Tropical Biomedicine 37(1): 237–250 (2020)

Global genetic diversity of *Spirometra* tapeworms

Hong, X., Liu, S.N., Xu, F.F., Han, L.L., Jiang, P., Wang, Z.Q., Cui, J.* and Zhang, X.*
Department of Parasitology, School of Basic Medical Sciences, Zhengzhou University, 40 Daxue Road, Zhengzhou 450052, People's Republic of China
*Corresponding author e-mails: zhangxi@zzu.edu.cn (Zhang, X.), cuij@zzu.edu.cn (Cui, J.)
Received 17 April 2019; received in revised form 16 November 2019; accepted 20 November 2019

Abstract. *Spirometra* larvae are etiological agents of human sparganosis. However, the systematics of spirometrid cestodes has long been controversial. In order to determine the current knowledge on the evolution and genetic structure of *Spirometra*, an exhaustive population diversity analysis of spirometrid cestodes using the mitochondrial gene: cytochrome *c* oxidase subunit 1 (*cox*1) was performed. All publicly available *cox*1 sequences available in the GenBank and 127 new sequencing genes from China were used as the dataset. The haplotype identify, network, genetic differentiation and phylogenetic analysis were conducted successively. A total of 488 sequences from 20 host species, representing four spirometrid tapeworms (*S. decipiens*, *S. ranarum*, *S. erinaceieuropaei* and *Sparganum proliferum*) and several unclassified American and African isolates from 113 geographical locations in 17 countries, identified 45 haplotypes. The genetic analysis revealed that there are four clades of spirometrid cestodes: Clade 1 (Brazil + USA) and Clade 2 (Argentina + Venezuela) included isolates from America, Clade 3 contained African isolates and one Korean sample, and the remainders from Asia and Australia belonged to Clade 4; unclassified *Spirometra* from America and Africa should be considered the separate species within the genus; and the taxonomy of two Korea isolates (*S. erinaceieuropaei* KJ599680 and *S. decipiens* KJ599679) was still ambiguous and needs to be further identified. In addition, the demographical analyses supported population expansion for the total spirometrid population. In summary, four lineages were found in the spirometrid tapeworm, and further investigation with deeper sampling is needed to elucidate the population structure.

INTRODUCTION

The *Spirometra* tapeworm belongs to the family Diphyllobothriidae and order Diphyllobothriidea. Adults live in the intestine of felids and canids, however larvae (plerocercoids or spargana) parasitize in body cavity and tissues of all tetrapode groups (Okamoto *et al*., 2007). In humans, the plerocercoid (sparganum) invades mainly subcutaneous tissues, but also eyes, brain, and central nervous system, causing a serious parasitic zoonosis known as sparganosis (Cui *et al*., 2011, 2017; Liu *et al*., 2015). Although with medical importance, the identification and taxonomy of *Spirometra* species has long been controversial (Faust *et al*., 1929; Mueller, 1937; Okamoto *et al*., 2007; Jeon *et al*., 2016a, 2018a; Zhang *et al*., 2017). The most recent review concluded only 4 valid species of the genus (Kuchta and Scholz, 2017), however, many more nominal *Spirometra* species have been described. In addition, the sparganum isolates from America (Argentina, Brazil and USA) and Africa (Ethiopia and South Sudan) were still unclassified (Eberhard *et al*., 2015; Almeida *et al*., 2016; Waeschenbach *et al*., 2017). Therefore, there is a pressing requirement to investigate the genetic diversity and phylogenetic relationship among the *Spirometra* species in order to establish the taxonomy of spirometrid tapeworm.

Generally, species identification of *Spirometra* tapeworm is based on the morphology of adult worms, however the typical specimens obtained in practice were *Spirometra* larval forms. It is hard to

distinguish the morphological differences in the plerocercoids among spirometrid tapeworms. Therefore, more species identification has preferred DNA borcoding methods. Among all genetic markers that have been applied in the molecular studies of *Spirometra*, the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1) has been the most commonly used. This gene has been widely used for the species identification (Lee *et al*., 1997; Miyadera *et al*., 2001; Liu *et al*., 2010; Simpson *et al*., 2012; Boonyasiri *et al*., 2013, 2014; Jongthawin *et al*., 2014; Jeon *et al*., 2015, 2016a, 2018a; Holldorf *et al*., 2015; Petrigh *et al*., 2015; Thanchomnang *et al*., 2016; Tang *et al*., 2017), genetic variation (Okamoto *et al*., 2007; Zhang *et al*., 2014, 2015a; Jeon *et al*., 2016b, 2018b) and phylogeny (Lee *et al*., 2007; Dai *et al*., 2012; Wei *et al*., 2015; Zhang *et al*., 2015b, 2016).

To date, 366 sequences of the *cox*1 gene of spirometrid tapeworms have been deposited in the GenBank database, which represent the isolates collected from different hosts and originated from more than 84 geographical locations in 17 countries. These publicly available datasets offer good opportunities to compare the intra- and interspecific diversity of *Spirometra* tapeworms. In this study, we intend to perform an exhaustive genetic diversity analysis of spirometrid cestodes using these available datasets, as well as with 127 newly added sequences of spargana isolated from wild frogs in 29 geographical locations in mainland China.

MATERIALS AND METHODS

Sequencing the *cox*1 gene of Chinese isolates

Wild frogs were collected from different locations in China from May 2014 until September 2018. The presence of spargana was examined as the methods described by Wei *et al*. (2015). In total, 127 spargana were collected from 29 geographical locations (Table 1). Genomic DNA was extracted from individual plerocercoids using the EasyPure Genomic DNA Kit (Transgen, China) according to the manufacturer's instructions. The *cox*1 gene was amplified by PCR using the primers designed by Yanagida *et al*. (2010). The PCR products were purified using the EasyPure PCR Purification Kit (Transgen, China) and sequenced in both directions at the Genwiz Company (Suzhou, China). All sequences were deposited in the GenBank database under accession numbers MK085765-K085891.

Genetic diversity analysis

To perform a worldwide genetic variable comparative analysis of *Spirometra* tapeworms, all available sequences of *cox*1 in the GenBank database were included in this study (Table 1). More specifically, 366 sequenced *cox*1 genes of spirometrid tapeworms have been published, representing five species and several unclassified species: *S. decipiens* (n=1), *S. ranarum* (n=1), *S. erinaceieuropaei* (n=349), *Sparganum proliferum* (n=1) and *Spirometra* sp. (n=14). For *S. erinaceieuropaei*, 11 sequences were from Australia, three from Indonesia, one from India, 12 from Thailand, 16 from Laos, one from Myanmar, three from Korea, seven from Japan, one from Vietnam, one from Iran and 293 from mainland China. Two diphyllobothriid tapeworms, *Diphyllobothrium scoticum* (KY552883) and *Adenocephalus pacificus* (KY552867) were used as outgroups according to the report by Waeschenbach *et al*. (2017).

The alignment of *cox*1 sequences was performed in MEGA v.6.06 (Tamura *et al*., 2013) with the default settings. The variable sites, nucleotide compositions and pairwise distances were also estimated in MEGA v.6.06. The number of haplotypes, haplotype diversity (Hd), and nucleotide diversity (Pi) were analysed with DnaSP v.6 (Rozas *et al*., 2017). The software Network v.5.0 (Bandelt *et al*., 1999) was employed to draw a median-joining network to explore the relationships among the detected haplotypes. To explore the levels of genetic differentiation among the geographical populations, the pairwise F_{ST} values between populations were calculated by using Arlequin v.3.5 (Excoffier *et al*., 2010).

Phylogenetic analysis

The phylogenetic pattern of all *cox*1 haplotypes was estimated through two methods: maximum likelihood (ML) and Bayesian inference (BI). The sequence evolution model was selected by jModelTest v0.2 (Darriba *et al.*, 2012) under the Akaike information criterion (AIC). The ML analysis was performed in MEGA v.6.06. Confidence in each node was assessed by boot-strapping (1000 pseudo-replicates). BI was performed in MrBayes v.3.2 (Ronquist *et al.*, 2012). The analysis consisted of two runs, each with four MCMC chains running for 5 000 000 generations, and sampling every 100th generation. The neutrality tests using Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997) were also applied through Arlequin v.3.5 as an assessment of possible population expansion. In addition, to estimate changes in population size over time, and the time to the most recent common ancestor (tMRCA), a Bayesian Skyline Plot analysis (BSP) implemented in BEAST v1.8.2 (Drummond *et al.*, 2012) was performed. Using the piecewise-constant skyline model, the molecular evolutionary rate of *cox*1 was fixed at 0.0225 substitutions per site per million years ago (Mya) as described previously (Michelet *et al.*, 2010).

RESULTS

Haplotypic variability

Six sequences (AF096239, KT375455, KT375456, KF656743, KF656744 and KP738256) were excluded due to ambiguous alignment. The final alignment contained 487 sequences representing one *Sparganum proliferum*, one *S. decipiens*, one *S. ranarum*, 12 unclassified *Spirometra* and 473 *S. erinaceieuropaei*. A total of 45 haplotypes (Haps) were identified from these 488 sequences (Table 1). Among the haplotypes, *Sparganum proliferum* originated from Venezuela (AB015753), *S. decipiens* from Korea (KJ599679), three unclassified isolates from Argentina (KF572950), Ethiopia (KM248530), and the USA (KY552892) were identified as separate Haps; two unclassified Brazil isolates (KF740506 and KF740507) and six isolates from South Sudan were identified 2 Haps; and the remaining *S. erinaceieuropaei* isolates and a *S. ranarum* sample constituted another 36 Haps. All samples had high haplotype diversity (0.652±0.023) accompanied by low nucleotide diversity (0.01619±0.00156). The median-joining network analysis showed that Hap12 was the most prominent haplotype represent 55.3% of all sequences and originated from China, Thailand, Japan, Australia, Korea and Iran (Fig. 1). Hap2 was shared by isolates from China, Thailand and Myanmar. Hap13 was shared by isolates from China, Australia, Japan and Indonesia. Hap14 was shared by isolates from China and Indonesia. The Chinese isolates (82.9% of the sequences represented China samples) showed the most diversity of haplotypes (24 Haps). Samples from Japan and Korea shared 5 (Haps12-13, Haps19-21) and 4 (Hap3, Hap12, Haps17-18) Haps, respectively. Isolates from Thailand (Hap2, Hap12 and Hap25), Indonesia (Haps13-15) and Laos (Haps22-24) revealed 3 Haps. Worms from Australia (Haps12-13), Brazil (Haps6-7) and South Sudan (Happs9-10) possessed 2 Haps. The species from Venezuela (Hap1), Argentina (Hap5), Ethiopia (Hap8), the USA (Hap11), India (Hap16) and Vietnam (Hap45) were identified as separate single haplotype. The pairwise *F*st values between isolates from different countries were calculated to estimate levels of genetic differentiation (Table 2). Most of the *F*st values between American countries (Brazil, Argentina, Venezuela and USA) and Asian countries + Australia, American and African countries, African countries and Asian countries + Australia were very high (above 0.5), although with little statistically significance ($p < 0.05$).

Phylogeny

The model test suggested that the HKY+G model was most suitable for the *cox*1 sequences. Both maximum likelihood and Bayesian inference analyses revealed four clades: Clades 1–4 (Fig. 2). The earliest divergence gave rise to isolates from Brazil and the USA (Clade 1) and then to a sample from Argentina and a sample from Venezuela

Table 1. *Spirometra* tapeworms and the corresponding *cox*1 sequences used in this study. Asterisks indicate sequences newly reported in this study

Taxa	Country of origin	Host	*Cox*1 haplotype	No. of seqs	GenBank No.	Reference
Sparganum proliferum	Venezuela	*Homo sapiens*	Hap1	1	AB015753	Miyadera *et al.* 2001
Spirometra sp.	Argentina	*Lycalopex gymnocercus*	Hap5	1	KF572950	Petrigh *et al.* 2015
	Brazil	*Leopardus pardalis*	Hap6	1	KF740506	Almeida *et al.* 2016
		L. pardalis	Hap7	1	KF740507	Almeida *et al.* 2016
	Ethiopia	*Homo sapiens*	Hap8	1	KM248530	Eberhard *et al.* 2015
	South Sudan		Haps9-10	6	KM248531-KM248536	Eberhard *et al.* 2015
	USA	*Pantherophis obsoletus*	Hap11	1	KY552892	Waeschenbach *et al.* 2017
	China	N/a	Hap4	1	HQ699076	Direct submission
S. decipiens	Korea	*Rhabdophis tigrinus*	Hap3	1	KJ599679	Eom *et al.* 2015
S. ranarum	Myanmar	*Hoplobatrachus rugulosus*	Hap2	1	MH298843	Jeon *et al.* 2018a
S. erinaceieuropaei	Australia	*Felis silvestris*	Hap13	1	AJ308261	Okamoto *et al.* 2007
		Litoria caerulea	Hap12	2	AJ308264-AJ308265	Okamoto *et al.* 2007
		Python bivittatus	Hap13	1	AJ308263	Okamoto *et al.* 2007
		Tiger snake	Hap13	1	AJ308262	Okamoto *et al.* 2007
		Vulpes velox	Hap13	2	AJ308259-AJ308260	Okamoto *et al.* 2007
		Canis familiaris	Hap13	2	AJ308257-AJ308258	Okamoto *et al.* 2007
		C. familiaris	Hap13	1	JN367266	Simpson *et al.* 2012
		C. familiaris	Hap12	1	KY552886	Waeschenbach *et al.* 2017
	Vietnam	*Xenochrophis flavipunctatus*	Hap45	1	KY552887	Waeschenbach *et al.* 2017
	Indonesia	*C. familiaris*	Haps13-15	3	AB278575-AB278577	Okamoto *et al.* 2007
	India	*C. familiaris*	Hap16	1	AB278574	Okamoto *et al.* 2007
	Thailand	*H. sapiens*	Hap12	9	KF539833-KF539841	Boonyasiri *et al.* 2014
			Hap25	1	KC551943	Boonyasiri *et al.* 2013
		Ptyas korros	Hap2	2	KM099139-KM099140	Jongthawin *et al.* 2014

Table 1 continued...

Laos	*Ptyas korros*	Haps22-24	16	KM099122-KM099137	Jongthawin *et al.* 2014
Myanmar	*Hoplobatrachus rugulosus*	Hap2	1	KM099138	Jongthawin *et al.* 2014
Korea	*H. sapiens*	Hap17	1	KJ599680	Eom *et al.* 2015
	N/a	Hap18	1	AF096237	Lee *et al.* 2007
		Hap12	1	AF096238	Lee *et al.* 2007
Japan	*Elaphe climacophora*	Hap19	1	AB015754	Miyadera *et al.* 2001
	Elaphe quadrivirgata	Hap12	1	AB369249	Yamasaki *et al.* 2007
	H. sapiens	Hap20	1	AB369251	Yamasaki *et al.* 2007
		Hap21	1	AB369250	Yamasaki *et al.* 2007
		Hap13	1	AB480297	Abe *et al.* 2009
	E. quadrivirgata	Hap12	1	AB522603	Okino *et al.* 2017
	Felis catus	Hap12	1	AB278573	Okamoto *et al.* 2007
Iran	*F. catus*	Hap12	1	KY009916	Badri *et al.* 2017
China	*F. catus*	Hap12	2	KC561784, KF988137	Direct submission
	C. familiaris	Hap14	17	GU576892-GU576908	Direct submission
	Pelophylax nigromaculatus	Haps2, 4	22	GQ866863-GQ866884	Direct submission
	C. familiaris	Haps28-31	11	FJ886763-FJ886773	Dai *et al.* 2012
	P. nigromaculatus	Haps4, 12, 14, 26-27	12	GQ999946-GQ999957	Liu *et al.* 2010
	Rana rugulosa	Hap12	12	KF656735-KF656746	Wei *et al.* 2015
	Rana temporaria	Haps2, 12	16	KF745143-KF745158	Zhang *et al.* 2014
		Haps2, 12, 13, 32-38	88	KM605255-KM605342	Zhang *et al.* 2015a
		Haps12, 39,	61	KP738228-KP738288	Zhang *et al.* 2015b
		Haps2, 4, 12, 14, 33, 40-42	52	KT376494-KT376545	Zhang *et al.* 2016
		Haps2, 4, 12-14, 32, 39, 43, 44	127	**MK085765*-MK085891***	This study

Table 2. Genetic differentiation (*F*st) values among *Spirometra* isolates. Significant comparisons ($P < 0.05$) are in bold

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 Brazil	0.000																
2 Argentina	0.672	0.000															
3 USA	0.511	1.000	0.000														
4 Venezuela	0.669	1.000	1.000	0.000													
5 South Sudan	**0.935**	0.993	0.994	0.992	0.000												
6 Ethiopia	0.658	1.000	1.000	1.000	0.895	0.000											
7 China	**0.916**	**0.898**	**0.921**	**0.869**	**0.871**	**0.874**	0.000										
8 Korea	0.562	0.353	0.510	0.248	**0.676**	0.252	0.406	0.000									
9 Myanmar	0.824	1.000	1.000	1.000	**0.992**	1.00	0.241	0.020	0.000								
10 Thailand	**0.877**	0.874	0.903	0.841	**0.885**	0.847	0.001	0.177	0.261	0.000							
11 Japan	**0.846**	0.849	0.883	0.814	**0.890**	0.819	**0.124**	0.126	0.082	**0.107**	0.000						
12 Australia	**0.911**	0.924	0.940	0.903	**0.930**	0.907	**0.256**	**0.333**	0.151	**0.311**	0.059	0.000					
13 Indonesia	0.837	0.915	0.931	0.891	**0.960**	0.896	**0.369**	0.113	0.077	**0.377**	0.080	0.013	0.000				
14 India	0.654	1.000	1.000	1.000	0.991	1.000	0.217	0.487	1.000	0.179	0.132	0.147	0.091	0.000			
15 Laos	0.940	0.950	0.959	0.937	**0.949**	0.940	**0.545**	**0.568**	**0.587**	**0.625**	**0.420**	**0.413**	**0.325**	0.579	0.000		
16 Vietnam	0.651	1.000	1.000	1.000	0.990	1.000	0.121	0.558	1.000	0.084	0.159	0.086	0.091	1.000	0.597	0.000	
17 Iran	0.636	1.000	1.000	1.000	0.990	1.000	0.544	0.960	1.000	0.832	0.281	0.400	0.520	1.000	0.765	1.000	0.000

Table 3. Neutrality tests results of spirometrid tapeworms. Significant level = 0.05. Number in parentheses is *P* value

Phylogroups	Neutrality Tests	
	Fu's F_S	Tajima's *D*
Total population	-5.40180 (0.17)	-1.89942 (0.00)

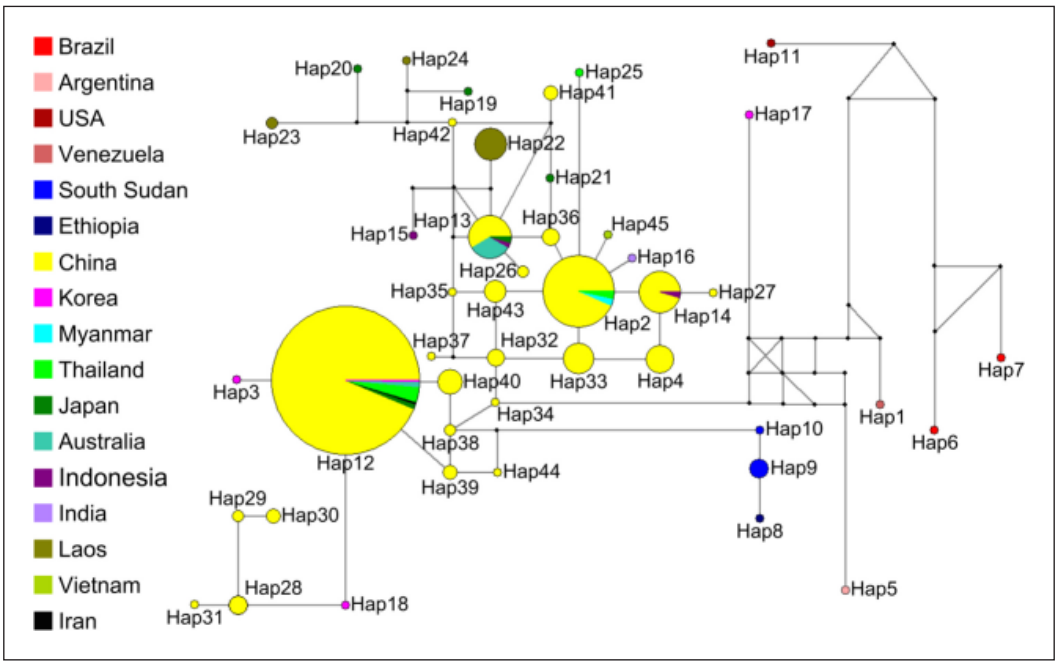

Figure 1. Median-joining network of haplotypes of spirometrid tapeworms. Each haplotype is represented by a circle, with the area of the circle proportional to its frequency. The median vector is indicated by a solid black circle.

(Clade 2), next to isolates from Africa (South Sudan and Ethiopia) and a sample from Korea (KJ599680) (Clade 3). The last divergence event separated isolates from Asia and Australia (Clade 4). However, within the cluster of Clade 4, a sample from Korea (KJ599679) and a sample from Myanmar (MH298843) were classified as *S. decipiens* and *S. ranarum* in the GenBank database respectively. Neutrality tests based on Tajima's *D* and Fu's F_S for the spirometrid tapeworms showed negative values, supporting a possible population expansion (Table 3). The result of Bayesian Skyline Plot analysis also supports a sudden population expansion for the total population: a sudden expansion was identified between 0.25–0.75 Mya by BSP (Fig. 3).

DISCUSSION

The systematics of *Spirometra* and the taxonomic position of species within it remain controversial (Faust *et al.*, 1929; Mueller, 1937; Okamoto *et al.*, 2007; Kuchta *et al.*, 2008; Zhang *et al.*, 2019). Molecular markers, especially the *cox*1, have played an important role in the studies on modern parasite identification, genetic variation and evolution over the past decade (Miyadera *et al.*, 2001; Okamoto *et al.*, 2007; Dai *et al.*,

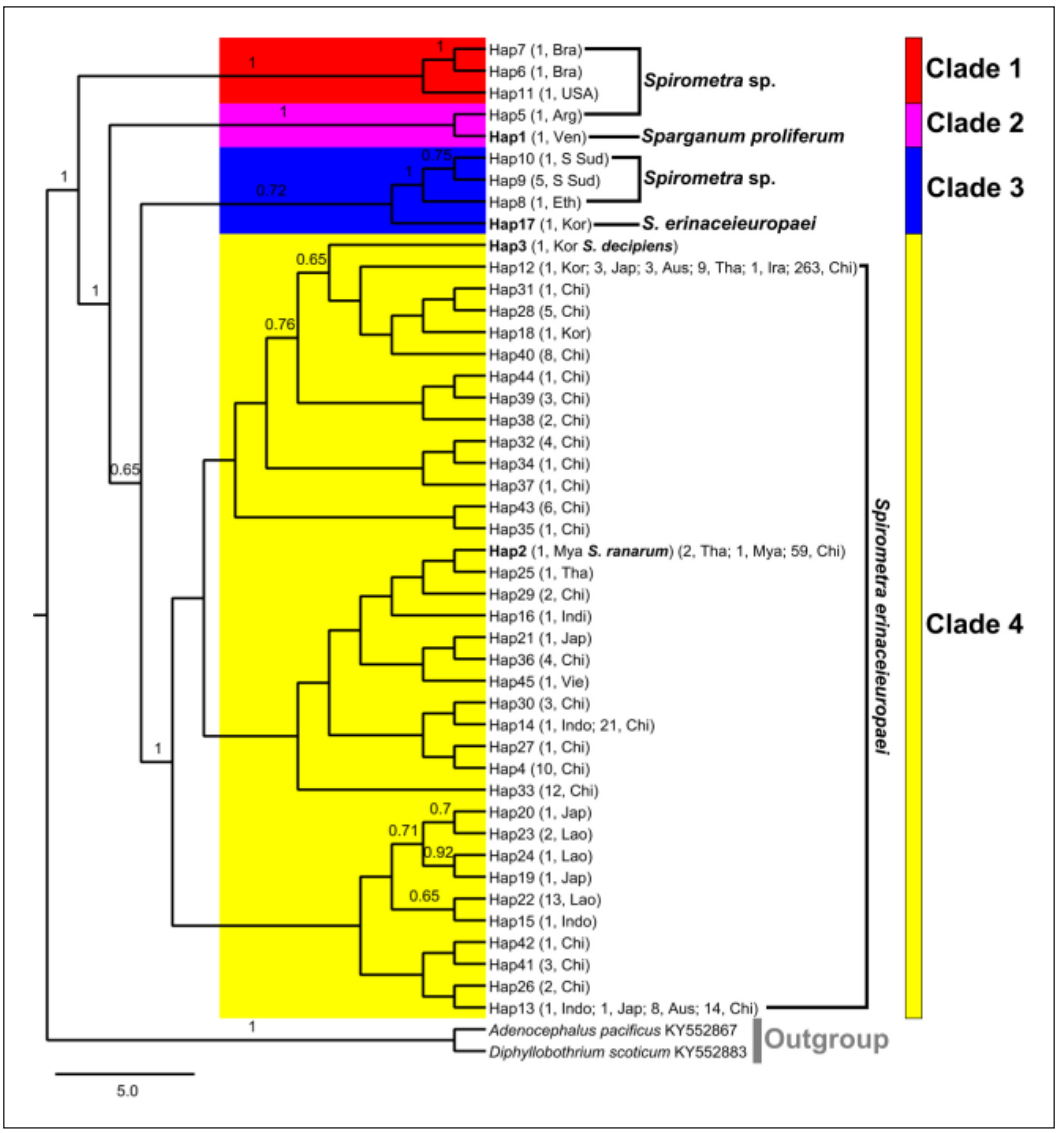

Figure 2. Bayesian phylogenetic tree of spirometrid tapeworms based on the data set of *cox*1. The numbers along branches indicate posterior probabilities and only posterior probabilities above 0.6 are shown. The number in the parenthesis indicates the sampling size of each haplotype. The abbreviations of countries are designated as follows: Bra, Brazil; Arg, Argentina; Ven, Venezuela; S Sud, South Sudan; Eth, Ethiopia; Kor. Korea; Jap, Japan; Ira, Iran; Tha, Thailand; Indi, India; Aus, Australia; Vie, Vietnam; Indo, Indonesia; Mya, Myanmar; Lao, Laos; Chi, China.

2012; Boonyasiri *et al.*, 2014; Eom *et al.*, 2015; Almeida *et al.*, 2016; Zhang *et al.*, 2018). Here, we performed the first comparative analysis of spirometrid cestodes from worldwide using all available *cox*1 sequences deposited in the GenBank as well as with 127 newly added sequences of spargana isolated from 29 geographical locations in mainland China.

A total of 488 sequences representing four species of spirometrid cestodes (*Sparganum proliferum*, *S. decipiens*, *S. ranarum*, and *S. erinaceieuropaei*) and several unclassified *Spirometra* from American and African identified 45 haplotypes. *Sparganum proliferum*, whose adult stage is still unknown, has been

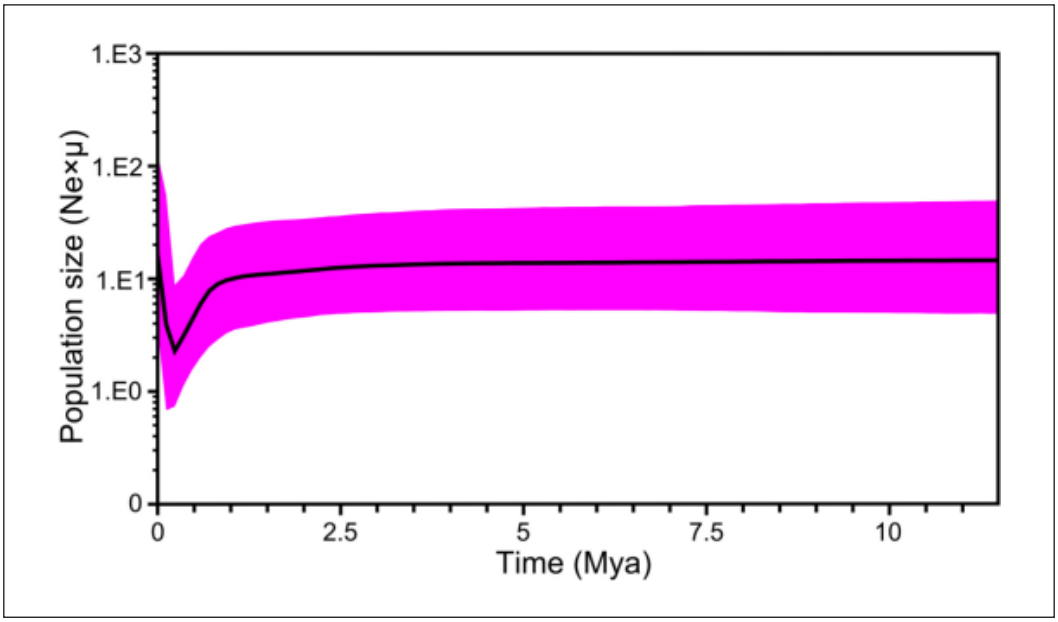

Figure 3. Estimated demographic expansion of the spirometrid tapeworm population. A Bayesian skyline plot derived from the data set of *cox*1. The X-axis is in units of million years in the past, and the Y-axis is Ne × μ (effective population size × mutation rate per site per generation). The median estimates are shown as thick solid lines, and the purple areas show the 95% HPD limits.

identified as a diphyllobothriidean cestode using *cox*1 and nuclear *sdh*B genes (Miyadera *et al.*, 2001). In this study, *S. proliferum* was identified as a separate Hap, indicating that this organism should be a valid species. However, the phylogenetic analysis showed that *S. proliferum* is a sister lineage to the Argentina isolate. Plerocercoid larvae collected from snake (*Rhabdophis tigrinus*) were identified as *S. decipiens* (KJ599679) in Eom *et al.* (2015). Although the *S. decipiens* (KJ599679) was revealed as a separate Hap, this species was inserted into a group containing mainly Asia and Australia *S. erinaceieuropaei* isolates in the phylogenetic tree, suggesting the ambiguous taxonomy of *S. decipiens*. The *S. ranarum* from Myanmar and isolates of *S. erinaceieuropaei* from Thailand and different locations in China were identified as a single haplotype (Hap2), confirming that *S. ranarum* is a synonym of *S. erinaceieuropaei*. However, Jeon *et al.* (2018a) confirmed a *Spirometra* species of Myanmar origin as *S. ranarum* based on *cox*1 and *nad*1 genes as well as morphological observations of an adult tapeworm. For the unclassified *Spirometra*, four isoaltes from America, one from Argentina, two from Brazil and one from the USA, were revealed as four separate Haps, and seven samples from Africa (South Sudan 6, Ethiopia 1) showed 3 Haps, indicating their specific taxonomy within the genus. The remaining sequences of *S. erinaceieuropaei* from 11 countries (China, Japan, Korea, Myanmar, Thailand, Indonesia, Laos, Vietnam, Iran, India and Australia) constituted 37 additional Haps. The haplotype diversity was less than that with smaller sampling of *S. erinaceieuropaei* in China (Zhang *et al.*, 2015a, 2016). A possible reason might be that the molecular marker used here is only a small portion of the *cox*1 (369 bp), and the markers for Chinese samples in previous studies were the concatenated complete genes of *cox*1 (1566 bp) and *cyt*b (1110 bp) (Zhang *et al.*, 2015a, 2015b, 2016).

The phylogenetic analyses supported the existence of four lineages among spirometrid tapeworms. America isolates belong to two clades: Clade 1 and Clade 2. Interestingly, in comparison with the Argentina isolate, the

USA isolate has a closer relationship with the Brazil isolates. In addition, sibling relationships were revealed between the Argentina isolate and *S. proliferum*. The third lineage only included African isolates, with the exception of a Korea isolate (KJ599680), indicating the specific phylogenetic position of the African *Spiromtra* species. In consideration of the above genetic analyses, the unclassified *Spirometra* from America and Africa should be separate species within the genus which could be due to high *F*st values between the different geographical isolates, identified as individual haplotypes and distinct phylogenetic patterns. Nevertheless, the precise taxonomy of the unclassified isolates requires further identification with more robust evidence in the future.

A Korean isolate (KJ599680), which has been classified as *S. erinaceieuropaei* (Eom *et al*., 2015), was identified as member of Clade 3 in the genetic analysis. The phylogenetic tree topology also indicated that this species had a close relationship with the African isolates. In addition, our previous mitogenomic comparative analysis of *Spirometra* also showed that the KJ599680 was distinct from other *S. erinaceieuropaei* mitogenomic sequences from China and Japan (Zhang *et al*., 2017). Therefore, the taxonomy of the Korea isolate (KJ599680) was probably imprecisely identified in GenBank and should be considered as an independent species within the genus. Although *S. decipiens* (KJ599679) was identified as a separate haplotype, its close relationship with most of the *S. erinaceieuropaei* isolates (with exception of KJ599680) has been firmly supported in the phylogenetic analysis, indicating that it is hard to distinguish *S. decipiens* from *S. erinaceieuropaei* using only the *cox*1 gene. For *S. erinaceieuropaei*, although high haplotype diversity has been found, no obvious phylogenetic patterns were revealed among isolates from different hosts and different geographical localities. Considering that the distribution of *Spirometra* species is cosmopolitan, the true pattern of the genetic structure of spirometrid tapeworms needs further investigation with deeper sampling, especially with samples from America, Africa and other scarce species, in the future.

CONCLUSION

In this study, all publicly available *cox*1 sequences in GenBank and 127 newly added sequences from China were used to explore the genetic diversity analysis of spirometrid cestodes worldwide. A total of 488 sequences of spirometrid tapeworms from 113 geographical locations with different hosts in 17 countries identified 45 haplotypes. The network, genetic differentiation and phylogenetic analyses revealed that (1) there are four clades of spirometrid cestodes: Clade 1 included isolates from Brazil and USA, Clade 2 included isolates from Argentina and Venezuela, Clade 3 contained African isolates and one Korean sample, Clade 4 contain the remaining samples from Asia and Australia; (2) unclassified *Spirometra* from America and Africa should be considered individual valid species within the genus; (3) The taxonomy of two Korean isolates (*S. erinaceieuropaei* KJ599680 and *S. decipiens* KJ599679) was still ambiguous and need to be further identified. In addition, the demographical analyses supported population expansion for all spirometrid tapeworms.

Acknowledgements. We thank Mr. QW Wu, DJ Zeng, CH Du, JW Rao, Y Chen, YH Lin and HH Huang, Ms. M Gao, HQ Li, M Peng, SZ Wang, F Wu, SS Wang, M Tao and SQ Yi for collecting valuable specimens used in this study. This work was supported by National Natural Science Foundation of China (U1704189), China Postdoctoral Science Foundation (2018T110740), Henan Province Science and Technology Key Project (182102310075) and Backbone Teachers Development Program in Henan Province (2017GGJS011).

REFERENCES

Abe, N., Kimata, I. & Uni, S. (2009). Identification by genetic examination of three cestodes isolated from patients: *Diphyllobothrium nihonkaiense*, larval *Spirometra erinaceieuropaei* and *Taenia saginata*. *Seikatsu Eisei* **53**: 169-176.

Almeida, G.G., Coscarelli, D., Melo, M.N., Melo, A.L. & Pinto, H.A. (2016). Molecular identification of *Spirometra* spp. (Cestoda: Diphyllobothriidae) in some wild animals from Brazil. *Parasitology International* **65**: 428-431.

Badri, M., Eslahi, A.V., Majidiani, H. & Pirestani, M. (2017). *Spirometra erinaceieuropaei* in a wildcat (*Felis silvestris*) in Iran. *Veterinary Parasitology*: Reg Stud Rep **10**: 58-61.

Bandelt, H.J., Forster, P. & Rohl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37-48.

Boonyasiri, A., Cheunsuchon, P., Srirabheebhat, P., Yamasaki, H., Maleewong, W. & Intapan, P.M. (2013). Sparganosis presenting as cauda equina syndrome with molecular identification of the parasite in tissue sections. *Korean Journal of Parasitology* **51**: 739-742.

Boonyasiri, A., Cheunsuchon, P., Suputtamongkol, Y., Yamasaki, H., Sanpool, O., Maleewong, W. & Intapan, P.M. (2014). Nine human sparganosis cases in Thailand with molecular identification of causative parasite species. *American Journal of Tropical Medicine and Hygiene* **91**: 389-393.

Cui, J., Lin, X.M., Zhang, H.W., Xu, B.L. & Wang, Z.Q. (2011). Sparganosis, Henan Province, central China. *Emerging Infectious Diseases* **17**: 146-147.

Cui, J., Wang, Y., Zhang, X., Lin, X.M., Zhang, H.W., Wang, Z.Q. & Chen, J.X. (2017). A neglected risk for sparganosis: eating live tadpoles in central China. *Infectious Diseases of Poverty* **6**: 58.

Dai, R.S., Liu, G.H., Song, H.Q., Lin, R.Q., Yuan, Z.G., Li, M.W., Huang, S.Y., Liu, W. & Zhu, X.Q. (2012). Sequence variability in two mitochondrial DNA regions and internal transcribed spacer among three cestodes infecting animals and humans from China. *Journal of Helminthology* **86**: 245-251.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**: 772.

Drummond, A.J., Suchard, M.A., Xie, D. & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29**: 1969-1973.

Eberhard, M.L., Thiele, E.A., Yembo, G.E., Yibi, M.S., Cama, V.A. & Ruiz-Tiben, E. (2015). Thirty-seven human cases of sparganosis from Ethiopia and South Sudan caused by *Spirometra* spp. *American Journal of Tropical Medicine and Hygiene* **93**: 350-355.

Eom, K.S., Park, H., Lee, D., Choe, S., Kim, K.H. & Jeon, H.K. (2015). Mitochondrial Genome Sequences of *Spirometra erinaceieuropaei* and *S. decipiens* (Cestoidea: Diphyllobothriidae). *Korean Journal of Parasitology* **53**: 455-463.

Excoffier, L. & Lischer, H.E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**: 564-567.

Faust, E.C., Campbell, H.E. & Kellogg, C.R. (1929). Morphological and biological studies on the species of *Diphyllobothrium* in China. *American Journal Hygiene* **9**: 560-583.

Fu, Y.X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**: 915-925.

Holldorf, E.T., Siers, S.R., Richmond, J.Q., Klug, P.E. & Reed, R.N. (2015). Invaded Invaders: Infection of invasive brown treesnakes on Guam by an exotic larval cestode with a life cycle comprised of non-native hosts. *PLoS One* **10**: e0143718.

Jeon, H.K., Park, H., Lee, D., Choe, S., Kang, Y., Bia, M.M., Lee, S.H., Sohn, W.M., Hong, S.J., Chai, J.Y. & Eom, K.S. (2018a). Genetic and morphologic identification of *Spirometra ranarum* in Myanmar. *Korean Journal of Parasitology* **56**: 275-280.

Jeon, H.K., Park, H., Lee, D., Choe, S. & Eom, K.S. (2018b). *Spirometra decipiens* (Cestoda: Diphyllobothriidae) collected in a heavily infected stray cat from the republic of Korea. *Korean Journal of Parasitology* **56**: 87-91.

Jeon, H.K., Park, H., Lee, D., Choe, S., Kim, K.H., Sohn, W.M. & Eom, K.S. (2016a). Genetic identification of *Spirometra decipiens* plerocercoids in terrestrial snakes from Korea and China. *Korean Journal of Parasitology* **54**: 181-185.

Jeon, H.K., Park, H., Lee, D., Choe, S., Sohn, W.M. & Eom, K.S. (2016b). Molecular detection of *Spirometra decipiens* in the United States. *Korean Journal of Parasitology* **54**: 503-507.

Jeon, H.K., Park, H., Lee, D., Choe, S., Kim, K.H., Huh, S., Sohn, W.M., Chai, J.Y. & Eom, K.S. (2015). Human infections with *Spirometra decipiens* plerocercoids identified by morphologic and genetic analyses in Korea. *Korean Journal of Parasitology* **53**: 299-305.

Jongthawin, J., Intapan, P.M., Sanpool, O., Sadaow, L., Laymanivong, S., Thanchomnang, T. & Maleewong, W. (2014). Molecular evidence of *Spirometra erinaceieuropaei* infection in snakes *Ptyas korros* from Lao PDR and Thailand and frogs *Hoplobatrachus rugulosus* from Myanmar. *The Southeast Asian Journal of Tropical Medicine and Public Health* **45**: 1271-1278.

Kuchta, R., Scholz, T., Brabec, J. & Bray, R.A. (2008). Suppression of the tapeworm order Pseudophyllidea (Platyhelminthes: Eucestoda) and the proposal of two new orders, Bothriocephalidea and Diphyllobothriidea. *International Journal for Parasitology* **38**: 49-55.

Kuchta, R. & Scholz, T. (2017). Diphyllobothriidea. In: Caira, J.N., Jensen, J. (Eds.). Tapeworms from vertebrate bowels of the earth 2008-2017. University of Kansas, Natural History Museum, Special Publication No. 25, Lawrence, Kansas, USA. pp. 167-189.

Lee, S.U., Chun, H.C. & Huh, S. (2007). Molecular phylogeny of parasitic Platyhelminthes based on sequences of partial 28S rDNA D1 and mitochondrial cytochrome *c* oxidase subunit I. *Korean Journal of Parasitology* **45**: 181-189.

Lee, S.U., Huh, S. & Phares, C.K. (1997). Genetic comparison between *Spirometra erinacei* and *S. mansonoides* using PCR-RFLP analysis. *Korean Journal of Parasitology* **35**: 277-282.

Liu, Q., Li, M.W., Wang, Z.D., Zhao, G.H. & Zhu, X.Q. (2015). Human sparganosis, a neglected food borne zoonosis. *The Lancet Infectious Diseases* **15**: 1226-1235.

Liu, W., Zhao, G.H., Tan, M.Y., Zeng, D.L., Wang, K.Z., Yuan, Z.G., Lin, R.Q., Zhu, X.Q. & Liu, Y. (2010). Survey of *Spirometra erinaceieuropaei* spargana infection in the frog *Rana nigromaculata* of the Hunan Province of China. *Veterinary Parasitology* **173**: 152-156.

Michelet, L., Carod, J.F., Rakontondrazaka, M., Ma, L., Gay, F. & Dauga, C. (2010). The pig tapeworm *Taenia solium*, the cause of cysticercosis: Biogeographic (temporal and spacial) origins in Madagascar. *Molecular Phylogenetics and Evolution* **55**: 744-750.

Miyadera, H., Kokaze, A., Kuramochi, T., Kita, K., Machinami, R., Noya, O., Alarcón de Noya, B., Okamoto, M. & Kojima, S. (2001). Phylogenetic identification of *Sparganum proliferum* as a pseudophyllidean cestode by the sequence analyses on mitochondrial COI and nuclear sdhB genes. *Parasitology International* **50**: 93-104.

Mueller, J.F. (1937). New host records for *Diphyllobothrium mansonoides* Mueller 1935. *Journal of Parasitology* **23**: 313-315.

Okamoto, M., Iseto, C., Shibahara, T., Sato, M.O., Wandra, T., Craig, P.S. & Ito, A. (2007). Intraspecific variation of *Spirometra erinaceieuropaei* and phylogenetic relationship between *Spirometra* and *Diphyllobothrium* inferred from mitochondrial CO1 gene sequences. *Parasitology International* **56**: 235-238.

Okino, T., Ushirogawa, H., Matoba, K., Nishimatsu, S.I. & Saito, M. (2017). Establishment of the complete life cycle of *Spirometra* (Cestoda:

Diphyllobothriidae) in the laboratory using a newly isolated triploid clone. *Parasitology International* **66**: 116-118.

Petrigh, R.S., Scioscia, N.P., Denegri, G.M. & Fugassa, M.H. (2015). Cox-1 gene sequence of *Spirometra* in Pampas foxes from Argentina. *Helminthologia* **52**: 355-359.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Höhna, S., Larget, B., Liu, L., Suchard, M.A. & Huelsenbeck, J.P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**: 539-542.

Rozas, J., Ferrer-Mata, A., Sanchez-DelBarrio, J.C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S.E. & Sánchez-Gracia, A. (2017). DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Molecular Biology and Evolution* **34**: 3299-3302.

Simpson, C., Jabbar, A., Mansfield, C.S., Tyrrell, D., Croser, E., Abraham, L.A. & Gasser, R.B. (2012). Molecular diagnosis of sparganosis associated with pneumothorax in a dog. *Molecular and Cellular Probes* **26**: 60-62.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**: 585-895.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725-2729.

Tang, T.H., Wong, S.S., Lai, C.K., Poon, R.W., Chan, H.S., Wu, T.C., Cheung, Y.F., Poon, T.L., Tsang, Y.P., Tang, W.L. & Wu, A.K. (2017). Molecular Identification of *Spirometra erinaceieuropaei* tapeworm in cases of human sparganosis, Hong Kong. *Emerging Infectious Diseases* **23**: 665-668.

Thanchomnang, T., Tantrawatpan, C., Intapan, P.M., Sanpool, O., Lulitanond, V., Tourtip, S., Yamasaki, H. & Maleewong, W. (2016). Rapid identification of nine species of diphyllobothriidean tapeworms by pyrosequencing. *Scientific Reports* **6**: 37228.

Waeschenbach, A., Brabec, J., Scholz, T., Littlewood, D.T.J. & Kuchta, R. (2017). The catholic taste of broad tapeworms – multiple routes to human infection. *International Journal for Parasitology* **47**: 831-843.

Wei, T., Zhang, X., Cui, J., Liu, L.N., Jiang, P. & Wang, Z.Q. (2015). Levels of sparganum infections and phylogenetic analysis of the tapeworm *Spirometra erinaceieuropaei* sparganum in wild frogs from Henan Province in central China. *Journal of Helminthology* **89**: 433-438.

Yamasaki, H., Nakaya, K., Nakao, M., Sako, Y. & Ito, A. (2007). Significance of molecular diagnosis using histopathological specimens in cestode zoonoses. *Tropical Medicine and Health* **35**: 307-321.

Yanagida, T., Matsuoka, H., Kanai, T., Nakao, M. & Ito, A. (2010). Anomalous segmentation of *Diphyllobothrium nihonkaiense*. *Parasitology International* **59**: 268-270.

Zhang, X., Cui, J., Liu, L.N., Jiang, P., Wang, H., Qi, X., Wu, X.Q. & Wang, Z.Q. (2015a). Genetic structure analysis of *Spirometra erinaceieuropaei* isolates from central and southern China. *PLoS One* **10**: e0119295.

Zhang, X., Wang, H., Cui, J., Jiang, P., Fu, G.M., Zhong, K., Zhang, Z.F. & Wang, Z.Q. (2015b). Characterisation of the relationship between *Spirometra erinaceieuropaei* and *Diphyllobothrium* species using complete *cytb* and *cox1* genes. *Infection Genetics and Evolution* **35**: 1-8.

Zhang, X., Cui, J., Wei, T., Li, L.Y., Jiang, J., Lu, J.C., Jiang, P., Liu, L.N. & Wang, Z.Q. (2014). Survey and genetic variation of *Spirometra erinaceieuropaei* sparganum in frogs and snakes from Guangxi of southern China. *Tropical Biomedicine* **31**: 862-870.

Zhang, X., Duan, J.Y., Shi, Y.L., Jiang, P., Zeng, D.J., Wang, Z.Q. & Cui, J. (2017). Comparative mitochondrial genomics among *Spirometra* (Cestoda: Diphyllobothriidae) and the molecular phylogeny of related tapeworms. *Molecular Phylogenetics and Evolution* **117**: 75-82.

Zhang, X., Hong, X., Duan, J.Y., Han, L.L., Hong, Z.Y., Jiang, P., Wang, Z.Q. & Cui, J. (2019). Development of EST-derived microsatellite markers to investigate the population structure of sparganum – the causative agent of zoonotic sparganosis. *Parasitology* **12**: 1-9.

Zhang, X., Shi, Y.L., Han, L.L., Xiong, C., Yi, S.Q., Jiang, P., Wang, Z.X., Shen, J.L., Cui, J. & Wang, Z.Q. (2018). Population structure analysis of the neglected parasite *Thelazia callipaeda* revealed high genetic diversity in Eastern Asia isolates. *PLoS Neglected Tropical Diseases* **12**: e0006165.

Zhang, X., Wang, H., Cui, J., Jiang, P., Lin, M.L., Zhang, Y.L., Liu, R.D. & Wang, Z.Q. (2016). The phylogenetic diversity of *Spirometra erinaceieuropaei* isolates from southwest China revealed by multi genes. *Acta Tropica* **156**: 108-114.