11.1% ± 10.3%) prevalence of this *T. polyacantha* like species were presented in Table S3. In addition, this *Taenia* species showed 98.8% in *cox1* (accession number: KM042890) and 99.1% in *nad1* (accession number: KM042889) identical to an unidentified *Taenia* specimen, *Taenia* sp. 2 YF-2014, which was collected from a Smokey vole (*Neodon fuscus*) in Jiuzhi County, Qinghai Province (Fan et al., 2014) (Table S2). Eggs of this *Taenia* species were not recovered in the four fecal samples, and its DNA was not detected the Sample A of the four fecal samples either.

Meanwhile, *cox1* DNA sequences amplified from two fecal samples by the CO1 ZQ primer pair were found to be 99.7% and 99.4% identical to a *T. asiatica* referential sequence (JQ517298) from Kanchanaburi, Thailand. Comparing with *T. asiatica* adult worm samples, the two fecal samples both showed 99.4% and 99.0%identical in *cox1* sequences

Table 2
The results of detecting Taeniidae spp. from Tibetan fox feces using DNA barcoding and specific-primers PCR methods.

Samples	Universal primers			Species specific primers			
	CO1 ZQ	ND1 JP	CO1 JP	CO1 Em	ND1 Em	CO1 Es	ND1 Es
F12001	\	\	*U.s.*(99.5%)	\	\	\	\
F12005	\	*E.m.* (99.1%)	*U.s.*(99.7%)	\	\	\	\
F12008	\	\	\	\	\	\	\
F12010	\	\	*U.s.*(99.7%)/*A.c.*(92.7%)	*E.m.*(97.7%)	\	\	\
F12014	\	\	\	\	\	\	\
F12017	\	\	\	\	\	\	\
F12019	\	\	*U.s.*(99.8%)*A.c.*(92.6%)/*P.m.*(91%)	\	\	\	\
F12020	\	\	*A.c.*(92.3%)/*U.s.*(99.5%)/*P.m.*(90.5%)	\	\	\	\
F12025	*E.s.*(98.3%)	\	\	\	\	*E.s.*(99.2%)	
F12027	\	\	\	\	\	\	\
F12035	*T.a.* (99.7%)	*Taenia.sp* (99.1%) *T.p.* (89.3%)	*A.c.*(92.6%)/*U.s.*(99.5%)	\		\	\
F12037	*Taenia.sp* (98.8%) *T.p.* (94.0%)	\		\	\	\	\
F12041	\	\	\	\	\	\	\
F12045	\	\	\	\	\	\	\
F12046	*E.m.* (98.3%)	*E.m.* (99.1%)	\	*E.m.* (98.6%)	*E.m.* (100.0%)	\	\
F12047	\	\	*U.s.*(97.0%)	\	\	\	\
F12048	*Taenia.sp* (98.8%) *T.p.* (94.0%)	*Taenia.sp* (99.1%) *T.p.* (89.3%)	\	\	\	\	\
F12052	\	\	\	\	\	\	\
F12055	*Taenia.sp* (98.8%) *T.p.* (94.0%)	*Taenia.sp* (99.1%) *T.p.* (89.3%)	\	\	\	\	\
F12056	*E.m.* (98.8%)	*E.m.* (98.1%)	*A.l.*(90.8%)	\	\	*E.s.* (98.9%)	\
F12057	\	\	\	\	\	*E.s.* (99.5%)	
F12058	*T.a.* (99.4%); *E.m.* (98.8%)		\	*E.m.* (95.8%)	*E.m.* (100.0%)		\
F12059	\	\	\	\	\	\	\
F12061	\	\	\	\	\	\	\
F12068	*E.m.* (98.3%)	\	\	*E.m.* (98.6%)	*E.m.* (100.0%)	\	\
F12069	\	\	\	\	\	\	\
F12071	*E.m.* (98.8%)	*E.m.* (99.5%)	\	*E.m* (98.1%)		\	\
F12077	*E.m.* (98.8%)	*E.m.* (99.2%)		\	\	\	\
F12078	*E.m.* (98.8%)	*E.m.* (100%)	\	\	\	\	\
F12079	\	*E.m.* (98.7%)	\	\	\	\	\
F12081	\	\	\	\	\	\	\
F12082	\	\	\	\	\	\	\
F12083	\	\	\	\	\	\	\
F12084	\	*E.m.* (99.1%)	\	\	\	\	\
F12104	*E.m.* (98.8%)	*E.m.* (99.0%)		*E.m.* (98.6%)	\	\	\
F12108	\	\	\	\	\	\	\

Abbreviations: T.*p.*; T. *polyacantha*; T.*a.*, T. *asiatica* strain, E.*m.*, E. *multilocularis*; E.*s.*, E. *shiqucus*; U.*s.*, *Uncinaria stenocephala*; P.*m.*, *Paranoplocephala macrocephala*; A.*c.*, *Ancylostoma ceylanicum*; *A.l. Anoplocephaloides lemmi*/.,Negative

(Table 2, Table S2). However, PCR using species-specific primers showed negative results in detecting *T. asiatica* in the two samples.

3.3. Phylogenetic analysis of Taenia species

To further confirm the existence of the *Taenia* species detected in this study, phylogenetic relationships of their *cox1* and *nad1* haplotypes with NCBI downloaded haplotypes were calculated and presented using ML trees. When building the ML tree for the *cox1* gene, one haplotype of *Taenia* species (*T. polyacantha*) and two of *T. asiatica* were aligned with 55 downloaded haplotypes of *Taenia* species, and a GTR + G substitution model was used. Regarding the *nad1* gene, one detected *Taenia* species (*T. polyacantha*) haplotype and 23 downloaded haplotypes of *Taenia* species were used to build the ML tree by setting a GTR + I + G substitution model. Both the *cox1* and *nad1* trees revealed that haplotypes of *Taenia species* (*T. polyacantha*) detected in this study and downloaded from NCBI belonged to the same *T. polyacantha* cluster supported by high bootstrap values (99 in the *cox1* tree and 100 in the *nad1* tree) (Fig. 1). Meanwhile, the two *T. asiatica cox1* haplotypes and downloaded *T. asiatica* haplotypes comprised one cluster with a bootstrap value of 93 (Fig. 1(a)). The details of the downloaded sequence information is

presented in Supplemental Table S1.

3.4. Discrepancy of primer pairs in detecting taeniid species

The results of different primer pairs were quite inconsistent in detecting taeniid species (Table 2). The universal primer pair CO1 JP failed to detect any taeniid species but detected many other non-taeniid helminthic species (Table 2), and thus had a significantly lower detection efficiency for taeniid species ($\chi2 = 15$, $P < 0.001$). The detection efficiency of the other universal and species-specific primer pairs were not statistically significantly different in detecting *E. multilocularis* ($\chi2 = 3.714$, $P = 0.294$), *E. shiquicus* ($\chi2 = 3.333$, $P = 0.343$), and *Taenia species* (*T. polyacantha*) ($\chi2 = 3$, $P = 0.223$), respectively.

4. Discussion

Echinococcus spp. are the main taeniid species detected in this study (Table 2). There are three *Echinococcus* species, *E. granulosus*, *E. multilocularis*, and *E. shiquicus* distributed in the Tibetan plateau (Craig et al., 2019). *Echinococccus granulosus* and *E. multilocularis*, two globally distributed taeniid species, cause a potentially lethal zoonosis,
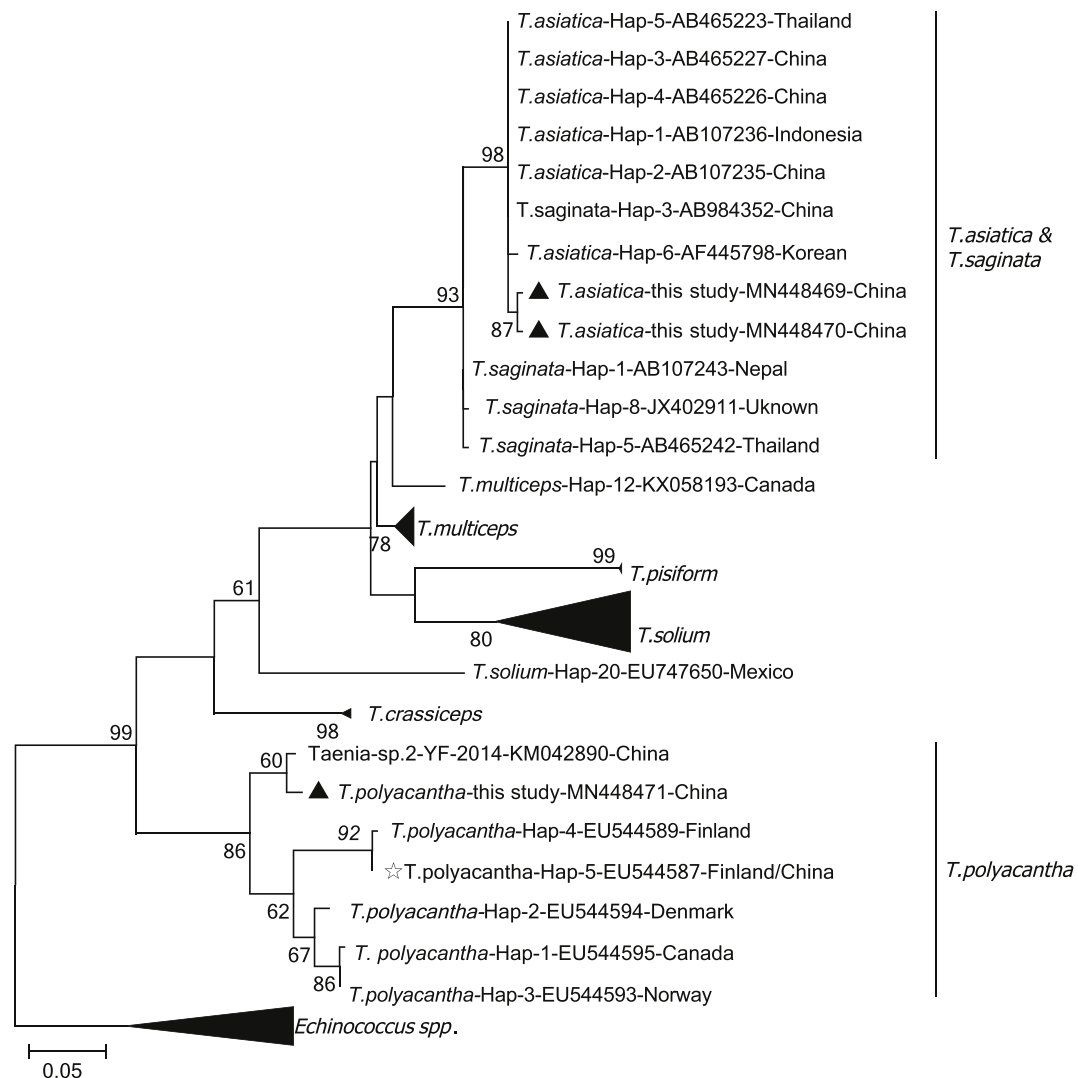

Fig. 1. Phylogram of *Taenia* species, which were found in China, especially in Tibetan Plateau, using the maximum likelihood method generated from partial *cox1* (a) and *nad1* (b) gene haplotypes. Isolates from Shiqu County were marked with ▲. Sequences from the referential *T. polyacantha* larva sample collected in Xinyuan County, Xinjiang Autonomous Region, China, shared the same haplotypes with published sequences from Finland marked with ☆. To each sequence, its accession number and geographic region were presented following the taeniid species name and haplotype number. Scale bar was represented in substitutions per nucleotide, and bootstrap values (with 1000 replicates) were marked at the nodes of branches.
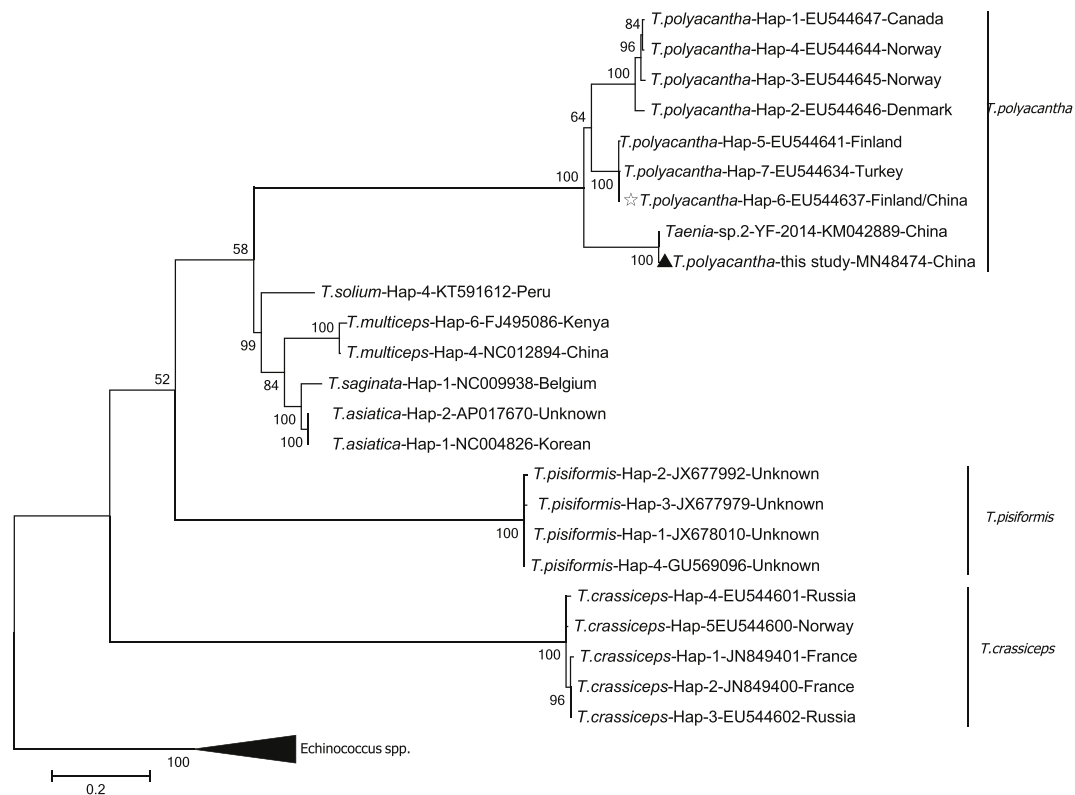
Fig. 1. (*continued*).

echinococcosis, threatening more than 50 million people in west China (Wang, 2016). The eastern Tibetan plateau area is one of the most serious endemic regions of the world (Li et al., 2005, 2010). *Echinococcus shiquicus* is a newly discovered *Echinococcus* species reported endemic to the eastern Tibetan plateau (Xiao et al., 2005; Craig et al., 2019). Although *E. granulosus* was confirmed mainly transmitting between cattle and dogs in this area, transmission of *E. multilocularis* and *E. shiquicus* involve complex wildlife cycles including small mammals (e. g., rodents and lagomorphs) as intermediate hosts and canids as definitive hosts. Tibetan foxes are the most abundant wild canid species in the Tibetan plateau (Schaller, 1998), and has been regarded as the main wildlife definitive host of *E. multilocularis* and *E. shiquicus* (Jiang et al., 2012; Craig et al., 2019). Thus, monitoring the *Echinococcus* spp. prevalence in Tibetan fox populations provides imperative knowledge of the zoonotic burden in the local wildlife reservoir, which is pivotal to the management and prevention of echinococcosis.

However, although the Tibetan fox is listed as least concern species by IUCN (Harris, 2014), regarding its population in China, habitat deterioration and high pressure of poaching are main threats for a long time. Consequently, the Tibetan fox has been listed as an endangered species by the Chinese Red list since 2005 (Wang and Xie, 2009), which resulted in the limitation on the application of the traditional hunting and post-mortem screening techniques (Qiu et al., 1995) for echinococcosis surveillance purposes. Meanwhile, as a wild canid species, it will always be challenging to catch enough fox individuals for epidemiological studies. Therefore, non-invasive sampling techniques like fox feces sampling and copro-DNA analysis are more convenient and feasible methods to epidemiological studies of taeniid cestodes in wild definitive host species.

Unlike the *Echinococcus* species, information about the *Taenia* infection in Tibetan foxes is limited. *Taenia polyacantha* has a wide distribution from the northern part of the Eurasia continent, from the UK to Japan (Ihama et al., 2000). Several canid species have been confirmed as definitive hosts including the red fox (*Vulpes vulpes*) (Al-Sabi et al.,

2014; Lavikainen, 2008), the arctic fox (*Vulpes lagopus*) (Stien et al., 2010), the gray wolf (*Canis lupus*) (Ćirović et al., 2015), the raccoon dog (*Nyctereutes procyonoides*) (Thiess et al., 2001), and the domestic dog (*C. familiaris*) (Umhang et al., 2014). Both *cox1* and *nad1* DNA fragments were amplified from Tibetan fox fecal samples (Table 2), and haplotypes of these amplicons (including the sequence from the larva sample from Yili, Xinjiang, China) were also grouped with published *T. polyacantha* sequences with high bootstrap values support in the ML phylogenetic trees (Fig. 1). Meanwhile, although eggs were not isolated from Tibetan fox fecal samples, the copro-PCR results of the four negative A samples and the four positive B samples suggested that *T. polyacantha* eggs were detected in the four Tibetan fox feces. The procedure of mechanical disruption (PMD) of taeniid eggshells is imperative in detecting DNA in taeniid eggs in Tibetan fox feces.

Rausch and Fay (1988a, b) recognized the existence of two subspecies of *T. polyacantha*, the temperate Eurasian subspecies *T. p. polyacantha* and the Holarctic tundra subspecies *T. p. arctica*. The three-division pattern under the *T. polyacantha* cluster in both *cox1* and *nad1* trees (Fig. 1) suggested that a more complex subspecies geographic distribution pattern of *T. polyacantha* may exist. Sequences of *cox1* and *nad1* from the larva sample collected in Xinyuan County, Xinjiang Autonomous region were highly identical with sequences from Turkey and UK (Table S2), and were grouped to the same genotypes with EU544587 (*cox1* tree, Fig. 1(a)) and EU544637 (*nad1* tree, Fig. 1(b)) respectively. The *Taenia-sp.*2-YF-2014 sequences (Table S2) were collected in Jiuzhi County, Qinghai Province (Fan et al., 2014), 275 km away from our sampling site in Shiqu County, Sichuan Province. Molecular data from our samples and Fan et al. (2014) established an eastern Tibetan plateau branch under the *T. polyacantha* cluster in both *cox1* (Fig. 1(a)) and *nad1* trees (Fig. 1(b)), and had larger differences to haplotypes in other branches of the species (Tables 2 and S2).

Taenia polyacantha is mainly transmitted between canids and small mammals (Boufana et al., 2012). *Taenia-sp.*2-YF-2014 samples were collected from the Smokey vole (*Lasiopodomis fuscus*) (Fan et al., 2014)

which is also abundant in our sampling site in Shiqu County (Wang et al., 2018). Therefore, similar to sylvatic transmission cycles of *E. multilocularis* and *E. shiquicus* following the food chain composed of Tibetan foxes and small mammals (mainly voles and pikas) (Jiang et al., 2012; Wang et al., 2018), a *T. polyacantha* transmission cycle may also be established between Tibetan foxes and voles. Meanwhile, the Tibetan fox has been confirmed as the definitive host species for *T. crassiceps*, *T. pisiformis*, *T. multiceps*, and *T. taeniaeformis* (*H. taeniaeformis*, as suggested by Nakao et al., 2013) in Qinghai Province, China (Li et al., 2013; Cui et al., 2015). Therefore, including our report of infecting *T. polyacantha*, Tibetan foxes are known to be the definitive host of at least seven taeniid species, including five *Taenia* species and two *Echinococcus* species in the Tibetan Plateau area.

In this study, DNA fragments of *T. asiatica cox1* gene were detected in two Tibetan fox fecal samples (Table 2). Our study area is located in western Sichuan Province where the three human *Taenia* species, *T. solium*, *T. saginata*, and *T. asiatica* (Hoberg, 2002), are co-endemic in local Tibetan communities (Li et al., 2006). Although the ML phylogenetic tree analysis revealed that *T. asiatica* sequences retrieved in this study grouped well with other *T. asiatica* sequences from western China (Fig. 1(a)), we failed to detect other mitochondrial and nuclear DNA markers using species-specific primers. Tibetan foxes can forage and defecate around villages (Vaniscotte et al., 2011). Therefore, *T.asiatica* sequences detection might be the result of Tibetan foxes' occasional feeding on feces from infected humans and defecating without truly infected, or was simply because of the short DNA fragment compared. No matter how, the *T.asiatica* instance indicated the importance of species-specific primers and multiple genes cross validation.

Combined with the noninvasive sampling technique, fecal DNA species identification techniques are becoming powerful tools in large-scale epidemiological studies of helminthic parasites, especially when screening wild and protected definitive host species. Species-specific DNA markers were developed and widely used in the identification of recognized species in the *Echinococcus* genus (Hüttner et al., 2009; Knapp et al., 2009; Jiang et al., 2012; Boufana et al., 2013). However, among the roughly 50 species in the *Taenia* genus (Nakao et al., 2013a), except for species specific methods developed for the three human *Taenia* species (Anantaphruti et al., 2007; González et al., 2004; Oka-moto et al., 2010; Li et al., 2017), DNA barcoding techniques using universal primers for specific gene fragments remain the main molecular species identification methodology (Lavikainen, 2014). However, the discrepancy of the detecting abilities of the same universal primer pairs in different scenarios must emphasized. For example, Galimberti et al. (2012) used the universal primer pair CO1 JP (JP3/4.5) (Bowles et al., 1992) with adult worm tissues to identify 16 *Taenia* species, while the same primer pair failed to detect any infection of *Taenia* species when copro-DNA was used (Table 2). Indeed, JP3/4.5 was designed based on *Fasciola hepatica* (Bowles et al., 1992) and thus has a wide detecting spectrum. The complex composition or the low quality of the Tibetan fox copro-DNA may limit the ability of CO1 JP (JP3/4.5) to detect *Taenia* species. To fix this issue, we designed a CO1 ZQ primer pair, which is exclusively focused on Taeniidae species. Nevertheless, the discrepancy-detecting results using CO1 ZQ and ND1 JP, respectively, hampered us from ascertaining the true infection burden of the *Taenia* species. Therefore, to make the copro-DNA species identification techniques more effective, efficient species-specific molecular markers to relevant the *Taenia* species should be developed.

Declaration of competing interest

The authors declared that they have no conflicts of interest to this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.06.008.

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