Molecular identification of *Clonorchis sinensis* and discrimination with other opisthorchid liver fluke species using multiple Ligation-dependent Probe Amplification (MLPA)

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**Abstract**

**Background:** Infections with the opisthorchid liver flukes *Clonorchis sinensis*, *Opisthorchis viverrini*, and *O. felineus* cause severe health problems globally, particularly in Southeast Asia. Early identification of the infection is essential to provide timely and appropriate chemotherapy to patients.

**Results:** In this study we evaluate a PCR-based molecular identification method, Multiplex Ligation-dependent Probe Amplification (MLPA), which allows rapid and specific detection of single nucleotide acid differences between *Clonorchis sinensis*, *Opisthorchis viverrini* and *O. felineus*. Three probe pairs were derived from the Internally Transcribed Spacer 1 (ITS1) of three opisthorchid liver flukes using a systematic phylogenetic analysis. Specific loci were detected in all three species, yielding three amplicons with 198, 172 and 152 bp, respectively, while no cross reactions were observed. A panel of 66 *C. sinensis* isolates was screened using MLPA. All species were positively identified, and no inhibition was observed. The detection limit was $10^3$ copies of the ITS gene for the three liver flukes, or about 60 pg genomic DNA for *Clonorchis sinensis*. Amplification products can be detected by electrophoresis on agarose gel or in a capillary sequencer. In addition, genomic DNA of *Clonorchis sinensis* in fecal samples of infected rats was positively amplified by MLPA.

**Conclusion:** The flexibility and specificity make MLPA a potential tool for specific identification of infections by opisthorchid liver flukes in endemic areas.

**Background**

*Clonorchis sinensis* (*C. sinensis*), *Opisthorchis viverrini* (*O. viverrini*) and *Opisthorchis felineus* (*O. felineus*) (Opisthorchiidae) are among the most frequent endemic food-borne liver flukes, causing severe clonorchiasis and opisthorchiasis. Humans contract the disease through consumption of raw or inadequately cooked freshwater fish containing the infective metacercariae. About 35 million people are infected with *C. sinensis* globally. Main endemic areas are located in southern Asia including China, Korea, Japan, Taiwan and Vietnam [1]. In China the estimated infection by *C. sinensis* is 15 million [2,3]. Approximately 9 million people are infected with *O. viverrini* in Thailand, Cambodia, and Laos [4-6]. In eastern Europe 1.2 to 1.5 million patients are infected with *O. felineus* [7]. In recent years, endemic areas of liver flukes are expanding to North America and Europe due to fish import and immigration [7-9].

Current clinical diagnosis of liver fluke infection is by direct microscopy of eggs in feces. However, this procedure is time-consuming and inaccurate, resulting in false-negatives due to the difficulty to distinguish eggs from each other or from those of closely related heterophyids [10-12]. As a result, appropriate chemotherapy may be delayed. Hence there is an urgent need for a novel tool to diagnose the infection.
A number of methods have been developed to identify or detect liver flukes using DNA, mRNA or protein. Among PCR-based molecular methods, nested-PCR [13] and loop-mediated isothermal amplification (LAMP) [14] are particularly promising [15-19]. However, simple PCR amplification carries the risk of false-negative data, due to PCR inhibitors involved in complex samples [20,21]. Moreover, mixed liver fluke infection may also hamper the application of simple PCR [22]. As an alternative, multiplex PCR may achieve high efficiency with simultaneous amplification of two or more genetic loci in one reaction, while this also may reduce the number of false-negative or false-positive results [23]. Although multiplex PCR has been reported to discriminate between C. sinensis and O. viverrini [24], it is technically difficult to optimize PCRs for amplification of multiple genes or loci, because each primer pair requires a different optimal combination of reagents and annealing temperatures. To overcome this problem we developed a multiplex PCR amplification technique using multiplex ligation-dependent probe amplification (MLPA).

MLPA is a simple, robust and fast method designed for simultaneous detection of specific genomic sequences targeting multiple mutations to amplify specific MLPA probes rather than target DNA [25]. In MLPA (Figure 1), two oligonucleotides (up to 45 pairs in one reaction) hybridize immediately on the target DNA. In addition to a target-specific sequence, each of the oligonucleotides contains one of two sequences recognized by a universal PCR primer pair. After denaturing and hybridization, two parts of each MLPA probe are ligated by a specific ligase enzyme, followed by PCR amplification using a universal primer pair. Non-hybridized probes are not removed, enabling a high throughput ‘one-tube’ method. MLPA probes are designed in such a way that each amplification product is identified by size using separation by capillary electrophoresis. Differences in relative probe signals between samples reflect differences in the probe target sequences. MLPA is widely used to identify point mutations [26], insertions [27], deletions [28], duplications [29], and recombination events [30] and is also applied for quantification of mRNAs [31] and determination of methylation status of CpG islands [32].

The elegance and simplicity of MLPA makes it applicable to various types of clinical samples, such as blood [33], amniotic fluid [34] or tumor tissue [35]. In pathogen detection, MLPA has only been applied to Mycobacterium tuberculosis [36], bacterial species in oral biofilms [37], respiratory viruses [38] and Penicillium marneffei [39], but not to parasites. In this study, we evaluate MLPA for the rapid identification of the opisthorchid liver flukes C. sinensis, O. viverrini and O. felineus, and establish specificity of the method to discriminate these three liver flukes in a single-tube reaction.

Results
In this study, the MLPA assay was adapted to identify C. sinensis, and discriminate with other opisthorchid liver flukes, O. viverrini, and O. felineus. We first performed a haplotype analysis of the three liver flukes and of phylogenetically related species to search for unique loci for MLPA probe design (Figure 2). Three specific loci were selected for designing species-specific pairs of oligonucleotide probes for MLPA (Table 1). Web-based BLAST analysis showed a low degree of similarity of the specific probes compared with other parasites. Three artificial templates of C. sinensis, O. viverrini and O. felineus...
were used to evaluate the specificity and sensitivity of the MLPA assay. MLPA reactions without artificial templates were used as negative controls. Artificial templates of padlock probes were used to evaluate the detection limits of the MLPA assay. These pairs of probes allowed specific amplification of the ITS1 gene of *C. sinensis*, *O. felineus* and *O. viverrini*, and yielded three amplicons of sizes 198, 170, and 152 bp, respectively (Figure 3). The amplicons were 100% consistent with the initial DNA used in each MLPA reaction. The detection limit of the MLPA assay for artificial DNA was found to be approximately 10^3 copies of the ITS gene for the three liver flukes analyzed (Figure 4) or 60 picogram genomic DNA for *C. sinensis* (data not shown) after the amplification products were visualized by electrophoresis on a 5% agarose gel. A total of 66 different DNA samples of adult liver flukes were tested and showed that the pairs of MLPA probes were able to amplify all loci presented in the samples (Table 2). No inhibition was observed after the addition of the same concentration of *Opisthorchis viverrini* or *Opisthorchis felineus* artificial template DNA (Table 2).

The MLPA products could be successfully detected using a capillary sequencer (Figure 5). Moreover, specific amplification was also achieved by the use of fecal samples from infected rats (Table 3).

**Discussion**

The use of ligated oligonucleotide probes in MLPA, and a number of other methods, allows specific detection of changes in single nucleic acids of targeted genes [40-42]. These probes hybridize to and capture these areas, which are then enriched through rolling circle amplification or by PCR. Using these probes we were able to simultaneously detect gene loci and characterize different strains in a single reaction. In the present study we evaluate the MLPA assay to identify and discriminate three opisthorchid liver flukes. The sensitivity and specificity of MLPA highlights that the method is a useful tool for prompt and accurate diagnosis, pathogen characterization, and epidemiological studies of parasite infections.

In our initial test, we evaluated the MLPA assay to identify and discriminate three liver flukes using artificial templates. All of the three probe pairs used allow specific amplification of the ITS1 locus of each respective species. The MLPA reaction was sensitive enough to detect 10^3 copies of artificial template DNA. This is consistent with previous studies on MLPA in oral biofilm, where DNA was detected at picogram levels [37]. The size of the *C. sinensis* genome varies from 500 to 700 Mbp (Wang et al., unpublished data), and 1 pg of DNA is equal to 978 Mbp of genomic DNA [43]. The weight of *C. sinensis* DNA is approximately 0.511-0.716 pg and the 10^3 copies of *C. sinensis* DNA detectable by MLPA is then roughly equivalent to 0.5-70 pg of genomic DNA. These results were consistent with the 60 pg genomic DNA of *C. sinensis* mentioned above. Results were comparable to a previous study indicating that the sensitivity of MLPA is equivalent to real-time PCR [38], while similar results were obtained in some studies focusing on *O. viverrini* [24] and *C. sinensis* [18]. However, our results deviate from a previous report where with simple PCR a detection limit of 10^{-12} ng was
obtained [17]. This might be explained by the use of different target genes or by different copy numbers of target genes in the genome.

Furthermore, the results of the inhibition test indicated that the MLPA assay was not inhibited by the presence of non-target DNA. These data demonstrate a considerable potential for MLPA in future clinical applications, which normally involve complex DNA mixtures. Although enhancement of sensitivity requires further optimization to capture low copy numbers of template DNA, the alternative strategy would be to increase the number of probe pairs.
Table 2 Evaluation of the three probe pairs in 66 different strains of *C.sinensis* isolates

| No. | GenBank   | Source                  | CsPL+OfPL+OvPL+Ov−T+Of−T | CsPL+OfPL+OvPL+Of−T | CsPL+OfPL+OvPL |
|-----|-----------|-------------------------|--------------------------|---------------------|----------------|
|     |           |                         | 198bp | 170bp | 152bp | 198bp | 170bp | 152bp | 198bp | 170bp | 152bp |
| 1   | HQ874538  | Cat, Anhui, China       | +     | +     | +     | +     | +     | -     | +     | -     | -     |
| 2   | HQ874523  | Cat, Anhui, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 3   | HQ874584  | Cat, Anhui, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 4   | HQ874537  | Cat, Anhui, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 5   | HQ874599  | Cat, Anhui, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 6   | HQ874585  | Cat, Anhui, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 7   | HQ874586  | Cat, Anhui, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 8   | HQ874588  | Cat, Anhui, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 9   | HQ874540  | Cat, Guangdong, China   | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 10  | HQ874535  | Cat, Guangdong, China   | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 11  | HQ874541  | Cat, Guangdong, China   | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 12  | HQ874602  | Cat, Guangdong, China   | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 13  | HQ874587  | Cat, Guangdong, China   | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 14  | HQ874532  | Cat, Guangdong, China   | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 15  | HQ874581  | Cat, Guangdong, China   | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 16  | HQ874582  | Cat, Guangdong, China   | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 17  | HQ874542  | Cat, Guangxi, China     | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 18  | HQ874536  | Cat, Guangxi, China     | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 19  | HQ874543  | Cat, Guangxi, China     | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 20  | HQ874529  | Cat, Guangxi, China     | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 21  | HQ874580  | Cat, Guangxi, China     | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 22  | HQ874533  | Cat, Guangxi, China     | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 23  | HQ874525  | Cat, Guangxi, China     | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 24  | HQ874579  | Cat, Guangxi, China     | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 25  | HQ874544  | Cat, Hubei, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 26  | HQ874545  | Cat, Hubei, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 27  | HQ874593  | Cat, Hubei, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 28  | HQ874578  | Cat, Hubei, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 29  | HQ874539  | Cat, Hubei, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 30  | HQ874592  | Cat, Hubei, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 31  | HQ874546  | Cat, Hubei, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 32  | HQ874547  | Cat, Hubei, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 33  | HQ874524  | Cat, Hubei, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 34  | HQ874601  | Cat, Henan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 35  | HQ874530  | Cat, Henan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 36  | HQ874597  | Cat, Henan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 37  | HQ874595  | Cat, Henan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 38  | HQ874573  | Cat, Henan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 39  | HQ874572  | Cat, Henan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 40  | HQ874571  | Cat, Henan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 41  | HQ874589  | Cat, Henan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 42  | HQ874598  | Cat, Hunan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 43  | HQ874590  | Cat, Hunan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 44  | HQ874591  | Cat, Hunan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 45  | HQ874551  | Cat, Hunan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 46  | HQ874534  | Cat, Hunan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 47  | HQ874552  | Cat, Hunan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 48  | HQ874553  | Cat, Hunan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 49  | HQ874554  | Cat, Hunan, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
| 50  | HQ874555  | Dog, Jilin, China       | +     | +     | +     | +     | -     | +     | -     | -     | -     |
efficiency of the MLPA reaction, or the employment of more sensitive detection equipment. The first option might be achieved by the addition of more efficient amplification facilitators such as dimethyl sulfoxide [44], dithiothreitol [45], betaine [46], bovine serum albumin and single-stranded DNA binding T4 gene 32 protein (gp32) [47]. For the later option, a real time detector could be used to track the limited fluorescent-labeled amplicons [38]. The results would be comparable with those of capillary electrophoresis or of fragment analysis of fluorescent-labeled amplicons. However, the electrophoresis maybe the optimized method to detect MLPA products for unequipped laboratory or lab of local hospital [25,48].

Table 2 Evaluation of the three probe pairs in 66 different strains of _C. sinensis_ isolates  

(Continued)

|   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|
| 51 | HQ874556 | Dog, Jilin, China | + | + | + | + | + | + | + |
| 52 | HQ874557 | Dog, Jilin, China | + | + | + | + | + | + | + |
| 53 | HQ874570 | Dog, Jilin, China | + | + | + | + | + | + | + |
| 54 | HQ874528 | Dog, Jilin, China | + | + | + | + | + | + | + |
| 55 | HQ874527 | Dog, Jilin, China | + | + | + | + | + | + | + |
| 56 | HQ874558 | Cat, Jiangsu, China | + | + | + | + | + | + | + |
| 57 | HQ874566 | Cat, Jiangsu, China | + | + | + | + | + | + | + |
| 58 | HQ874559 | Cat, Jiangsu, China | + | + | + | + | + | + | + |
| 59 | HQ874530 | Cat, Jiangsu, China | + | + | + | + | + | + |
| 60 | HQ874583 | Cat, Jiangsu, China | + | + | + | + | + | + |
| 61 | HQ874569 | Cat, Jiangsu, China | + | + | + | + | + | + |
| 62 | HQ874604 | Cat, Jiangsu, China | + | + | + | + | + | + |
| 63 | HQ874565 | Cat, Jiangsu, China | + | + | + | + | + | + |
| 64 | HQ874560 | Cat, Jiangsu, China | + | + | + | + | + | + |
| 65 | HQ874561 | Cat, Jiangsu, China | + | + | + | + | + | + |
| 66 | HQ874564 | Cat, Jiangsu, China | + | + | + | + | + | + |
| 67 | HQ874567 | Negative control | - | - | - | - | - | - | - |

CsPL: _Clonorchis sinensis_ Padlock probe pairs  
OrPL: _Opisthorchis felineus_ Padlock probe pairs  
OvPL: _Opisthorchis viverrini_ Padlock probe pairs  
Cs-T: Artificial template of _Clonorchis sinensis_ Padlock probe pairs  
Ov-T: Artificial template of _Opisthorchis viverrini_ Padlock probe pairs  
Of-T: Artificial template of _Opisthorchis felineus_ Padlock probe pairs

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Figure 5 Electropherogram showing peaks generated by MLPA. Template DNA from individual species is used in the reaction. The MLPA reaction included all the 3 probes designed, Rox 500 as internal molecular standard. Fragment sizes (bp) correspond to: 198 = _C. sinensis_, 170 = _O. felineus_, 152 = _O. viverrini_.

Table 3 Fecal samples of infected rats and data from the three different detection methods

| Fecal Samples no. | Microscopy detection | ITS PCR First Run | ITS PCR Second Run | CsPL | OvPL | OfPL |
|-------------------|----------------------|-------------------|-------------------|------|------|------|
| 1                 | +                    | -                 | +                 | +    | -    | -    |
| 2                 | +                    | -                 | +                 | +    | -    | -    |
| 3                 | +                    | -                 | +                 | +    | -    | -    |
| 4                 | +                    | -                 | +                 | +    | -    | -    |
| 5                 | +                    | -                 | +                 | +    | -    | -    |
| 6                 | +                    | -                 | +                 | +    | -    | -    |
| 7                 | +                    | -                 | +                 | +    | -    | -    |
| 8                 | +                    | -                 | +                 | +    | -    | -    |
| 9                 | +                    | -                 | +                 | +    | -    | -    |
| 10                | +                    | -                 | +                 | +    | -    | -    |
| 11                | +                    | -                 | +                 | +    | -    | -    |
| 12                | +                    | -                 | +                 | +    | -    | -    |
| 13                | +                    | -                 | +                 | +    | -    | -    |
| 14                | +                    | -                 | +                 | +    | -    | -    |
| 15                | +                    | -                 | +                 | +    | -    | -    |
| 16                | +                    | -                 | +                 | +    | -    | -    |
| 17                | +                    | -                 | +                 | +    | -    | -    |
| 18                | +                    | -                 | +                 | +    | -    | -    |
| 19                | +                    | -                 | +                 | +    | -    | -    |
| 20                | +                    | -                 | +                 | +    | -    | -    |
| 21                | +                    | -                 | +                 | +    | -    | -    |
| 22                | +                    | -                 | +                 | +    | -    | -    |
| 23                | +                    | -                 | +                 | +    | -    | -    |
| 24                | +                    | -                 | +                 | +    | -    | -    |
| 25                | +                    | -                 | +                 | +    | -    | -    |
| 26                | +                    | -                 | +                 | +    | -    | -    |
| 27                | +                    | -                 | +                 | +    | -    | -    |
| 28                | +                    | -                 | +                 | +    | -    | -    |
| 29                | +                    | -                 | +                 | +    | -    | -    |
| 30                | +                    | -                 | +                 | +    | -    | -    |
| 31                | +                    | -                 | +                 | +    | -    | -    |
| 32                | +                    | -                 | +                 | +    | -    | -    |
| 33                | +                    | -                 | +                 | +    | -    | -    |
| 34                | +                    | -                 | +                 | +    | -    | -    |
| 35                | +                    | -                 | +                 | +    | -    | -    |
| 36                | +                    | -                 | +                 | +    | -    | -    |
| 37                | -                    | -                 | -                 | -    | -    | -    |
| 38                | -                    | -                 | -                 | -    | -    | -    |
| 39                | -                    | -                 | -                 | -    | -    | -    |
| 40                | -                    | -                 | -                 | -    | -    | -    |
| 41                | -                    | -                 | -                 | -    | -    | -    |
| 42                | -                    | -                 | -                 | -    | -    | -    |
| 43                | -                    | -                 | -                 | -    | -    | -    |
| 44                | -                    | -                 | -                 | -    | -    | -    |
| 45                | -                    | -                 | -                 | -    | -    | -    |
| 46                | -                    | -                 | -                 | -    | -    | -    |
| 47                | -                    | -                 | -                 | -    | -    | -    |
| 48                | -                    | -                 | -                 | -    | -    | -    |

CsPL: Clonorchis sinensis Padlock probe pairs
OvPL: Opisthorchis felineus Padlock probe pairs
OfPL: Opisthorchis viverrini Padlock probe pairs
Conclusion
In the current study the MLPA assay was adapted to identify and discriminate three liver flukes in a ‘one-tube’ reaction, which was proven to be a sensitive and specific tool with high efficiency. Multiplex amplification makes this assay useful for high throughput analysis of pathogens in large clinical or ecological samples [48]. The flexible arms of the probes allow for minimal fluorescent labeling. The advantages of this method have a potential for wider application, e.g. to the detection of other parasites or to diagnostics of mixed infections in severely ill patients.

Material and Methods
Ethical Standards
All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies. Procedures involving vertebrate animals were reviewed and approved by Sun Yat-Sen University’s Animal Care and Use Committee.

Parasite sampling and genomic DNA extraction
Sixty-six *C. sinensis* individuals were collected from infected cats or dogs, the most common reservoir hosts, in 9 provinces in China mainland (Table 4). Genomic DNA from adult worms was extracted using a commercial DNA extraction kit (Dong sheng Biocompany, Guangdong, China) according to manual instruction. Briefly as: single adult was ground in a 1.5 ml microcentrifuge tube containing 200 μl of extraction buffer I, after shortly homogenizing, proteinase K (New England Biolabs, U.K.) and RNase A (New England Biolabs, U.K.) were added to final concentrations of 100 μg/ml and 20 μg/ml, respectively, and incubated for 3 h at 37°C. Following this, 200 μl Buffer II was added to the mixture, and incubated for 10 min at 65°C. Then, 200 μl ethanol was added to the mixture. Totally mixture was moved into the spin column after tightly vortex, after spin for 1 min at 8000 rpm, extra protein was removed using Buffer III, and then the column was washed twice with 70% ethanol, followed by centrifuge at 12000 rpm for 2 min to remove extra ethanol, DNA was recovery using 50 μl buffer EB. RNase (5 μl each, 10 mg/ml in pH7.4 NaAC) treatment was performed at 37°C for 30 min. The DNA quantification was done at 260 nm in a UV spectrophotometer (Shimadzu, Japan).

Fecal sampling, DNA extraction and qualification
Thirty-six fecal samples were collected from 36 infected rats at 8 weeks after infection with metacercariae. Twelve fecal samples from 12 uninfected rats were used as control. The feces of rats were firstly examined by FECT methods[49]. One gram of feces was taken for FECT. The pellet after centrifugation was resuspended

| Species              | GenBank     | Source                      |
|----------------------|-------------|-----------------------------|
| *Clonorchis sinensis*| EU038112    | Shenyang, China             |
|                      | EU038113    | Shenyang, China             |
|                      | EU038114    | Shenyang, China             |
|                      | EU038115    | Shenyang, China             |
|                      | EU038116    | Shenyang, China             |
|                      | EU038117    | Shenyang, China             |
|                      | EU038118    | Shenyang, China             |
|                      | EU038119    | Shenyang, China             |
|                      | EU038120    | Gimhae-si, Gyeongsangnam-do, South Korea |
|                      | EU038121    | Gimhae-si, Gyeongsangnam-do, South Korea |
|                      | EU038122    | Gimhae-si, Gyeongsangnam-do, South Korea |
|                      | EU038123    | Gurye-gun, Jeollanam-do, South Korea |
|                      | EU038124    | Gurye-gun, Jeollanam-do, South Korea |
|                      | EU038125    | Gurye-gun, Jeollanam-do, South Korea |
|                      | EU038126    | Jinju-si, Gyeongsangnam-do, South Korea |
|                      | EU038127    | Jinju-si, Gyeongsangnam-do, South Korea |
|                      | EU038128    | Jinju-si, Gyeongsangnam-do, South Korea |
|                      | EU038129    | Jinju-si, Gyeongsangnam-do, South Korea |
|                      | EU038130    | Jinju-si, Gyeongsangnam-do, South Korea |
|                      | EU038131    | Jinju-si, Gyeongsangnam-do, South Korea |
|                      | HQ874538    | Cat, Anhui, China           |
|                      | HQ874539    | Cat, Anhui, China           |
|                      | HQ874540    | Cat, Anhui, China           |
|                      | HQ874541    | Cat, Guangdong, China       |
|                      | HQ874542    | Cat, Guangdong, China       |
|                      | HQ874543    | Cat, Guangxi, China         |
|                      | HQ874544    | Cat, Guangxi, China         |
|                      | HQ874545    | Cat, Guangxi, China         |
|                      | HQ874546    | Cat, Guangxi, China         |
|                      | HQ874547    | Cat, Guangxi, China         |
|                      | HQ874548    | Cat, Guangxi, China         |
with 1 ml of 10% formalin and 20 μl of suspension was used for microscopy detection.

DNA was extracted from fecal samples as described previously[50]. Briefly, 800 mg feces were washed twice with 1 ml PBS. After centrifugation, the pellet was resuspended into 200 μl of 2% polyvinylpolypyrrolidone (PVPP, Sigma, St. Louis, MO) and heated for 10 min at 100°C. After sodium dodecyl sulfate-proteinase K treatment (2 h at 55°C), DNA was isolated using QIAamp Tissue Kit spin columns (QIAgen, Hilden, Germany), and eluted using 100 μl of elution buffer.

The quality of the DNA of C.sinensis was confirmed by successful PCR amplification with universal fungal primers ITS1F and ITS1R [51]. The first run of PCR to detect fungal DNA was performed as follows: an initial 95°C for 5 min and then 25 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 7 min. Then one microliter amplicons of first run was used as the PCR template for the second run under the same reaction program. Amplicons were analyzed by electrophoresis (Bio-Rad, Hercules, CA) on 2% agarose gels.

Nucleotide polymorphism analysis of ITS gene of C. sinensis isolates

ITS1 rDNA regions of C.sinensis were amplified using primers ITS1F and ITS1R [51]. PCR was performed as follows: 95°C for 5 min; 25 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 1 min, with final extension at 72°C for 7 min. Amplicons were detected by electrophoresis
DNA or artificial templates were used as negative controls. Artificial template of padlock probe was used to evaluate the detection limit of the MLPA assay. Two microlitres of each 10-fold serial diluted artificial template mixture and genome DNA of C.sinensis was used as templates for MLPA reaction. Amplified products were analyzed by electrophoresis on 5% agarose gels, stained with ethidium bromide and photographed. 20bp DNA ladder was used as molecular weight standard.

**Results detection using capillary sequencer**
One microliter of the products was dissolved in 9 μl of deionized formamide, 0.2 nM Gene-Scan® -ROX 500 size standards, and 0.5 μl loading dye (all from Applied Biosystems, Foster City, CA, USA) and denatured for five minutes at 95°C. The products were electrophoresed on an ABI Prism® 3730XL Genetic Analyzer model capillary sequencer (Applied Biosystems) in the GeneScan mode. Analysis of the products was performed using Gene-Scan 3.7 and Genotyper® 3.7 software (Applied Biosystems) consecutively.

**Evaluation of MLPA in fecal samples of infected rats**
Crude-extracted DNA of 2 μl each from 48 fecal samples was used as a template for MLPA assays. The amplified products were analyzed by electrophoresis.

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**Authors’ contributions**
XY, JX and JS designed the present experiments. JS carried out these experiments and drafted the manuscript. PL, QM and CL collected the isolates using in this study. YH, XL and CD give crucial reviews of this manuscript. All authors read and approved the final version of the manuscript.

**Competing interests**
The authors declare that they have no competing interests.

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