Analysis of Internal Transcribed Spacer Regions II Gene and Morphology of Paragonimus From Yunnan Province, China

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Research

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Abstract

For a long time, there is no clear-cut to identify some species of paragonimus in Yunnan Province, China. This paper involved the distribution of Paragonimus in Jinping country and Baoshan city, Yunnan province. In this experiment, the metacercariae, excysted metacercariae, eggs, adult worms were obtained from different hosts were observed and measured. Cats have been described as the appropriate definitive host of *paragonimus* sp., which are closed to *P. cheni* according to morphology. Especially the ovaries of adult worms are few and has no third branches. With SEM observation, their spines in the surface are single, a sharp-pointed knife or half-moon in shape and the end of a few of spines are bifurcate. While the clustered sequences strains of this study in the ITS2 tree clustered of Yunan is outsider of *P. skrjabini* complex and have the genetically greater distances than other isolates of the *P. skrjabini* complex. Therefore, the *Paragonimus* sp. of this study from Jinping County and Baoshan city are the same subspecies of *P. skrjabini* complex.

Introduction

*Paragonimus* is the pathogen of paragonimiasis that is a severe zoonosis. Around 50 species have been described since this genus was erected by Braun in 1899[1]. Among the *Paragonimus* most species are found in Asia. And 32 species have been reported in China. As the province that located in the southwestern border of China Yunnan’s climate and geography are profit to the life cycle of paragonimus. So It had been reported some species of paragonimus in Yunnan, like *Paragonimus heterotremus*[2], *P. proliferus*[3], *P. microrchis*[4], *P. bangkokensis*[5], *P. cheni*[6] and *P. skrjabini*[7].

*Pagumogonimus skrjabini* is reported in 1959 by Xintao Chen[8], regional distribution is very broad, mainly in Sichuan, Yunnan, Guizhou, Hubei, Fujian and other 14 provinces of China. *P. skrjabini* can cause cutaneous larva migrancy and visceral larva migrancy, the main performance is migratory subcutaneous nodules, but also can invade the liver, brain and other tissues, easy to cause clinical misdiagnosis. Although the sequences of *P. skrjabini* collected from Hubei province, Sichuan province, Fujian province, Guangdong province, Guangxi province and Yunnan Province had been done[8], the phylogenetic trees had been built. But only one samples from Yunnan and not described in detail about morphology, However, about species of *P. skjarbini* is lacking detailed research data collected from Yunnan. And the same condition happened in 2002, it was reported that there are *P. skjarbini* in Jinping county by researching little samples and Not described in detail.

Given the relative paucity of morphological characters available for distinguishing among Paragonimus species, the morphotypes were indistinguishable using light microscopy. Scanning electron microscopy has often been used to reveal details of subtle morphological features, such as tegumentary papillae, that might assist in taxonomy[9,10]. Given this background, it is important to discern whether different morphotypes of metacercariae represent different species, a task well suited to molecular studies. This approach has been used only rarely for metacercariae of Paragonimus species in Latin America.
Hernández and Cavaleiro[11,12] reported two different morphotypes of metacercariae in an ultrastructural study of the papillae on the ventral sucker.

To date, molecular studies have identified four complexes of Paragonimus species[13]. Among these are the *P. westermani* complex, including *P. westermani* and *P. siamensis*, while the *P. ohirai* complex, including numbers of nominal species such as *P. ohirai* and *P. harinasutai*[14]. Sequence data of the ribosomal RNA (rRNA) gene, in particular the two highly variable internal transcribed spacer regions (ITS1 and ITS2), have been successfully used to resolve taxonomic questions and to determine phylogenetic affinities among closely related paragonimius species and other parasites. At present, morphology is an important method to the taxonomy of the paragonimus. The size and layer of metacercaria, shape of ovary and spines of adult worms are still the main basis for the identification and classification of the species.

We then collected the crabs in distribution of *Paragonimus* in Jinping country and Baoshan city, Yunnan province, China. Metacercariae, excysted metacercariae, eggs, adult worms were observed and measured. The surface structures of adult worms were observed with scanning electron microscope (SEM). The sympathism of two hosts was compared. From these we can understand the infection condition of *Paragonimus* host (rats, cats and crabs). We sequence analyses of ITS2 genes of *Paragonimus* in Jinping county and Baoshan city in Yunnan province. All these were done to definite the genus relationship and genetic relationship with other *Paragonimus*.

**Methods**

**Parasitology**

Metacercariae were separated from crabs which collected collected at Jinping county and Baoshan city, Yunnan province, China. Freshly isolated metacercariae were used for morphological observation and experimental infection. Nine crabs were examined individually in detail to determine the prevalence of each *Paragonimus* species and their distribution in crab tissues. 20 rats and 3 cats were previously known to be free from Paragonimus. The feces of animals were examined 30 days from post infection and animals were sacrificed after 110 days of infection. Morphology of living metacercariae, eggs and the stained samples of excysted metacercariae and adult worms were carefully investigated and compared.

**Molecular analysis**

The metacercariae of Paragonimus were isolated from individual crabs which were collected from Jinping county and Baoshan city, Yunnan province, China. The metacercariae which are all taken from above places and the adult and the egg obtain from the cat that was infected by the metacercariae collected from Jinping county and Baoshan city as the molecular samples. These samples were immediately fixed in 100% ethanol and stored at 4°C before extracting the genomic DNA.
To amplify the nuclear ribosomal second internal transcribed spacer region 2 (ITS2), the following primers were used 3S (5′-GGT ACC GGT GGA TCA CTC GGC TCG TG-3′) and BD2 (5′-TAT GCT TAA ATT CAG CGG GT-3′) also previously described by Bowles and Bowles et al. (1993). The initial denaturazed step of ITS2 (95°C for 1 min) was followed by 35 cycles at 95°C(50 s), 68°C(2 min) and extension of 72°C(10 min). The quality of PCR products was indicating by electrophoresis in a 1% agarose gel. The amplified DNA products were separated by electrophoresis in a 1% low melting point agarose gel in TAE buffer. The polymerase chain reaction (PCR) products were purified using PCR purification kit, primed using Big-Dye terminator cycle sequencing kit v3.1 (ABI), and both strands directly sequenced by Sangon Biotech (Shanghai) Co..

**Phylogenetic analyses**

For one thing the first or last 24-29 base pairs (bp) of each sequence were uniformly excluded because in our sequences the first 24-29 base pairs beyond each primer were often rich in ambiguities. We got the ITS2 sequence which had 463 bp. All sequences are available from GenBank (Accession # EU769097-EU769103). Then all Aligned ITS2 sequences obtained in this study were compared with 39 sequences of each ITS2 genes of several Paragonimus species, respectively, obtained from previous studies species were obtained from previous study. Phylogenetic tree was constructed based on sequences. Phylogenetic tree was reconstructed based on sequence data sets ITS2. They were aligned using Clustal-X v1.83[15]with default options. The aligned matrix from this procedure was verified to have the same length, and minor adjustments were then made manually using SeaView v.4.2.5[16]. The data matrices are available from the corresponding author. The haplotype analyses were performed to 39 sequences using DAMBE software for 50 sequences[17]. Distances from the predicted amino acid sequences were determined with the p-distance models which were computed by MEGA v. 4.1[18].

Phylogenetic hypotheses of Leishmania were generated with ITS2 rRNA segments using two types of commonly applied phylogenetic method: heuristic searches using equally weighted maximum parsimony (MP) analyses performed with the program PAUP*[19] and Bayesian inference (BI) with the program MrBayes v.3.2[20]. For heuristic searches under parsimony, invariant characters were removed from the dataset, and all remaining characters were treated as equally weighted. Each search involved ten random addition replicates, one tree held at each step, TBR branch swapping, steepest descent on, and a maximum of 10,000 saved trees; all other search settings were left at default values. Non-parametric bootstrapping was used to generate phylogeny confidence values[21], with 1,000 pseudoreplicates using a heuristic tree search for each pseudoreplicate. Euparagonimus cenocopious (AF159601). Because intraspecific gene evolution cannot always be represented

Phylogenetic hypotheses of paragonimus were generated with ITS2 segments using Bayesian inference (BI) using the MrBayes v.3.2 program. In BI analyses, gaps were treated as missing data. Euparagonimus cenocopious (AF159601) was used to root the trees. Prior to Bayesian analyses, the best-fit model of evolution, HKY+G, was selected using Modeltest 3.7 under the Bayesian information criterion, following recent recommendations. We estimated the posterior probability distributions by allowing four
incrementally heated Markov chains (default heating values) to proceed to four million generations, and with samples were taken every 200 generations. Analyses were repeated beginning with different starting trees to ensure that the analyses were not restricted from the global optimum. Convergence was first tested by examining the average deviation of the split frequencies of the two runs, in order to determine whether the two runs had converged. MCMC convergence was also explored by examining the potential scale reduction factor (PSRF) convergence diagnostics for all model parameters (provided by the sump and sumt commands). The first one million generations before this chain became stationary were discarded, and the remaining samples from the independent runs were pooled to obtain the final approximation of the posterior tree distribution.

Because intraspecific gene evolution cannot always be represented by a bifurcating tree, haplotype networks may more effectively portray the relationships among haplotypes within species[22]. Therefore, we constructed unrooted parsimony networks of haplotypes for P. skrjabini complex and Paragonimus sp. (see below) using TCS v.1.21[23].

Results

The morphology of paragonimus

The shape of metacercaria from Jinping country and Baoshan city close to spherical. The capsule wall of metacercariae almost is monoptychial and the size of its from Jinping country are 0.401 mm×0.395mm, from Baoshan city are 0.435mm×0.433mm (Fig. 1a-b). Adult worms are all elliptic in shape and the average size from Jinping country and Baoshan city are 10.107 mm×4.064mm and 9.59mm×43.577mm respectively (Fig. 2). The rate of width to length of each adult worm from Jinping country and Baoshan city are 1:2.49 and 1:2.63 respectively. The size of their ventral sucker larger than oral sucker. The ovarys of these adults are all near the ventral sucker, the branches are simple. The testes’ centrosomes are small, and the length are about 1/6 of the body. With SEM observation there are single spines in the surface of adult worms, the spines before the ventral sucker are triangle and a sharp-pointed knife in shape, after the ventral sucker are almost half-moon in shape and the end of a few of spines are bifurcate (Fig. 3-4). The morphology of metacercariae, excysted metacercariae, eggs from Jinping country are nearly resemble but there are few different from the morphology of its from Baoshan city (Fig. 1c-f). The morphology of adult worms is identical.

The natural infection rate of the second intermediate host with Paragonimus from Jinping country and Baoshan city are 79.9% and 100%. The quantity of each crab infects metacercariae which were collected from Jinping country and Baoshan city are 1.8 and 13.3 respectively. The rate of worms recovered from experimentally infected rats and cats which infected metacercaria from Jinping country are 24% and 55%. The rate of worms recovered from rats and cats which infected metacercaria from Baoshan city are 5% and 67.7%.
Molecular phylogenetic analyses

We obtained the complete 7 ITS2 of *Paragonimus* collected from Jinping county gene sequences in the GenBank nucleotide sequence databases with the accession numbers (EU769097- EU769103), from the metacercariae, adult and eggs samples. The ITS2 alignment was 463 bases in length separately. All the above sequences were completely identical with each other. These sequences and several related species within genus Paragonimus obtained from GenBank which had been aligned by us constructed the phylogenetic trees separately in the sequence data set of ITS2 (Table 1-2) genes. P distances among the Paragonimus species except *Paragonimus* sp. ranged from 0.004 (between *P. proliferus* and *P. miyazakii*) to 0.107 (between *P. vietnamensis* and *P. ohirai*). Most pairwise comparisons mentioned above had divergence values of less than 0.107, with 0.073 on average. Meanwhile, the divergence between *Paragonimus* sp. and other species ranged from 0.013 (*Paragonimus* sp. versus *P. skrjabini India*) to 0.019 (*Paragonimus* sp. vs. *P. westermani*), with an average of 0.017 (Table 3). P distances among subspecies of *P. skrjabini complex* (*P. skrjabini, P. miyazakii and P. proliferus*) ranged from 0.004 (between *P. proliferus* and *P. miyazakii*) to 0.009 (between *P. skrjabini* and *P. proliferus*), with an average of 0.006 (Table 3).

Phylogenetic relationships

The heuristic search of the ITS2 matrix resulted in 10,000 equally parsimonious trees of 146 steps, with high values of CI (0.8220) and RI (0.8779). In the strict consensus phylogram (Fig. 5), 14 isolates in China formed a branch (H2 and H3 BP=64%), *Paragonimus* sp. (13 isolates from China shared a haploid H2 with 2 isolates from Xishuangbanna, Yunnan and 9 isolates from Vietnam) that was sister to the remaining members of subspecies *P. skrjabini India* (BP=52%). The haplotype H6 of proliferus was clustered with six haploids (H1, H5, H7-10) of *P. skrjabini (P. miyazakii)* (BP = 69%), and then, forming a cluster with Paragonimus sp., that formed a clade with *P. vietnamensis* (BP = 52%), juxtaposition with *P. heterotremus (P. pseudoheterotremus)*. This branch went on with forming one clade with *P. ohirai, P. bangkokensis* and *P. harinasutai. P. westermani* is the base of Paragonimus.

For the BI analyses, the likelihood value of the 50% majority consensus tree (Fig. 6) was ln L=-1377.24. The average PSRF was 1.001. Overall, as well with maximum parsimony analyses, Paragonimus sp. which is composed of 14 geographical strains from Jinping and Baoshan in Yunnan, China, 2 strains from Xishuangbanna in Yunnan and 9 strains from Vietnam forms an evolutionary clade with a high posterior probability of 0.99, was sister to the *P. skrjabini India* strain from India (PP = 0.80). Similarly, the subspecies of *P. skrjabini complex* include H6 (*P. proliferus*), one of the six haploids (H1, H5, h7-10) of *P. skrjabini (P. miyazakii)* cluster (PP = 0.79) is juxtaposed with Paragonimus sp. The relationship of the remaining Paragonimus species is similar to that of Fig. 5.

Haplotype network

To get additional insight into the relationships among the *P. skrjabini complex* strains, we analyzed our data set, using the coalescent-based statistical parsimony network approach. The network of 10
haplotypes was shown as Fig. 7. H5 (strains from Fujian, China) and H1 seemed to be central haplotypes with 2 mutational steps. *P. skrjabini* (isolates from Sichuan and Hubei, China) and *P. miyazakii* (isolates from Japan and Hubei, China) share the one haplotype H1. *P. skrjabini* (H4, H5, H7, H10 and parts of H1) have a wider geographical distribution and revealed much more polymorphism than *P. miyazakii* (H8, H9 and other part of H1). The strains of this study and other strain of Yunnan reported by Blair previously share one haplotype H2 with that from Vietnam. And H6, shared by *P. proliferus* from Yunnan, China and Vietnam. H1 (*P. skrjabini* and *P. miyazakii*) was most closely related to the H9 (*P. skrjabini* from Fujian, China), H7 (*P. skrjabini* from Guangxi, China) and H8 (*P. miyazakii* from Japan, with one mutational step. H10, shared by four strains from Guangdong, China, was also closely related to H9, with two mutational steps. Having an advantage over the bifurcating tree in detail at the intraspecific level, the haplotype network could intuitively reflect the genetically greater distances between the singleton (H4 from India) and H2 (4 mutational steps, see Fig. 7). *P. proliferus* (H6) has 3 mutational steps genetically distances with H1 and one mutational step genetically distances with H5. Strains of this study (H2) have the genetically greater distances with the two-central haplotype (H1 with 7 mutational steps, H5 with five mutational steps; see Fig. 7).

Numbers of haplotypes correspond to Table 1. In the network, solid circles indicate sampled haplotypes; small hollow circles indicate unsampled or extinct haplotypes. Each mutation step is shown as either a short or longer line connecting neighboring haplotypes (including observed and unobserved one). The size of the solid circles roughly represents the numbers of strains carrying the haplotype, with the scale given beside the network; different filled patterns represent the corresponding geographical origin from which the haplotype was sample.

**Discussion**

Up to now, it has 150 years in history since we study *Paragonimus*. The shape of metacercariae is important to the taxology of *Paragonimus*[36]. The capsule wall of metacercariae from Jinping and Baoshan are almost monoptychial. We changed the method that separate metacercariae under platoscope directly, which Zhou Benjiang has been used[37]. Then, the metacercariae. We had are all most monoptychial. Metacercariae with the single cyst wall are *Piloktsuenensis* Chen, 1940, *Paragonimus fukienensis* Tang and Tang, 1962, *P. cheni*, *P. proliferus*, *Pcaliensis*, *Puterobilateralis*, but there are only first 4 species distributing in China [38]. These four species are difference in the size. The size of metacercariae from Jinping and Baoshan are near to *P. cheni*.

The adult worms from Jinping and Baoshan are similar which figures are oval-shap and every rate of width to length are over 1:2.4. *Paragonimus* have been divided into 3 species according to figure and rate of width to length of adult worms[39]. Among those, which figures are fusiform shape and every rate of width to length are over 1:2.4, are *Pagumogonimus skrjabini*, *P. cheni*, *P. macrorchis* and *P. proliferus* and so on. But the size of metacercariae from Jinping and Baoshan are very difference from *P. macrorchis* and *P. proliferus*, which size are 0.280mm×0.259mm[36] and over 1mm respectively. The type of ovaries of adult worms from Jinping are similar with *P. skrjabini* and *P. cheni*[40] which branches are few and has
no third branches. We observed 4 adult worms from Jinping and Baoshan respectively and the spines are resembled. There are single spines in the surface, and the spines before the ventral sucker are triangle and a sharp-pointed knife in shape, after the ventral sucker are almost half-moon in shape and the end of a few of spines are bifurcate.

Different species of *Paragonimus* have different infection efficiency across various animal host. In this experiment, rats and cats have been infected, and cats have been described as the appropriate definitive host of *paragonimus* from Jinping and Baoshan. Cats are suitable definitive host of *P. cheni* [36]. It indicates that the *Paragonimus* from Jinping and Baoshan are the same species according to that the morphology and the result of SEM. Although the morphology are both similar with *P. skrjabini* and *P. cheni*.

In the present study, we determined the DNA sequences of ITS2 genes of *Paragonimus* obtained from Jinping and Baoshan in Yunnan province where is in southern. Farther more our samples form one clade with *P. skrjabini* harvested from Xishuangbanna Yunnan and *P. skrjabini* harvested from Vietnam [8, 26]. In case of ITS2 gene, our samples of two places and the sequences of *P. skrjabini* yn are 100% identified with each other except the egg samples. So, the *Paragonimus* from three places of Yunnan are one species. In our previous study, *P. hokuoensis* [8] suggesting that *P. hokuoensis* is highly likely be a synonymy of *P. proliferus* [3]. And Blair et al. recognise two species within the *P. skrjabini* complex: *P. hokuoensis* in southern Yunnan and *P. skrjabini* in Guangdong, Sichuan, Hubei, Fujian and Japan (and other Chinese Province not sampled by them.) before this our research [8]. And in our study the clustered sequences of Yunnan include our samples and samples in Xishuangbannan of Yunan in the ITS2 tree clustered of Yunan is out sider of *P. skirjabini* complex. While haplotype networks show that Strains from this study, Xishuangbannan of Yunan and Vietnam (H2) have the genetically greater distances with the two-central haplotype (H1 with 7 mutational steps, H5 with five mutational steps) than that H6, shared by *P. proliferus* from Yunnan, China and Vietnam. *P. proliferus* (H6) has 3 mutational steps genetically distances with H1 and one mutational step genetically distances with H5. Therefore, the clade of our sequences with the sequences of *P. skrjabini* yn [8] is a distinct subspecies in the *P. skirjabini* complex like *P. proliferus*.

**Conclusions**

The results of morphology indicate that the *Paragonimus* from Jinping and Baoshan are the same species and both similar with *P. skrjabini* and *P. cheni*. Meanwhile, the analysis of phylogenetic and network show that the clade of our sequences with the sequences of *P. skrjabini* yn is a distinct subspecies in the *P. skirjabini* complex like *P. proliferus*. Thus, according to our morphology and molecular result, the *Paragonimus* sp. of this study from Jinping County and Baoshan city and the strain of Xishuangbanna are the same species, which is a distinct subspecies in the *P. skirjabini* complex like *P. proliferus* and belong to the *P. skirjabini* complex.

**Declarations**
Availability of data and materials

Raw sequence data are available in NCBI GenBank.

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Contributions

BBY participated in Molecular analysis, Phylogenetic analyses, the analytical design, interpretation, writing the draft of the manuscript and revisions. JL participated in collecting specimen, animal experiment and morphological analysis. BJZ contributed to the conception and design of the project, analytical design, interpretation, writing the draft of the manuscript and revisions. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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Tables

Due to technical limitations, table 1 to 3 is only available as a download in the Supplemental Files section.

Figures
Figure 1

Metacercariae: Excysted metacercariae and Eggs from Host a Metacercariae from Jinping single cyst wall ×100, b Metacercariae from Baoshan single cyst wall ×100, c Excysted metacercariae from Jinping ×100, d Excysted metacercariae from Baoshan ×100, e Egg from Jinping (×400, From cat's stool, 80 days), f Egg from Baoshan (×400, From cat's stool, 80 days).
Figure 2

Adults from Two Places of Yunnan, China a Adult worm from Jinping (×10, From cat’s lung, 110 days) b Adult worm from Baoshan (×10, From cat’s lung, 110 days)
Figure 3

SEM of adult worms a Abdominal view of anterior portion×35, Jinping b Oral sucker×200, Jinping c Abdominal view of anterior portion×35, Baoshan d Oral sucker×200, Baoshan e Ventral sucker×180, Jinping f Genital pore×500, Jinping g Ventral sucker and genital pore×70, Baoshan h Genital pore×400, Baoshan

Figure 4

SEM spines in the surface of adult worms a Spines after oral sucker (×2000, Jinping) b Spines of later part between two suckers (×3500, Jinping) c Spines between two testes (×4500, Jinping) d Spines near the posterior end of body (×3500, Jinping) e Spines after oral sucker (×2000, Baoshan) f Spines between two suckers (×1800, Baoshan) g Spines between two testes (×4000, Baoshan) h Spines near the posterior end of body (×1000, Baoshan)
Figure 5

Maximum parsimony consensus tree from 1,000 bootstrap replicates of ITS2 dataset by using PAUP*. Numbers above the branch represent percent recovery in bootstrap analysis (1,000 pseudoreplicates). Tree length=146, CI=0.8220, RI=0.8779
Figure 6

The 50% majority-rule consensus tree inferred from Bayesian inference of ITS2 dataset by using MrBayes v. 3.2. Numbers at nodes represent Bayesian posterior probabilities.
Figure 7

Statistical parsimony network showing genetic relationships and distance among 10 haplotypes of P. skrjabini complex from different countries. Numbers of haplotypes correspond to Table 1. In the network, solid circles indicate sampled haplotypes; small hollow circles indicate unsampled or extinct haplotypes. Each mutation step is shown as either a short or longer line connecting neighboring haplotypes (including observed and unobserved one). The size of the solid circles roughly represents the numbers of strains carrying the haplotype, with the scale given beside the network; different filled patterns represent the corresponding geographical origin from which the haplotype was sampled.

Supplementary Files

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