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Full Length Research Paper

Epidemiological survey and phylogenetic analysis of *Paragonimus westermani* isolates in Jinhua, China

Lou, H. Q.[1], Hu, Y.[1], Zhang, L.[2]*, Zhang, Y. J.[2]*, Tu, P. G.[1], Yu, X. T.[3], Jin, Y. J.[1] and Wang, L.[1]

[1]Institute of Zoonosis, Jinhua College of Profession and Technology; Jinhua Zhejiang 321007, P. R. China.
[2]Co-first unit: Center for Disease Control and Prevention of Zhejiang Province, Hangzhou Zhejiang 310051, P. R. China.
[3]Jinhua Municipal Center for Disease Control and Prevention; Jinhua Zhejiang 321002, P. R. China.

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Paragonimiasis has previously been reported in many zones of China, which is the infestation of the trematode genus *Paragonimus*. The aim of this study was to investigate the presence and epidemiology of paragonimiasis in Jinhua which is next to Fujian, China. The differences of *Paragonimus westmani* isolates from Jinhua and other areas of the world were also analyzed. Freshwater crabs and snails from streams located in each of the villages were dissected for isolation of *Paragonimus metacercariae.* The epidemiology data were further analyzed by using geographic information systems (GIS). We found that the infestation rate for the *Paragonimus cercariae* in snails was as low as 2.3%, while that was as high as 76.67% in crabs. So, freshwater crabs were demonstrated as one of the main hosts for *P. metacercariae* in Jinhua. Moreover, phylogenetic analysis indicated that the sequences of the second internal transcribed spacer (ITS2) and cytochrome coxidase subunit I (COI) were conserved among different isolates, especially for the ITS2 gene. Based on the phylogenetic trees generated from the comparison results of COI gene, it was interesting to find that *Paragonimus* strains from different areas were present in different cluster. We conclude that the COI gene of *Paragonimus* can be a molecular marker contributing to the typing and source tracking for Paragonimiasis.

Key words: *Paragonimus westermani*, epidemiological survey, second internal transcribed spacer (ITS2), cytochrome coxidase subunit I (COI) gene.

INTRODUCTION

Paragonimiasis is an endemic disease caused by trematode parasites of the genus *Paragonimus* (Blair et al., 1999; Vélez et al., 2003). The disease is common in Southeast Asia, South America, and Africa, with an associated 21 million people infected around the world (Aka et al., 2008; Cho et al., 1997; De et al., 2000). The parasite is transmitted via snails to freshwater crabs or crayfish, then to humans and other mammals, such as cats and dogs, and causes paragonimiasis (Abdul-hadi et al., 2008). Patients with paragonimiasis usually present with signs and symptoms in the lower respiratory tract. The parasite can migrate to several other vital tissues including brain (Ashitani et al., 2000; Calvopina et al., 2003; Chen et al., 2008). It is estimated that over 20 million people are infected worldwide due to several species of *Paragonimus*. Over 40 species are known to infect the lung of different mammalian hosts (representing as many as eleven families) throughout the world and approximately 15 species are known to infect humans (Devi et al., 2007; Lane et al., 2009). *P. westermani* is an important pathogen in Southeast Asia and China (Liu et al., 2008; Nakamura-uchiyama et al., 2002).

As described previously, there have been many reports on the existence of paragonimiasis in different areas of China, such as Fujian and Sichuan province (Ming-gang et al., 2001). Since there have been also several clinical *Paragonimus* infection cases found in Jinhua City which is next to Fujian Province, and it was suspected to be a foci of this disease. However, no or scanty information is available about the prevalence of the parasite among its hosts here.

*Corresponding authors. E-mail: lzhang@cdc.zj.cn, slatni_tarek@yahoo.fr Tel: 00 216 79 325 848. Fax: 00 216 79 325 638.

Geographic information systems (GIS) have the power to relate a disease or process to a particular location. Importantly, they can also link significant non-spatial data (example, Medical record information, claims data, neighborhood measures of poverty, or environmental pollutant measurements) to that same point in space, creating a rich picture between the relationship of exposure, outcome and its variation across space (Berke et al., 2010; Bowman et al., 2010). As an endemic disease, it is important to understand the relationships between the prevalence of paragonimiasis and the environment. At the same time, they provide information facilitating the continuous process of planning, monitoring and control of paragonimiasis. In this research, geographic information systems (GIS) were applied to visually depict the association between the distribution of *Paragonimus* and the geographic environment. The challenges, lessons learned, and future directions for paragonimiasis research have also been analyzed.

Morphology of the encysted and excysted metacercariae, which occur as the infective stage in the muscle tissue of the crustacean second intermediate host, has been conventionally used in identification of species of *Paragonimus*. In recent times, the amplification of specific DNA regions via the polymerase chain reaction (PCR) and improved sequencing techniques have been applied to resolve taxonomic issues related to various helminth parasites by comparing their DNA sequences (Singh et al., 2009). The majority of studies undertaken to distinguish species and subspecies have used sequences of nuclear ribosomal second internal transcribed spacer (ITS2) or partial sequences of the mitochondrial cytochrome coxidase subunit I (COI) gene. In this study, we tried to describe the phylogenetic relationship among different *Paragonimus* isolates, based on the COI gene and the ITS2 gene sequences (Blair et al., 1996, 1997).

MATERIALS AND METHODS

Parasitologic investigations

In order to investigate the presence of *Paragonimus* in different districts of Jinhua, crabs were collected from streams in each of the villages using fishing baskets tufted with palm nuts and cassava. As described previously, the baskets were placed at various points in the streams overnight and only checked the next day for the presence of crabs. Some crabs were dug out of small borrows along the stream beds or picked under some stones in the streams. The collected crabs were transported in perforated plastic buckets each with a lid to the laboratory where they were fed with palm nuts and slices of cassava until dissected. By using a small metal hammer, hard shell of each crustacean was removed and the muscle tissues beneath extracted with a small metallic spatula (Moyou-somo et al., 2003). The obtained sample was then examined under a dissecting microscope. According to the methods described previously, the infestation is mean calculated as (infestation ratios ×metacercariae number per-crab ×metacercariae number per-gram crab). The infestation mean of different cites were generated and then applied for making distribution map of intermediate hosts by ArcViewGIS 3.3 software.

DNA isolation, amplification and sequencing

P. westermani metacercariae were recovered from naturally infected freshwater edible crabs, which was collected from a mountain stream of Baisha, Babai, Shafan and Baimu District of Jinhua City. Metacercariae were isolated from the muscles of the crustacean host by digestion technique in artificial gastric juice. The sediments were examined for *Paragonimus* metacercariae under a dissecting stereoscopic microscope. Metacercariae were orally fed to dogs, which would have been killed after 3 months post-infection. Fully developed adult flukes from each isolate were used for DNA extraction following the earlier standardized procedure and protocol.

Polymerase chain reaction (PCR) was employed for gene fragments amplification. A High Fidelity PCR Kit (TaKaRa, China) was used to amplify the target gene. The total volume per PCR was 100 µl which included 20 pmol of each of the primers, 2.5 U Taq-Pfu DNA polymerase, and 100 ng DNA templates. The reaction mixture was initiated by incubation at 94°C for 5 min, followed by 30 cycles of amplification at 94°C for 30 s, 50°C for 30 s and 72°C for 30 cycles, and then incubation at 72°C for 10 min. The products were detected in 1.5% ethidium bromide pre-stained agarose gel after electrophoresis. After purified by gel extraction, the PCR products were ligated into a T cloning vector. Clones were generated by transforming *Escherichia coli* DH 5α competent cells. The blue and white color selection was used for recombinant plasmid identification. Plasmid DNA from the positive recombinants were first identified by enzyme digestion and then sequenced by Invitrogen.

Molecular phylogenetic analysis using bioinfomatic tools

The DNA sequences were put to further analysis with the usage of bioinformatics tools including similarity search using BLAST (Basic Local Alignment Search Tool); http://www.ncbi.nlm.nih.gov/blast, and phylogenetic prediction using ClustalW provided at the http://www.ebi.ac.uk/clustalw for query DNA sequence. Gene sequences of *P. westermani* collected from various localities were used for sequence similarity analysis. Gene sequences from isolates of neighbor countries were selected depending upon their BLAST hits and E-value. Only unique sequences were used in tree construction; COI, ITS2 sequences arranged with MEGA format were entered in the MEGA for construction of phylogenetic trees that were inferred using distance method like Neighbor Joining and character state method maximum parsimony. Test of phylogenetic accuracy was done by bootstrapping.

RESULTS

Epidemiological analysis combined with GIS technology

The infestation rates for the cercariae of *Paragonimus* among the snails collected in the vicinity of streams in Jinhua, Zhejiang province is presented in Table 1. It could be conclude that the infestation rate for the *Paragonimus* cercariae in the snails was very low. In every site, the approximate number of snails collected for detection was 80, with 3 exceptions. And it was found that only 0 to 2.3% snails were infested with *Paragonimus*.

We investigated the infestation rates for the

Table 1. Infestation rate of the intermediate hosts in the different zones of Jinhua.

Site of investigation		Snail			Fresh crab			Time of investigation
		Total number	Positive number	Positive ratio (%)	Total number	Positive number	Positive ratio (%)	
Wucheng	Langya	83	1	1.20	60	32	53.33	2009.09
	Shafan	88	2	2.27	60	46	76.67	
	Andi	72	0	0	65	0	0	
Jindong	Caozhai	80	0	0	60	0	0	2010.09
	Yuandong	86	0	0	50	0	0	
	Xiaoshun	85	0	0	70	0	0	
Yiwu	Suxi	80	0	0	60	0	0	2009.09
	Fotang	87	0	0	55	0	0	
	Beiyuan	75	0	0	0	0	0	
Yongkang	Tangxian	85	0	0	50	0	0	2009.10
	Xinlou	80	0	0	45	0	0	
	Shizhu	0	0	0	0	0	0	
Wuyi	Baimu	75	0	0	60	18	30.00	2009.10
	Tongqin	80	0	0	0	0	0	
	Xilian	0	0	0	63	0	0	
Lanxi	Huangdian	85	0	0	55	0	0	2008.08
	Yongchang	0	0	0	53	0	0	
	Hengxi	87	0	0	63	0	0	
Dongyang	Qianxiang	85	0	0	55	0	0	2010.10
	Nanma	78	0	0	60	0	0	
	Hulu	85	0	0	0	0	0	
Panan	Xiwo	87	0	0	0	0	0	2009.09
	Lengshui	86	0	0	55	0	0	
	Dapan	83	0	0	60	0	0	
Pujiang	Tanxi	84	0	0	56	0	0	2008.10
	Hangping	85	0	0	37	1	3	
	Huangzhai	86	0	0	60	0	0	
Total number		1987	3	0.15	1252	81	6.47	

metacercariae of *Paragonimus* among the freshwater edible crabs collected. It was found that the infestation rate for the *Paragonimus* metacercariae in the crabs varied considerably. As presented in Table 1, approximate number of crabs collected in every site for examination was 60. In Langya, Shafan and Baimu cites, the infestation rate for the *Paragonimus* metacercariae was 53.33, 76.67 and 30.00%, respectively however, the infestation rate of other cites was near zero. Results also show that infestation means for Langya, Shafan, and Baimu was 2.00, 0.87 and 0.03, respectively. Infestation means for other cites was negative. As shown in Figure 1, we observed that the potential prevalence areas were located in a small district.

PCR amplification results of ITS2 and COI genes presented in *Paragonimus*

With the templates of genome DNA from different isolate, the fragments of ITS2 and COI from different adults of *Paragonimus* were amplified by PCR and visualized on a

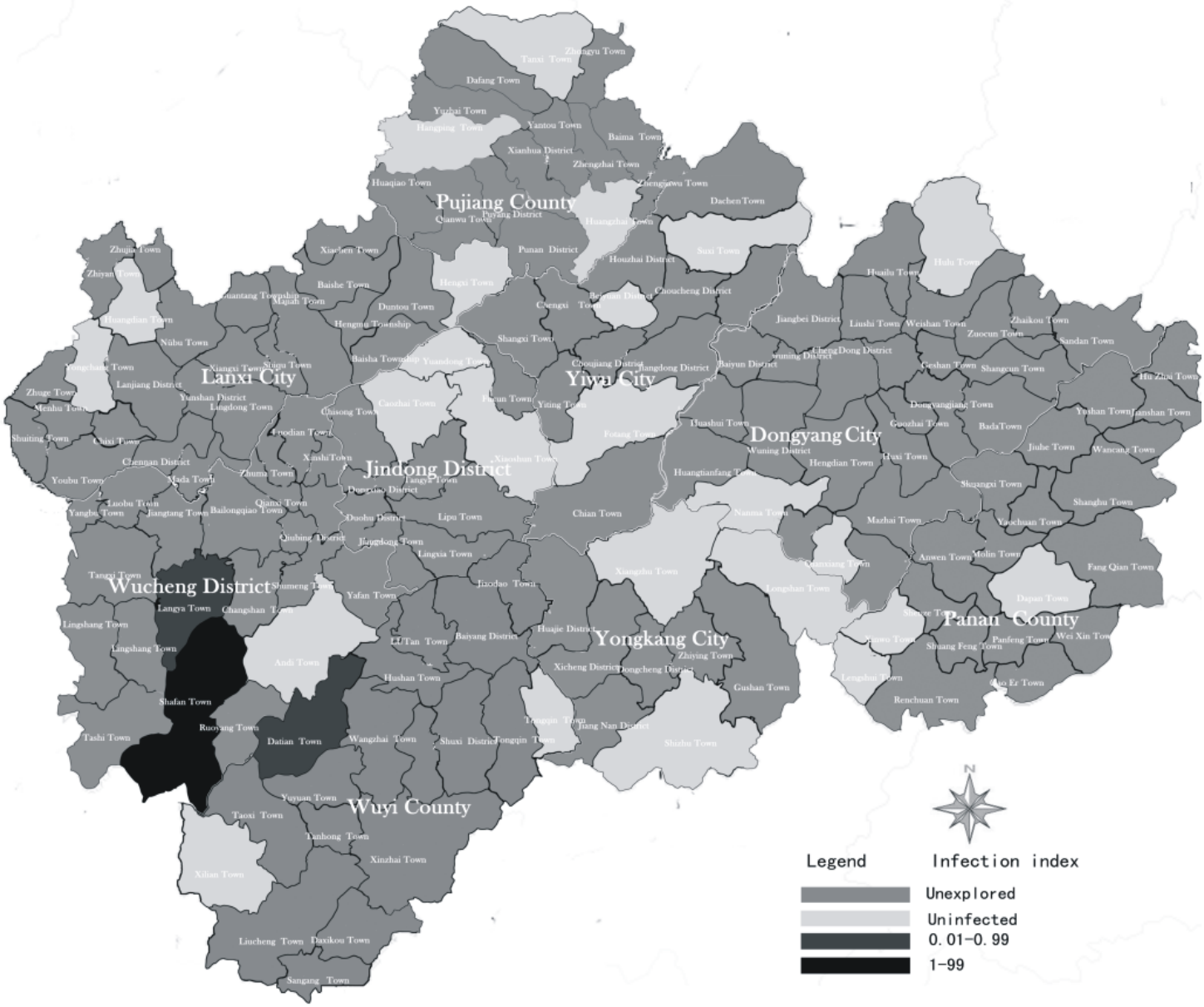

Figure 1. Distribution map of the intermediate hosts of *P. westermani* in Jinhua. All these three zones with high prevalence potential are located in the Niutoushan Hill Area. The altitude of Niutoushan Hill Area is significantly higher than other areas of Jinhua.

1.5% agarose gel. The results of agarose gel electrophoresis are shown in Figure 2A and C and Table 2. According to the size of these genes described previously, it showed that the size of amplification product was the expected size.

Analysis on the identity of ITS2 and COI genes from different *Paragonimus* isolates

After sequencing, the results of these DNA sequences were checked with chromas software and preserved. Then, the sequences were put into BioEdit software for comparison of their identity. The sequences alignments results and identity of ITS2 and COI genes from these isolates are 98.1 to 99.7% and 89.5 to 99.2%, respectively.

Phylogenetic trees of different *Paragonimus* isolates based on ITS2 and COI genes, respectively

Phylogenetic trees were constructed for several major *Paragonimus* species and isolates used in this study. The ITS2 and COI genes sequences from *P. skrjabini*, *P. westermani*, *P. veocularis*, *P. heterotremus* and so on

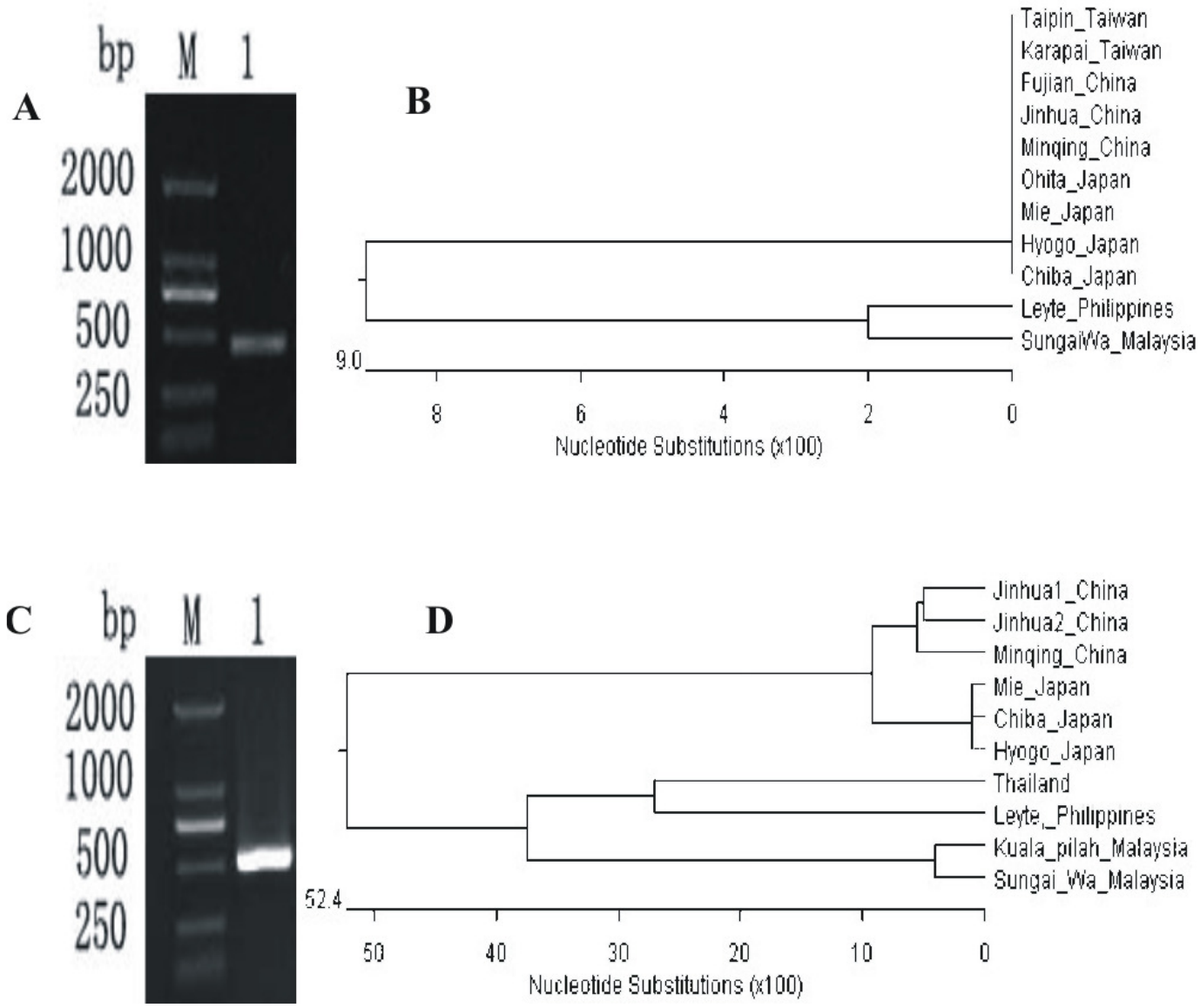

Figure 2. ITS2 and COI gene amplification and sequence analysis. A and C: PCR amplification results of ITS2 and COI genes from *P. westermani* isolated in Jinhua, China, M: Marker; 1: PCR amplification product of ITS2 and COI genes, respectively. B and D: Phylogenetic analysis of *P. westermani* among different strains from Taiwan, China, Japan, Philippines and Malaysia based on ITS2 and COI genes sequences, respectively.

Table 2. Nucleotide sequences of primers used in this study.

Primer	Sequence (5' to 3')	PCR product size (bp)
COIF	CCTGATTTTGCCTGGGTTTG	390
COIR	ACGACGAACCACGTATCAT	
ITS2F	ATATTGCGGCCACGGGTTA	363
ITS2R	GGTACTCACGTCTGATCCG	

were retrieved from GeneBank. These gene sequences were arranged with MEGA format and entered into MEGA for construction of the phylogenetic trees, as shown in figure 2B and D. The method for the construction was minimal evolution (ME) as applied previously. The ITS2 and COI sequences were highly conserved among three

isolates. However, the diversity of the COI gene among different isolates was higher than that of ITS2.

DISCUSSION

Previous studies indicated the presence of paragonimiasis in several areas of China, such as Guangdong, Fujian, Yunnan province and so on. This study was aimed to investigate the presence and epidemiology of paragonimiasis in the district of Jinhua which is next to Fujian Province. A total of 1987 snails and 1252 crabs from 27 villages of Jinhua were examined for the presence of *Paragonimus* eggs under a dissecting microscope. The results show an overall positive ratio of 0.15 and 6.47% for paragonimiasis in these zones. However, another interesting result could be noted from the Table 1. Infection ratios of the species from Langya, Shafan and Baimu villages were 53.33, 76.67 and 30.00%, respectively, which were significantly higher than that of other villages. This observation indicated the potential prevalence areas of Jinhua.

The rapid advancements in geographic information science allow us to better understand the spatial associations between a pathogen and its survival condition. After been analyzed by using geographic information system, geographical difference in prevalence could be traced to the rate of infestation of crabs. All of the streams in these three prevalence zones are located in the Niutoushan Hills Area. The altitude of Niutoushan Hill Area is significantly higher than other zones of Jinhua. What is more, vegetation of Niutoushan Hills Area has been kept well protected for a long time. The pollution of other areas was severer than Niutoushan Hills Area. It was indicated that the survival of *Paragonimus* may have some relationship with the altitude of the stream. Good environment protection and high water quality could be another key point for the presence of *Paragonimus*. These conclusions could also be used for the prediction of prevalence possibility of *Paragonimus* of other areas.

ITS2 and COI sequences have been widely used in intraspecific variation study in *Paragonimus* species (Lee and Huh, 2004; Nagaraja and Ranganath, 2004; Park et al., 2003). They tried to compare the difference of isolates of *Clonorchis sinensis* from one Korean (Kimhae) and two Chinese areas (Guangxi and Shenyang). The sequences of ITS 2 gene and CO1 gene from different isolates were compared respectively. The ITS2 and COI sequences were highly conserved among three isolates. These findings indicate that the Korean and two Chinese isolates are similar at the DNA sequence level, which is consistent with previous study that the genetic distances between three populations of Korean diploid and triploid *P. westermani* showed no significant difference in the nucleotide sequences of the mitochondrial cytochrome coxidase subunit I (mtCOI) and ribosomal second internal transcribed spacer (ITS2) genes.

In this research, ITS2 and COI genes were applied for *Paragonimus* identification and phylogenetic trees analysis. ITS2 and COI genes in *Paragonimus* from Jinhua were used to compare with that from other areas. As shown in Figure 2D, COI nucleotide sequences exhibited a few variable sites between *Paragonimus* strains from different areas. According to the diversity of COI gene, it was interesting to find that *Paragonimus* strains from different area were present in different cluster. Genetic position of *P. westermani* in Jinhua is especially close to the Mingqing strain, which was also isolated in China. However, the ITS2 gene sequences from different strains were almost the same (Figure 2B). There are only two clusters, three branches could be seen. Based on ITS2 gene sequences, we cannot distinguish *Paragonimus* strains from China, Taiwan and Japan. These conclusions show good accordance with that generated by Blair et al. (1996) They have reported that COI gene sequences from *Paragonimus skrjabini* and *Paragonimus miyazakii* showed a number of differences. While, ITS2 gene sequences were the same between *P. skrjabini* and *p. miyazakii*. So, these findings indicate that ITS2 gene is useful for *Paragonimus* strains identification, while COI gene is more suitable for *Paragonimus* strains typing.

In conclusion, our study makes much sense on the investigation of the potential prevalence areas of Jinhua. By using GIS, we observed that the survival of *Paragonimus* was associated with the altitude and environment of its habitation. Suitable altitude and good water quality are important for the presence of *Paragonimus*. Intraspecific difference between *Paragonimus* from Jinhua and other areas was analyzed by COI and ITS2 genes comparison. We suggest that both ITS2 and COI gene are useful in *Paragonimus* strains identification, however, COI gene can be more useful in *Paragonimus* typing and further be applied for the source tracing.

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