Original Article

Interspecies Phylogenetic Analysis of *Clonorchis sinensis* in High-incidence Areas of Hunan Province, China

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Abstract

Objective This study aims to investigate the infection of *Clonorchis sinensis* (*C. sinensis*) in high-incidence areas of Hunan Province, China. The phylogenetic analysis of the *C. sinensis* species in the highly infected areas was carried out.

Method Infection of the definitive human host and intermediate fish host by *C. sinensis* was investigated, and the mitochondrial genes *cox1* and *Nad1* were used as genetic markers for phylogenetic analysis.

Results In 2016–2020, the average population infection rate of Hunan was 1.38%, while in Tongdao County the rate was up to 26.90%, and the highest fish infection rate was detected in Qiyang County (99.44% in the dorsal fin of *crucian carp*). High genetic sequence similarity was observed in the samples from Qiyang and Lengshuitan which exhibited high homology with those from Guangdong and Gansu, whereas the parasitic species from Tongdao was highly homologous with those located in high-latitude areas. Moreover, no significant difference was found in the gene sequence of the parasitic species in definitive hosts dogs and cats.

Conclusion The systematically study of *C. sinensis* infection in the high-incidence areas will contribute greatly to the prevention and effectively controlling the spread of *Clonorchis sinensis* in Hunan Province The endemic of *C. sinensis* infection in Hunan Province is the result of co-action of local and foreign parasite species.

Key words: *Clonorchis sinensis*; Mitochondrial genes; *Cox1*; *Nad1*; *Phylogeny*

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INTRODUCTION

The Chinese liver fluke, *Clonorchis sinensis* (*C. sinensis*), generally parasitizes the liver and hepatic duct causes a serious food-borne parasitic disease[1,2]. *C. sinensis* causes mechanical stimulation and obstruction, and its metabolites and secretions[3] lead to local expansion, hyperplasia, inflammation, and even nodules in the hepatic ducts. This results in fatty degeneration, atrophy, and necrosis of adjacent liver cells and even liver cirrhosis and cancer[4-6].

C. sinensis is mainly prevalent in China, South Korea, northern Vietnam, and parts of Russia[7,8]. Around 15 million humans have been estimated to be infected worldwide, with 85% in China[9]. The prevalence of *C. sinensis* is subject to the combined effects of social, environmental, and control factors, and the main reason is human ingestion of *C. sinensis metacercaria*[10].

Except for Xinjiang, Inner Mongolia, Gansu, Qinghai, Tibet, Ningxia, and other provinces and

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autonomous regions in China, *C. sinensis* has been spread or reported in 25 provinces and cities, with the number of patients as high as 13 million. In some areas of Hunan Province, the infection rate of *C. sinensis* has always been high because of the consumption of sashimi and salted fish by the local residents and cross-pollution of raw and cooked food and tool containers[11]. In 1990, *C. sinensis* was reported in 20 counties (cities and districts), including Qiyang County and Lengshuitan District, in Hunan Province. Approximately 2.1%–85.2% of the population in these areas were infected, and the age of the infected people ranged from 2 to 75 years[12]. The infection rate in children (less than 15 years of age) was 19.8%, and that in adults was 2.5%. In Tongdao Coungty, Huaihua, the infection rate of *C. sinensis* has always been high. In 2011, a survey of *C. sinensis* in humans was conducted in Tongdao County, and the infection rate in the population was more than 70%. However, no studies have investigated the prevalence of *C. sinensis* or the types of epidemic strains in this county, in fact, no *C. sinensis* cases have been reported in the counties and cities around Tongdao County.

Between 2016 and 2020, many surveys and studies have been conducted on human parasitic diseases in 122 counties (districts) and 14 cities (prefectures) across Hunan Province. In addition, a detailed investigation was performed on the infection status of the human population and fish metacercariae in Qiyang County, Lengshuitan District, and Tongdao County, where the infection rate was high.

The mitochondrial genome has a simple structure, maternal inheritance, lack of recombination, and rapid evolution rate; therefore, it is particularly suitable as a marker for genetic research, and it has been used by many scholars to study genetic variations between and within a variety of parasites[13,14]. Two gene fragments, partial sequences of the mitochondrial cytochrome c oxidase subunit I (*Cox1*) gene of *C. sinensis* and nicotinamide adenine dinucleotide dehydrogenase (NADH) subunit I gene, have been used to study the genetic diversity of *C. sinensis* in Qiyang County, Lengshuitan County[15,16], and Tongdao District. In this study, the genetic relationship after the sequencing was evaluated[17-20].

MATERIALS AND METHODS

Population Infection Rate

Between 2016 and 2020, *C. sinensis* was monitored in 14 cities (prefectures) and 122 counties (districts) in Hunan Province. Fecal samples (> 30 g) were collected from the subjects, and a modified Kato-Katz method was used to evaluate the infection rate and infectiosity of *C. sinensis*[21]. *C. sinensis* eggs identified in the smears were counted using microscopy[22].

Take a part out from the fresh fecal sample to cover a 8 cm × 8 cm, 80-mesh nylon silk; scrape the fine fecal sample and place it in the round table hole of the plastic quantitation plate until it is filled up, about 38.75 mm^3 (4 cm × 3 cm × 1 cm); after mixing thoroughly, cover it with hydrophilic cellophane containing malachite green (mixed evenly and soaked according to the ratio of 100 mL distilled water, 100 mL pure glycerin, 1 mL malachite green (or methylene blue, 3%) and flatten it to spread the feces under the hydrophilic cellophane without overflowing the glass slide and form a uniformly thick round fecal film with a diameter of about 2 cm[22]; place the prepared modified Kato-Katz thick smear at room temperature to make it transparent before microscopic examination. If *C. sinensis* eggs are discovered in the smear, it is determined to be positive, and the *C. sinensis* eggs are counted by microscopy.

Infection Rate of Fish Metacercariae

A preliminary investigation of the infection rate of *C. sinensis* was conducted among the human population and metacercariae of commercial freshwater fish in three counties (districts) south of Hunan, Tongdao County, Qiyang County, and Lengshuitan District, where the infection rates were relatively high. Small wild fish, especially *crucian carp*, *parabramis pekinensis*, *cyprinuscarpio,* and *grass carp* (often made into sashimi), were collected from fish ponds around the epidemic area. The direct compression method was used to check for fish metacercariae: A piece of muscle (about 0.5 g) was cut from the dorsal, pectoral, and caudal fins of each fish, placed between two glass slides, and squeezed until a translucent mist was obtained, and active metacercariae were detected under a microscope and counted.

Sequencing of Cox1 and Nad1

Cats and dogs (more than 5 years of age) livers were purchased from counties and towns located to the east, west, south, north, and middle of Qiyang County, Lengshuitan District, and Tongdao County, one final host was collected from each (Figure 1)[23,24]. The animal liver and bile ducts were dissected, the

C. sinensis adults were washed out with distilled water, counted, and stored in 90% ethanol.

The *C. sinensis* adults were puted into the ultra-low temperature refrigerator for freeze-thaw, *the* adults was then cut into pieces with scissors, and the broken adults *C. Sinensis* were puted into ice for tissue homogenization. Nucleic acid was extracted from the homogenized tissue using the DNA Mini Kit QIAGEN Nucleic Acid Extraction Kit[25].

The partial sequences of *C. sinensis Cox1* and *Nad1* were used as the amplification sites for PCR[26,27], and the amplification conditions were as follows: 5 min pre-denaturation at 94 °C, followed by 30 s denaturation at 94 °C, 30 s annealing at 55 °C, and 30 s extension at 72 °C and finally 5 min extension at 72 °C. The PCR reaction system were Forward Primer (10 μm) 1 μL, Reverse Primer (10 μm) 1 μL, 2xTaq Master Mix 10 μL, DNA 1 μL, and finally ddH_2O was added to 20 μL. The amplified products were electrophoresed with the QIAxcel Advanced automatic electrophoresis apparatus (Qiagen Germany). The selected PCR products underwent recovery and purification, and they were sent to Invertrogen Biotechnology Co., Ltd (USA). For bidirectional sequencing.

The complete mitochondrial genome sequence

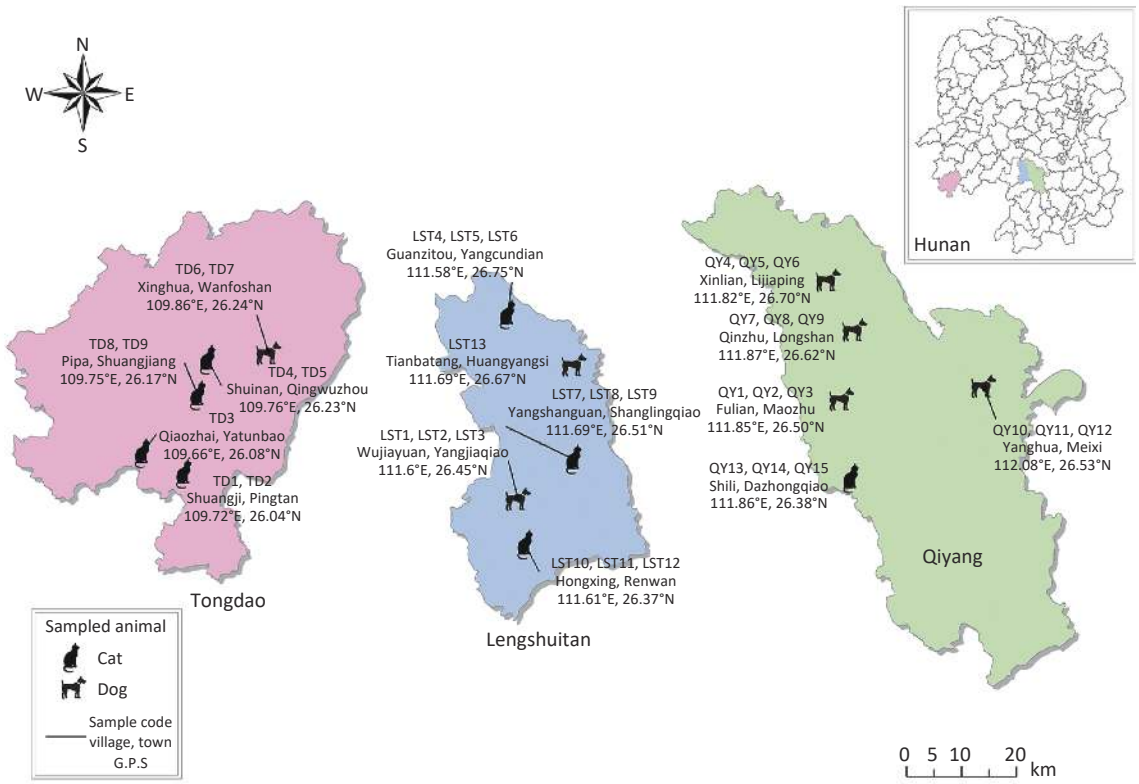

Figure 1. Sampling map for Tongdao, Qiyang, and Lengshuitan. Samples were obtained from the following towns in Tongdao: Shuangji Village of Pingtan Township (cats, sample numbers TD1 and TD2), Qiaozhai Village of Yatunpu Town (cat, sample number TD3), Shuinan Village of Jingwuzhou Town (cats, sample numbers TD4 and TD5), Xinghua Village of Wanfoshan Town (dogs, sample numbers TD6 and TD7), and Pipa Village of Shuangjiang Town (cats, sample numbers TD8 and TD9). Samples were collected from the following towns in Lengshuitan: Wujiayuan Neighborhood Committee of Yangjiaqiao Sub-district (dogs, sample numbers lST1, lST2, and lST3), Guanzitou of Yangcundian Township (cats, sample numbers lST4, lST5, and lST6), Yangshanguan Village of Shanglingqiao Town (cats, sample numbers lST7, lST8, and lST9), Hongxing Village of Renwan Street (cats, sample numbers lST10, lST11, and lST12), and Tianbatang Village of Huangyangsi Town (dog, sample number lST13). Samples were obtained from the following towns in Qiyang: Fulian Village of Maozhu Town (dogs, sample numbers QY1, QY2, and QY3), Xinlian Village of Lijiaping Town (dogs, sample numbers QY4, QY5, and QY6), Qingzhu Village of Longshan Street (dogs, sample numbers QY7, QY8, and QY9), Yanghua Village of Meixi Town (dogs, sample numbers QY10, QY11, and QY12), and Shili Village of Dazhongqiao Town (cats, sample numbers QY13, QY14, and QY15).

of *C. sinensis* from Tongdao (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) was used to design primers COX-F (-TTGGTTATGGGGG-CTTGGTG-), COX-R(-CGCTCAGATCTCAGCAGGTT-), NAD-F(-GAGCGGCTAGATCGGGTTAG-), and NAD-R (-GTGTTGCACGCGACCAAATA-). These primers were synthesized by Invertrogen Biotechnology Co., Ltd (USA).

Data Statistics and Analysis

Excel 2019, SPSS 18.0, Instata 10.0 and other software were used for statistical analysis. In the analysis, homogeneity test of variance was conducted first. If homogeneity of variance was observed, one-way ANOVA was used for overall comparison, and then pair one-factor *t*-test between the two groups was conducted by Dunnett method after differences were found. If the variance is not uniform, the original data should be converted into appropriate variables to meet the homogeneity test of variance, and then the converted data will be used for statistics. If the purpose of homogeneity of variance is still not achieved after the conversion of variables, the rank sum test is used for statistics, and the difference in population comparison is found, then the pair comparison is performed by Tamhane's ST2 test, which does not require homogeneity of variance.

Pair chi-square χ^2 test was used to compare the difference in the detection rate of *C. sinensis* infection which detected by the three methods, and Kappa test was used for consistency analysis. $P < 0.05$ was considered statistically significant.

Analysis of Genetic Diversity and Phylogeny

All the obtained sequences were checked using chromatograms and Contig Express and manually corrected for scoring errors. Chromas Lite version 2.1 was used to detect the sequences. DNAMAN was used to splice the sequences in both directions.

The unique haplotype was found using DNASP 5.10, and the following organisms were searched in GenBank: *C. sinensis* from Russia (FJ381664), *C. sinensis* from Guangdong (JF729303), *C. sinensis* from Gansu (JF729304), *C. sinensis* from USA (NC_012147), *Opisthorchis felineus* (EU921260), *Opisthorchis viverrini* (JF739555), *Fasciola hepatica* (NC_002546), *Paragonimus westermani* (NC_027673), *Schistosoma haematobium* (DQ157222), *Schistosoma japonicum* (NC_002544), *Trichobilharzia regenti* (NC_009680), *Taenia solium* (NC_004022) *Schistosoma mekongi* (NC_002529), and the outgroup *Gyrodactylus* (NC_004022). Then, the Clustal W algorithm was used for alignment in MEGA 6.

Two independent genetic marker *Cox1* and *Nad1* were analyzed with the Bayesian method and BEAST 2 software to construct a phylogenetic tree, The maximum likelihood tree inferred with MEGA 6 with the best model (GTR+G+I) was used as in-put. Additionally, the maximum likelihood analysis (by J Modeltest v. 2.1.7) was performed with MEGA 6 and bootstrap resampled 1,000 times, for phylogroups. With every 1,000 generations recorded among 10,000,000, a burn-in of 25%, and final 10,000 trees summarized using the tree annotator. GTR+G+I was estimated to be the best fit substitution model. The maximum likelihood was estimated with MAGE 6 and 1,000 bootstrap values. The clades were collapsed using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) with the collapse module[28].

RESULTS

C. sinensis Infection Status across Hunan Province in 2016–2020

The infection rate of *C. sinensis* in Hunan Province between 2016 and 2020 is shown in Table 1. The infection rate in the province, except in Yongzhou and Huaihua, was less than 1.5%. The infection rate in Yongzhou and Huaihua year-by-year showed a downward trend because of prevention, intervention, health education, and other reasons. Tongdao county, Lengshuitan District, and Qiyang County were monitored ashigh infection areas in Yongzhou and Huaihua; the systematic investigation of population infections in these three regions was of great significance in controlling the spread of *C. sinensis* (Table 2).

The results showed that 1,449 people were infected in Tongdao County, with an infection rate of 26.90%; 713 people in Qiyang County, with an infection rate of 18.96%; and 699 people in Lengshuitan District, with an infection rate of 21.80%. Significant differences ($\chi^2 = 82.91$, $P < 0.01$) were observed in the infection rates in these three regions.

Infection Status of Fish Metacercariae in Qiyang, Lengshuitan, and Tongdao

The infection of fish metacercariae by *C. sinensis* in Qiyang County, Lengshuitan District, and Tongdao County was investigated (Table 3). Common freshwater fish cultured in fish ponds were purchased from Tongdao County, Qiyang County, and Lengshuitan District. Differences in infectiosity

Table 1. Infection status of *C. sinensis* in Hunan Province from 2016 to 2020

Item	2016		2017		2018		2019		2020		5 years in total	
	Sample size	Infection rate (%)	Sample size	Infection rate (%)	Sample size	Infection rate (%)	Sample size	Infection rate (%)	Sample size	Infection rate (%)	Sample size	Infection rate (%)
Changsha	2,017	0	0	0	2,156	0	3,227	0	2,017	0.05	9,417	0.01
Zhuzhou	3,205	0	1,000	0	1,000	0	3,033	0	2,413	0	10,651	0
Xiangtan	3,014	0	0	0	1,041	0	987	0	0	0	5,042	0
Hengyang	2,554	0.04	0	0	1,000	0	5,000	0	0	0	8,554	0.01
Shaoyang	4,103	0	3,014	0	2,000	0	4,002	0	0	0	13,119	0
Yueyang	9,768	0	2,013	0.15	2,000	0	4,000	0	1,000	0	18,781	0.02
Changde	4,099	0	0	0	1,000	0	3,004	0	3,000	0	11,103	0
Zhangjiajie	0	0	1,000	0	1,109	0	1,006	0	1,000	0	3,115	0
Yiyang	3,731	0	0	0	2,619	0.04	3,500	0	1,000	0	10,850	0.01
Chenzhou	7,031	0	1,029	0.29	2,052	0.05	5,023	0	4,000	0	19,135	0.02
Yongzhou	5,979	7.26	1,006	5.47	4,166	5.59	5,035	5.54	10,102	2.15	26,288	4.63
Huaihua	7,233	7.47	1,000	23.80	5,072	3.21	5,229	4.25	8,294	2.50	26,828	5.11
Loudi	4,635	0.02	0	0	3,580	0	4,500	0	1,000	0	13,715	0.01
Xiangxi	2,276	0	2,006	0.15	2,135	0	3,006	0	2,018	0	11,441	0.03
Hunan	59,645	1.64	12,068	2.50	30,930	1.29	50,552	0.99	35,844	1.19	188,039	1.38

Table 2. Infection status of *C. sinensis* among populations in Tongdao, Qiyang, and Lengshuitan

Region	Number of examinees	Number of infected people	Infection rate (%)
Tongdao County	5,385	1,449	26.90
Qiyang County	3,761	713	18.96
Lengshuitan District	3,200	699	21.80
Total	12,346	2861	23.17

Table 3. Infection of fish metacercaria in Tongdao, Qiyang, and Lengshuitan ($\bar{x} \pm s$)

Region	Category	Number of examinees (mantissa)	Number of infected (mantissa)	Infection rate (%)	Average infectiosity (piece/g)
Tongdao County	Crucian	25	22	88.00	72.32 ± 38.05
	Parabramispekinensis	12	10	83.33	46.08 ± 22.89
	cyprinuscarpio	7	4	57.14	7.14 ± 5.82
	Grass carp	4	1	25.00	0.75 ± 1.00
Qiyang County	Crucian	18	17	94.44	77.78 ± 26.95
	Parabramispekinensis	10	9	90.00	49.10 ± 18.56
	cyprinuscarpio	8	5	62.50	10.25 ± 6.20
	Grass carp	5	1	20.00	1.40 ± 2.09
Lengshuitan District	Crucian	30	27	90.00	70.52 ± 33.82
	Parabramispekinensis	24	17	70.83	43.28 ± 26.00
	cyprinuscarpio	12	7	58.33	16.58 ± 10.17
	Grass carp	7	2	28.57	2.00 ± 2.40
Total	Crucian	73	66	90.41	73.54 ± 33.43
	Parabramispekinensis	46	36	78.26	46.16 ± 23.21
	cyprinuscarpio	27	16	59.26	11.33 ± 8.33
	Grass carp	16	4	25.00	1.38 ± 1.95

invarious freshwater fish metacercariae were investigated; specifically, both infection rate and average infection rate were the highest in *crucian carp* (90.41% and 73.54 piece/g, respectively), followed by *Parabramis pekinensis* (78.26% and 46.16 piece/g, respectively), *cyprinuscarpio* (59.26% and 11.33 piece/g, respectively), and *grass carp* (25.00% and 1.83 piece/g, respectively). The infection rates of these four fishwere significantly different (χ^2 = 103.56, P < 0.01); the infection rate and infectiosity were higherin Qiyang County than in the other regions.

Phylogenetic Analysis of C. sinensis

C. sinensis adults were collected from cats and dogs (age, more than 5 years) and observed under a microscope (Figure 2). The body of an adult *C. sinensis* long and narrow, with a flat dorsal abdomen, narrow front end, blunt and round rear end (like a sunflower seed), and no spines on the surface. The oral suckers are slightly larger than the abdominal suckers, which are located at the front 1/5 of the body. The alimentary canal is simple, with the mouth at the center of the oral sucker, the pharynx is spherical, and the esophagus is short, followed by the intestinal branches. The intestinal branches are divided into two, which reach the posterior end along both sides of the worm without confluence and with a blind end. Judging from morphology, it is the adults of *C. sinensis*.

Morphological differences were detected among the *C. sinensis* adults from the three regions. The some adults in Qiyang County are similar to those in Lengshuitan District. The some adults of Tongdao county was more slender and brighter in color.

The mitochondrial genes *Cox1* and *Nad1* of *C. sinensis* were used as genetic markers for amplification. The results showed that the length of *Cox1* and *Nad1* are approximately 1,000 bp and 800 bp, respectively, which isconsistent with the predicted values; the electrophoresis results showed clear bands with no tailing phenomenon or unspecific bands (Figure 3).

Analysis of Genetic Diversity and Phylogeny

To verify the amplification accuracy of the two genes, the PCR products were sequenced and compared with homologous sequences from GenBank, and the amplified products were the *Cox1* and *Nad1* sequences of *C. sinensis*.

A phylogenetic tree was constructed using *Cox1* as the genetic marker and *Gyrodactylus*as the outgroup for *C. sinensis* and other trematodes in Tongdao County, Qiyang County, and Lengshuitan District as well as Guangdong Province, Gansu Province, China, the United States, and Russia (Figure 4A). *C. sinensis*specimens from Qiyang and Lengshuitan were clustered together without any regional differences; both exhibited high homology with those from Guangdong Province and Gansu Province, China, with a high bootstrap value. The regional clustering in Tongdao County was high and demonstrated low homology with that in Qiyang County and Lengshuitan District but high homology with that in the United States and Russia. The relationships of the other liver flukes, schistosomes,

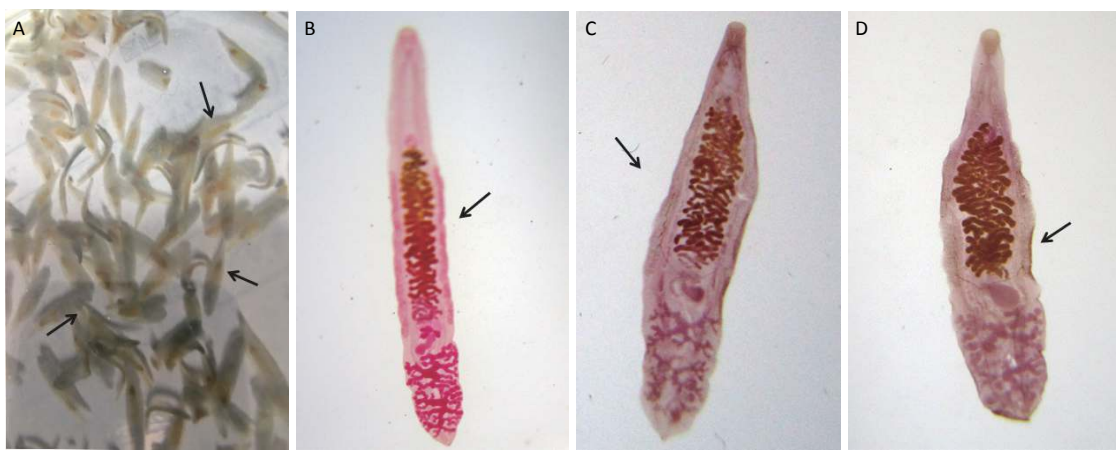

Figure 2. Adult Clonorchis sinensisobtained from hosts. (A) Naked eye of *C. sinensis*. (B) Clonorchis sinensisfrom Tongdaounder a microscope (magnification, 10×). (C) Clonorchis sinensisfrom Qiyang under a microscope (magnification, 10×). (D) Clonorchis sinensisfrom Lengshuitan under a microscope (magnification, 10×).

and *Gyrodactylus* with *C. sinensis* gradually decreased, which is consistent with the taxonomic records. No significant specificity was observed in the genetic relationships of the parasitic *C. sinensis* in different definitive hosts (dog and cat).

With *Nad1* as the genetic marker (Figure 4B), *C. sinensis* from Qiyang County and Lengshuitan District showed few regional differences and high homology with those from Guangdong Province and Gansu Province, China. the regional clustering in Tongdao County was high and demonstrated high homology with that in the United States and Russia. The relationships of the other liver flukes, schistosomes, and *Gyrodactylus* with *C. sinensis*gradually decreased, which is consistent with the taxonomic records. The parasitic *C. sinensis* in different definitive hosts (dog and cat) showed no significant specificity in the genetic relationship. This is

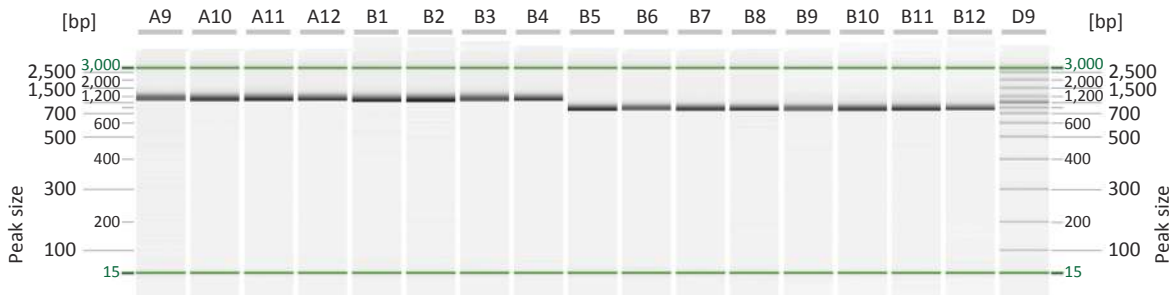

Figure 3. Electrophoresis map of *Cox1* gene and *Nad1* gene. Description: Lame: 3kb ladder, LameA9, A10, A11, A12, B1, B2, B3 and B4 are *Cox1* gene amplification results, and Lame B5, B6, B7, B8, B9, B10, B11, B12 are Nad1 gene amplification results.

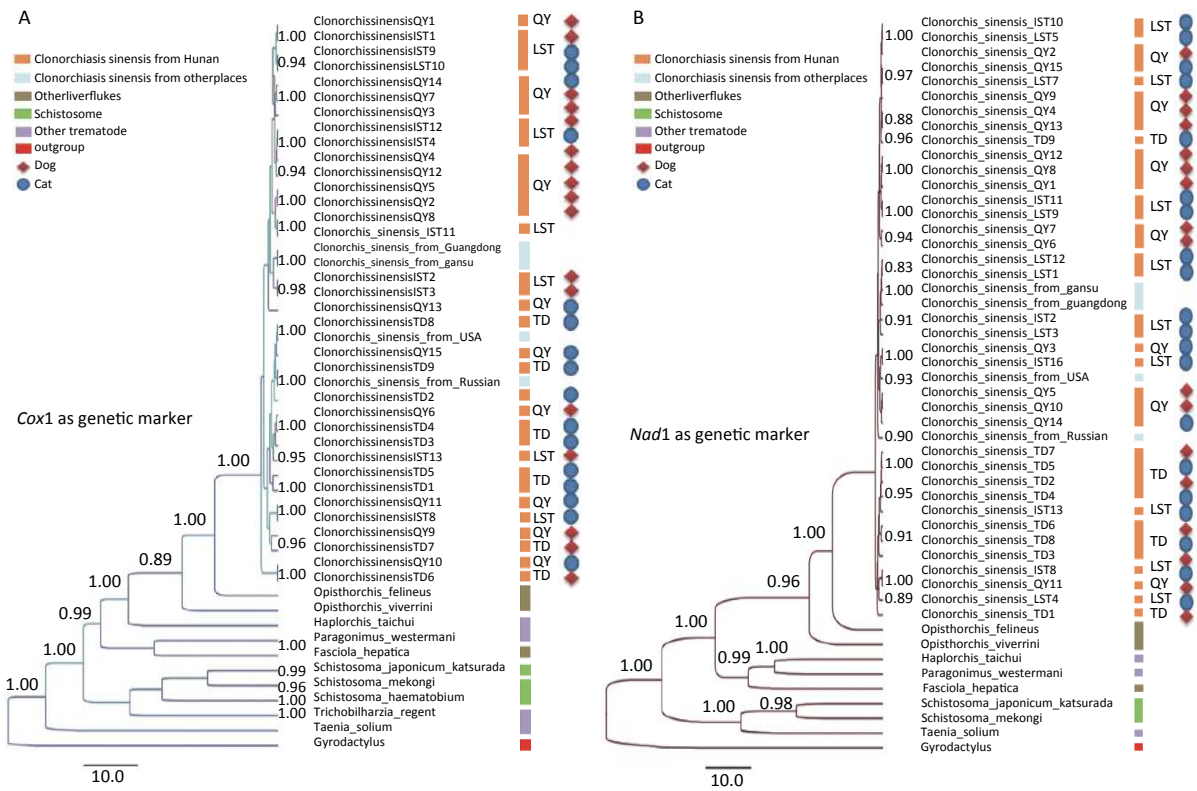

Figure 4. Phylogenetic relationships of *C. sinensis* with *Cox1* and *Nad1* as the genetic marker. (A) Phylogenetic relationships of *C. sinensis* with *Cox1* as the genetic marker. (B) Phylogenetic relationships of *C. sinensis* with *Nad1* as the genetic marker.

consistent with the phylogenetic relationship results with *Cox1* as the genetic marker.

DISCUSSION

According to the investigation results of *C. sinensis* infection in the population of 122 counties (districts), 14 cities (prefectures) in the Hunan Province from 2016 to 2020, it can be seen that the infection of *C. sinensis* has been decreasing year by year since 2016, The high infection rates of *C. sinensis* among the populations in Qiyang County and Lengshuitan District were related to the high consumption of sashimi and salted fish in these areas[29]. Recently, the infection rates of *C. sinensis* have decreased in these areas because of improvement indietetic hygiene and scientific prevention[30], but theyare still significantly higher than the infection rates in other areas of Hunan Province. Sashimi is part of the daily diet of people in Tongdao County, Huaihua, so it is important to investigate *C. sinensis* infection in the population and intermediate hosts. The results of this study showed that the infection rate of *C. sinensis* in Tongdao County is high. Of the four fish commonly used for making sashimi, the infection rate incruciancarp was the highest, which could be attributed to its small size and phototropism. This study filled the gap of systematic report on the infection of *C. sinensis* between the population and the intermediate host in Tongdao County.

To study the genetic relationships of *C. sinensis* in Tongdao County, Qiyang County, and Lengshuitan District, the mitochondrial genes *Cox1* and *Nad1* were selected as markers[31] and sequenced from adult specimens collected from different areas of Tongdao County, Qiyang County, and Lengshuitan District. The sequence alignment and phylogenetic analysis showed that the parasitic species in Qiyang County and Lengshuitan Distric had a high degree of homology, which could be attributable to the similar latitudes and regional closeness. However, the parasitic species in Tongdao were different from those in Qiyang and Lengshuitan, which have no high homology with the domestic parasitic species, but similar to those in the United States and Russia. This may be related to species invasion and needs to be further studied in the future. This is the first phylogenetic analysis of *C. sinensis* in the high-incidence area of Hunan Province, China.

The results obtained by designing primers for *Cox1* and *Nad1* showed a high degree of consistency, indicating that both *Cox1* and *Nad1* are stable genetic markers for the phylogenetic analysis of *C. sinensis*. The homology analysis of *C. sinensis* showed that latitude and longitude and geographical clustering are important. It is suggested that *C. sinensis* in the Tongdao County may have the biological characteristics of high latitude species, and have similar antigenicity. It is also suggested that *C. sinensis* in the Tongdao County may originate from the high latitude region of Eurasia, Russia. This will lay a foundation for further exploration of its control direction, gene traceability and detection methods.

The phylogenetic analysis of *C. sinensis* adults detected in different definitive hosts (dogs and cats) showed that *C. sinensis* has no specificity in the parasitism of mammalian hosts, and it does not have different gene homology because of changes in the hosts.

A combination of molecular biology and morphology is more reliable than morphological identification. Morphological description is greatly affected by subjective factors and measurement errors. Differences in the genomes of *C. sinensis* explain the differences in the morphology of *C. sinensis* in Tongdao County, suggesting that the antigenicity of *C. sinensis* in Tongdao County may be closer to that of the species in high-latitude regions[32]. The results of this study have laid the foundation for further natural selection and evolutionary analyses of *C. sinensis* in Tongdao. *C. sinensis* in Tongdao may have originated from high-latitude areas, providing clues for studying the evolution and mutation adaptation of *C. sinensis* in high- and low-latitude areas and research on group evolution, individual differences, detection methods, control drugs, and vaccines for *C. sinensis* in this area.

CONCLUSION

The infection rate of *C. sinensis* was higher in Qiyang County, Lengshuitan District and Tongdao County of Hunan province, China. Mitochondrial genes *Cox1* and *Nad1* were used as genetic markers to study the phylogeny of *C. sinensis*. The results showed that the *C. sinensis* in Tongdao county was far from the other area of Hunan Province, which might be related to the migration and invasion of the species. Based on this method, mitochondrial genes *Cox1* and *Nad1* were selected as good genetic markers for the phylogenetic analysis of *C. sinensis*. The results of molecular genetic marker analysis were basically the same, which again confirmed the reliability of the experimental study. Taking into

account the complexity of the species of *C. sinensis* in Hunan Province, it is recommended to reduce the infection rate of parasites in terms of controlling local transmission and preventing external sources.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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