

# Risk factors for *Calodium hepaticum* infection in small mammals along the China-Myanmar border: a study in Tengchong City and Longchuan County

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## Research Article

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# Abstract

Small mammals are the primary hosts of *Calodium hepaticum* (syn. *Capillaria hepatica*) and play a critical role in its transmission and parasite maintenance; however, there is a lack of comparative studies on the detection of *C. hepaticum* infection in small mammals using tissue compression microscopy and conventional PCR, and research on the associated risk factors for infection remains limited. This study was conducted in Tengchong City and Longchuan County, located along the China-Myanmar border in Yunnan Province, aiming to characterize the status of *C. hepaticum* infection in local small mammals, compare the detection performance between the two methods, analyze the risk factors for infection, and provide a theoretical basis for the scientific prevention and control of hepatic capillariasis. Small mammals were captured using the night trapping method, *C. hepaticum* eggs were observed via tissue compression microscopy, and the 18S rRNA target gene fragment was amplified and sequenced using conventional PCR; the McNemar test and Cohen's Kappa coefficient were applied to compare the detection consistency and differences between the two methods, while multivariate Logistic regression was performed to identify the independent risk factors for infection. Our results revealed that a total of 507 small mammals were captured (308 in Tengchong City, 199 in Longchuan County), belonging to 3 orders, 5 families, 15 genera, and 27 species, among which 20 species were confirmed to be infected with *C. hepaticum* with an overall infection rate of 34.91% (38.31% in Tengchong City, 29.65% in Longchuan County); *Rattus species* accounted for the highest proportion (59.76%) of the captured individuals with an infection rate reaching 42.90%, the detection rate of conventional PCR (34.91%) was significantly superior to that of tissue compression microscopy (15.19%) ( $\chi^2_{\text{McNemar}}=100, P < 0.001$ ), and region, rodent species, sex, and body length were identified as independent risk factors for infection:

the risk of *C. hepaticum* infection in small mammals from Tengchong City was 2.49-fold that in Longchuan County. Compared with *Rattus tanezumi*, *Suncus murinus* exhibited a 70% lower infection risk, while other non-dominant rodent species showed a 52% lower infection risk. Female small mammals had a 1.64-fold higher infection risk than males. Small mammals with a body length > 148 mm had a 1.68-fold higher infection risk than those with a body length  $\leq$  148 mm. This study demonstrates that the prevalence of *C. hepaticum* infection in small mammals is relatively high in Tengchong City and Longchuan County along the China-Myanmar border of Yunnan Province. The high proportional abundance and elevated infection rate of species in the genus *Rattus* increase the risk of pathogen spillover to human populations. The detection performance of conventional PCR is superior to that of tissue squash microscopy. Furthermore, *C. hepaticum* infection is shaped by regional and species-specific characteristics, suggesting that collaborative prevention and control strategies targeting humans, host animals and the environment should be implemented under the "One Health" framework.

## 1 Introduction

*Calodium hepaticum* is a zoonotic parasite whose transmission is closely associated with environmental contamination, drinking water safety, and personal hygiene practices[1]. Infection primarily occurs through the oral ingestion of food or water contaminated with embryonated eggs[2]. This parasite predominantly infects the liver of small mammals and various other mammalian species, causing hepatic capillariasis, a

zoonotic disease[3]. Once *C. hepaticum* establishes itself in the host liver, neither the adult worms nor their eggs can be excreted via the digestive tract. The persistent presence of these eggs and adult worms serves as a continuous antigenic stimulus to the host immune system, leading to infiltration of inflammatory cells such as eosinophils, epithelioid histiocytes, and multinucleated giant cells. This, in turn, induces granulomatous reactions in the liver and results in hepatic dysfunction[4]. Severe cases may progress to liver fibrosis or liver failure, directly threatening host survival[5, 6]. The primary hosts of *C. hepaticum* include small mammals [7, 8], cats [2], dogs [9], rabbits [10], *Enhydra lutris* [4], *Mandrillus sphinx* [11], and others. Human infection cases have also been reported [12, 13]. Among these, small mammals exhibit rapid reproduction, wide distribution, high species diversity, and strong adaptability [14], making them important reservoirs and transmission hosts for *C. hepaticum*. After infected small mammals die, eggs released from the liver during carcass decomposition contaminate the environment. These eggs, when ingested by other animals or humans, establish transmission chains among different hosts, allowing continuous circulation within ecosystems and posing a potential threat to both human and animal health. Therefore, investigating *C. hepaticum* infection status in small mammals is crucial for assessing its ecological transmission risk and elucidating the key role of small mammals in the transmission chain of this zoonosis.

High-risk distribution areas of *C. hepaticum* are closely related to host density, environmental humidity, and sanitation conditions: host aggregation facilitates pathogen spread, high humidity prolongs egg survival, and inadequate sanitation control further increases transmission risk[8]. Tengchong City and Longchuan County in Yunnan Province are located along the China–Myanmar border, representing key areas among the 18 counties and cities in Yunnan that border Myanmar. The unique geographical location and ecological environment create favorable conditions for the cross-border transmission of *C. hepaticum*. The complex ecological types and high diversity of small mammal species in these two regions provide abundant potential hosts for *C. hepaticum*, and the cross-border movement of small mammals further accelerates transboundary pathogen spread. Additionally, the warm and humid climate in these regions is conducive to egg maturation, shortening the developmental period of infective eggs and enhancing transmission capacity. Moreover, frequent cross-border human movement and trade facilitate the spread of the pathogen via the flow of people and goods, posing a serious threat to public health security in both regions. As important ecological transition zones along the China–Myanmar border, Tengchong City and Longchuan County may play a significant role in the cross-border transmission of zoonotic diseases. However, the population distribution characteristics, key influencing factors, and transmission risk assessment of *C. hepaticum* in small mammals in this region remain unclear. Therefore, investigating *C. hepaticum* infection in small mammals in these two areas is of considerable practical significance for elucidating the transmission patterns of zoonotic diseases in border regions and improving regional parasite control strategies. This study employed two detection techniques, tissue compression microscopy and polymerase chain reaction (PCR), to compare the infection status of *C. hepaticum* in small mammals, determine the detection discrepancies between the two methods, and explore risk factors associated with infection, thereby providing theoretical support for the scientific prevention and control of hepatic capillariasis.

## **2 Materials and Methods**

### **2.1 Ethics statement**

The experimental protocol for this study has been approved by the Medical Ethics Committee of Dali University (No. MECDU-201901-3) and complies with medical research ethics principles and relevant national regulations on the welfare of animals in medical research.

### **2.2 Selection of Sampling Areas**

This study selected Tengchong City and Longchuan County, located at the border between China and Myanmar in Yunnan Province, as the sampling areas. The sampling area in Tengchong City includes Mingguang Town, Jietou Town, Qushi Town, Hehua Town, and Xinhua Township, while the sampling sites in Longchuan County encompass Zhangfeng Town, Qingping Township, Chengzi Town, Husa Township, Longba Town, and Jinghan Town. The exact geographical locations of these sampling areas are shown in Fig. 1 and were mapped using ArcGIS 10.8.

### **2.3 Collection of Animal and Tissue Samples**

Using the night trap method, small mammals were captured in residential areas and surrounding farmlands, shrublands, and cultivated fields using peanuts as bait. Traps were set at night and collected in the morning. Captured individuals were numbered, individually placed in sealed bags, and transported to the laboratory. When collecting traps, small mammals that are still alive are euthanized by cervical dislocation. Small mammal species were identified through morphological examination, records were made of the sex, age, body length, and body weight of the small mammals. Perform aseptic dissection within a biosafety cabinet to collect liver tissue from small mammals. Store the tissue in an ultra-low-temperature freezer at -80°C for future use.

### **2.4 Liver tissue pressing microscopy**

In a biosafety cabinet, scissors and forceps were used to sample suspected lesion areas on the liver surface. If no obvious lesions were present, three rice-grain-sized tissue pieces were taken from different regions of the liver and placed on glass slides containing normal saline. Three pressed slides were prepared per sample. The tissue pieces were covered with clean coverslips, gently pressed vertically with the thumb, and excess normal saline was removed using absorbent paper. The pressed slides were observed under an optical microscope using a 10× objective; upon detection of suspected egg areas, the objective was switched to 40× for further observation.

### **2.5 DNA Extraction from Liver Samples**

Approximately 10 mg of liver tissue was taken and ground to complete fragmentation using a high-speed tissue grinder (OSE-Y30, Tiangen Biochemical Technology Co., Ltd., China). Subsequently, DNA was extracted using the column-based method according to the protocol provided with the Tissue DNA Extraction Kit (OSR-M401, Tiangen Biochemical Technology Co., Ltd., China). The concentration and purity

of each DNA sample were determined using a micro-ultraviolet-visible spectrophotometer (NanoReady F1100, Shenying Biotechnology Co., Ltd., China). Samples with concentrations greater than 50 µg/mL and a nucleic acid purity index (A260/A280) within the range of 1.8 to 2.1 were considered to meet the standards[14]. Compliant samples were stored at -80°C for future use, while non-compliant samples underwent secondary extraction.

## 2.6 PCR Amplification

The *C.hepaticum* 18S rRNA gene was selected as the target gene. Its specific primers were synthesized by Shanghai Sangon Biotech Co., Ltd. The primer sequences were referenced from previously published research findings[13]. The upstream primer sequence is 233F: 5'-CGGTTCTGCTGTTCAAGTTGTT-3'; the downstream primer sequence is 436R: 5'-TGCTGCCTTCCTTGGATGTA-3'. The amplified product length was 204 bp. The total reaction volume was 25 µL, comprising 12.5 µL Dream Taq Green PCR Master Mix (2X), 1 µL upstream primer, 1 µL downstream primer (10.0 µmol/L), 8.5 µL nuclease-free water, and 2 µL DNA template. The gradient PCR instrument program was set as follows: pre-denaturation at 95°C for 3 min; denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 40 s, repeated for 40 cycles; post-extension at 72°C for 5 min (RePure-A, Hangzhou BoHeng Technology Co., Ltd., China).

## 2.7 Gel Electrophoresis Imaging

The PCR products were detected using a 1.5% agarose gel containing Gelred in an electrophoresis apparatus (DYY-6C, Beijing Sixi Yitian Instrument Factory, China). The sample volume was 4 µL per well. Under the conditions of 120V voltage and 80mA current, the electrophoresis was conducted for 30 minutes. The results were observed on the gel imaging system (G: BOX F3, Syngene Company, USA). If a bright and single band appeared at approximately 204 bp, and there was no band in the negative control and blank control, it was determined as a preliminary positive result. The negative control was sterile double-distilled water.

## 2.8 Sequence Determination and Phylogenetic Analysis

The preliminary positive products were sent to Sangon Biotech Co., Ltd. for bidirectional sequencing via Sanger sequencing. Sequence assembly was performed using SeqMan software. After obtaining the nucleotide sequences, they were submitted to the National Center for Biotechnology Information (NCBI) website. The BLAST tool was used for comparison (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The alignment results indicated *C. hepaticum*, confirming the positive result. The 18S rRNA gene sequence of *C. hepaticum* was then retrieved from the GenBank database, downloaded, and saved. The homology analysis matrix diagram was constructed using the MegAlign program of DNASTar 7.1 software, and the multiple sequence alignment was performed using MEGA 11.0 software. The neighbor-joining method was selected, with 1000 bootstrap repetitions set. The P-distance parameter model was used to construct the phylogenetic tree. The outgroup was *Trichinella spiralis* (GenBank accession number: U60231). The tree was beautified on the iTOL website (<https://itol.embl.de/>). Representative 18S rRNA sequences of the samples have been submitted to GenBank, with accession numbers PX105951-PX105979.

## 2.9 Statistical Analysis

Small mammals were classified into dominant and non-dominant species based on their composition ratio, with dominant species defined as those with a composition ratio > 10% and non-dominant species as those with a composition ratio  $\leq 10\%$  [15]. The morphological characteristics of small mammals showed a skewed distribution and were converted into binary variables using the median as the cut-off. All statistical analyses were performed using R software (version 4.3.1). McNemar's test and Cohen's kappa coefficient were used to compare the detection rates of *C. hepaticum* in small mammals between tissue pressing microscopy and conventional PCR. The infection rate of *C. hepaticum* in small mammals was determined based on the PCR results and served as the dependent variable. The independent variables included region, habitat, species, sex, age, presence or absence of ectoparasites, body length, and body weight. Univariate analysis was conducted using the Pearson's chi-square test, and variables with  $P < 0.05$  in the univariate analysis were included in the multivariate logistic regression model. Variable selection was performed using the backward elimination method, and the final model was determined by the likelihood ratio test (LR-test), with the significance level set at  $\alpha = 0.05$ . For each categorical level of the independent variables in the final model,  $P$  values were calculated using the Wald test. The coefficients of each variable were exponentiated to obtain the odds ratios (OR) along with their 95% confidence intervals (CI).

## 3 Results

### 3.1 Results of tissue pressing microscopy for *C. hepaticum* infection in small mammals

Liver samples from 507 small mammals were examined, and eggs of *C. hepaticum* were detected in 77 samples, yielding a detection rate of 15.19%. Under microscopy, the eggs exhibited clear morphology and intact structure, appearing oval in shape; the eggshell consisted of two layers with striations visible between them, and each pole of the egg possessed a mucous plug, which is consistent with the typical morphological characteristics of *C. hepaticum* eggs[16]. The results are shown in Fig 2.

### 3.2 Molecular Identification and Phylogenetic Analysis of *C. hepaticum* Infection in Small Mammals

A total of 507 small mammal DNA samples were extracted, with both concentration and purity meeting requirements. The target 18S rRNA gene fragment was amplified using conventional PCR. Agarose gel electrophoresis analysis of the amplified products revealed a clear, single band at approximately 204 bp, consistent with the expected fragment size. No bands were detected in the negative control or blank control samples, as shown in Figure 3. The samples were sent to Sangon Biotech Co., Ltd. for sequencing. Ultimately, 177 sequences were successfully obtained and identified as *C. hepaticum* through BLAST sequence alignment against the NCBI database.

A phylogenetic analysis was conducted on representative 18S rRNA sequences. The homology analysis matrix diagram revealed that the nucleotide similarity between the studied sequences and the reference sequence of *C. hepaticum* deposited in GenBank ranged from 99.5% to 100.0%, with internal nucleotide similarity within the sequences ranging from 99.0% to 100.0%. A phylogenetic tree was constructed by aligning the study sequences with reference sequences, as shown in Fig 4. The study samples cluster with the Japanese *Rattus norvegicus* (LC425008) and Indian *Rattus norvegicus* (OR841356, PQ241655,

PQ764976) within the same branch, classified as *C. hepaticum*, while they diverge from the Indian *Baruscapillaria obsignata* (LC052336, LC425004) and Thailand *Paracapillaria najae* (OQ372228, OQ372229), sharing the same major branch. Additionally, German *Eucoleus aerophilus* (MF599385) and Australian *Eucoleus aerophilus* (MW709573) formed another major branch, while sequences from the outgroup *Trichinella spiralis* (U60231) belonged to a distinct branch.

### 3.3 Comparison of detection rates between tissue pressing microscopy and conventional PCR

The detection of *C. hepaticum* infection in small mammals using tissue pressing microscopy and conventional PCR is detailed in Table 1. All samples that tested positive for *C. hepaticum* by tissue pressing microscopy were successfully amplified for the 18S rRNA gene fragment by conventional PCR. McNemar's test was used to compare the detection rates of *C. hepaticum* in small mammals between the two methods, and the results showed that the detection rate of conventional PCR was superior to that of tissue pressing microscopy ( $\chi^2_{\text{McNemar}}=100$ ∕ $P<0.001$ ). Cohen's kappa analysis indicated a moderate level of agreement between tissue pressing microscopy and conventional PCR for detecting *C. hepaticum* infection in small mammals ( $\kappa=0.501$ ∕ $P<0.001$ ).

**Table 1.** Detection of *C. hepaticum* infection in small mammals by tissue pressing microscopy and conventional PCR

|          | Conventional PCR | Tissue pressing microscopy |          | Total |
|----------|------------------|----------------------------|----------|-------|
|          |                  | Positive                   | Negative |       |
| Positive |                  | 77                         | 100      | 177   |
| Negative |                  | 0                          | 330      | 330   |
| Total    |                  | 77                         | 430      | 507   |

### 3.4 Infection Status of Small Mammals with *C. hepaticum*

A total of 507 small mammals (308 in Tengchong City and 199 in Longchuan County) were captured, belonging to 27 species, including 17 species of Rodentia, 9 species of Eulipotyphla, and 1 species of Scandentia. The dominant species were *Rattus tanezumi*, *Rattus andamanensis*, and *Suncus murinus*, accounting for 44.58% (225/507), 12.03% (61/507), and 14.40% (74/507), respectively. A total of 20 different small mammal species were found to be infected with *C. hepaticum*, with an overall infection rate of 34.91% (38.31% in Tengchong City and 29.65% in Longchuan County). The genus *Rattus* (mainly including *R. tanezumi*, *R. andamanensis*, *R. nitidus*, and *R. norvegicus*) had the highest composition ratio (59.76%, 303/507) and an infection rate of 42.90% (130/303) (as shown in Table 2). The infection rates of *C. hepaticum* among different species ranged from 15.79% to 100%, but apart from the dominant species, the sample sizes of the remaining small mammals were small, making their infection rate data non-representative. Specifically, the infection rate of *R. tanezumi* was 42.67% (96/225), that of *R. andamanensis* was 44.26% (27/61), and that of *S. murinus* was 16.22% (12/74).

**Table 2.** Infection Status of *C.hepaticum* in Different Small Mammal Species

| Order    | Family     | Genus              | Species                       | Tengchong City    | Longchuan County  | Total              |
|----------|------------|--------------------|-------------------------------|-------------------|-------------------|--------------------|
| Rodentia | Muridae    | <i>Rattus</i>      |                               | 74/145<br>¥51.03¥ | 56/158<br>¥35.44¥ | 130/303<br>¥42.90¥ |
|          |            |                    | <i>Rattus nitidus</i>         | 5/11<br>¥45.45¥   | 1/4¥25.00¥        | 6/15<br>¥40.00¥    |
|          |            |                    | <i>Rattus norvegicus</i>      | 1/2¥50.00¥        | 0                 | 1/2<br>¥50.00¥     |
|          |            |                    | <i>Rattus tanezumi</i>        | 47/86<br>¥54.65¥  | 49/139<br>¥35.25¥ | 96/225<br>¥42.67¥  |
|          |            |                    | <i>Rattus andamanensis</i>    | 21/46<br>¥45.65¥  | 6/15<br>¥40.00¥   | 27/61<br>¥44.26¥   |
|          |            | <i>Mus</i>         |                               | 9/28<br>¥32.14¥   | 0/4¥0¥            | 9/32<br>¥28.13¥    |
|          |            |                    | <i>Mus pahari</i>             | 9/28<br>¥32.14¥   | 0/1¥0¥            | 9/29<br>¥31.03¥    |
|          |            |                    | <i>Mus caroli</i>             | 0                 | 0/3¥0¥            | 0/3¥0¥             |
|          |            | <i>Apodemus</i>    |                               | 2/4¥50.00¥        | 0                 | 2/4<br>¥50.00¥     |
|          |            |                    | <i>Apodemus draco</i>         | 2/3¥66.67¥        | 0                 | 2/3<br>¥66.67¥     |
|          |            |                    | <i>Apodemus chevrieri</i>     | 0/1¥0¥            | 0                 | 0/1¥0¥             |
|          |            | <i>Niviventer</i>  |                               | 5/19<br>¥26.32¥   | 1/1<br>¥100.00¥   | 6/20<br>¥30.00¥    |
|          |            |                    | <i>Niviventer confucianus</i> | 0/2¥0¥            | 1/1<br>¥100.00¥   | 1/3<br>¥33.33¥     |
|          |            |                    | <i>Niviventer fulvescens</i>  | 5/17<br>¥29.41¥   | 0                 | 5/17<br>¥29.41¥    |
|          |            | <i>Bandicota</i>   | <i>Bandicota indica</i>       | 0/1¥0¥            | 1/1<br>¥100.00¥   | 1/2<br>¥50.00¥     |
|          |            | <i>Dacnomys</i>    | <i>Dacnomys millardi</i>      | 0/1¥0¥            | 0                 | 0/1¥0¥             |
|          |            | <i>Leopoldamys</i> | <i>Leopoldamys edwardsi</i>   | 1/2¥50.00¥        | 0                 | 1/2<br>¥50.00¥     |
|          |            | <i>Micromys</i>    | <i>Micromys minutus</i>       | 1/3¥33.33¥        | 0                 | 1/3<br>¥33.33¥     |
|          | Cricetidae | <i>Eothenomys</i>  |                               | 3/14<br>¥21.43¥   | 0/3¥0¥            | 3/17<br>¥17.65¥    |

|              |             |                    |                                |              |             |              |
|--------------|-------------|--------------------|--------------------------------|--------------|-------------|--------------|
|              |             |                    | <i>Eothenomys eleusis</i>      | 0/500        | 0/300       | 0/800        |
|              |             |                    | <i>Eothenomys miletus</i>      | 1/616.67     | 0           | 1/616.67     |
|              |             |                    | <i>Eothenomys cachinus</i>     | 2/366.67     | 0           | 2/366.67     |
| Eulipotyphla | Soricidae   | <i>Suncus</i>      | <i>Suncus murinus</i>          | 12/4725.53   | 0/2700      | 12/7416.22   |
|              |             | <i>Crocidura</i>   |                                | 5/2420.83    | 1/250.00    | 6/2623.08    |
|              |             |                    | <i>Crocidura attenuata</i>     | 2/450.00     | 0           | 2/450.00     |
|              |             |                    | <i>Crocidura indochinensis</i> | 0/100        | 0           | 0/100        |
|              |             |                    | <i>Crocidura lasiura</i>       | 0            | 1/1100.00   | 1/1100.00    |
|              |             |                    | <i>Crocidura vorax</i>         | 0            | 0/100       | 0/100        |
|              |             |                    | <i>Crocidura dracula</i>       | 3/1915.79    | 0           | 3/1915.79    |
|              |             | <i>Anourosorex</i> | <i>Anourosorex squamipes</i>   | 3/1030.00    | 0/100       | 3/1127.27    |
|              | Erinaceidae | <i>Neotetracus</i> | <i>Neotetracus sinensis</i>    | 2/825.00     | 0           | 2/825.00     |
|              |             | <i>Hylomys</i>     | <i>Hylomys suillus</i>         | 0            | 0/200       | 0/200        |
| Scandentia   | Tupaiaidae  | <i>Tupaia</i>      | <i>Tupaia belangeri</i>        | 1/250.00     | 0           | 1/250.00     |
| Total        |             |                    |                                | 118/30838.31 | 59/19929.65 | 177/50734.91 |

Note: Numbers outside parentheses indicate positive cases/detected cases; numbers inside parentheses indicate rate/%.

### 3.5 Univariate Analysis of *C. hepaticum* Infection in Small Mammals

Univariate analysis was performed on the 507 small mammals, and variables with  $P < 0.05$  included region, habitat, species, sex, body length, and body weight. The infection rate of *C. hepaticum* in small mammals was higher in Tengchong City than in Longchuan County. Regarding habitat, the highest infection rate was observed in residential areas, followed by farmland, woodland, and shrubland. Among species, *R. andamanensis* had the highest infection rate, followed by *R. tanezumi*, other species, and *Suncus murinus*.

Females had a higher infection rate than males. Small mammals with a body length >148 mm showed a higher infection rate than those with a body length  $\leq$ 148 mm. Details are shown in Table 3.

**Table 3.** Univariate Analysis of Small Mammal Infection with *C. hepaticum*

| Factor                     | Detection samples | Positive samples | infection rate /% | $\chi^2$ | $P$    |
|----------------------------|-------------------|------------------|-------------------|----------|--------|
| Region                     |                   |                  |                   | 3.993    | 0.046  |
| Tengchong City             | 308               | 118              | 38.31             |          |        |
| Longchuan County           | 199               | 59               | 29.65             |          |        |
| Habitat                    |                   |                  |                   | 14.819   | 0.002  |
| Residential area           | 78                | 40               | 51.28             |          |        |
| Farmland                   | 224               | 76               | 33.93             |          |        |
| Shrubland                  | 66                | 14               | 21.21             |          |        |
| Woodland                   | 139               | 47               | 33.81             |          |        |
| Species                    |                   |                  |                   | 22.285   | <0.001 |
| <i>Rattus tanezumi</i>     | 225               | 96               | 42.67             |          |        |
| <i>Rattus andamanensis</i> | 61                | 27               | 44.26             |          |        |
| <i>Suncus murinus</i>      | 74                | 12               | 16.22             |          |        |
| Others                     | 147               | 42               | 28.57             |          |        |
| Sex                        |                   |                  |                   | 4.732    | 0.030  |
| Female                     | 253               | 100              | 39.53             |          |        |
| Male                       | 254               | 77               | 30.31             |          |        |
| Age                        |                   |                  |                   | 4.732    | 0.030  |
| minor                      | 44                | 100              | 39.53             |          |        |
| Adulthood                  | 463               | 77               | 30.31             |          |        |
| Ectozoon                   |                   |                  |                   | 0.036    | 0.851  |
| No                         | 258               | 87               | 33.72             |          |        |
| Yes                        | 249               | 82               | 32.93             |          |        |
| Body length                |                   |                  |                   | 19.629   | <0.001 |
| ≤148mm                     | 260               | 67               | 25.77             |          |        |
| >148mm                     | 247               | 110              | 44.53             |          |        |
| Body weight                |                   |                  |                   | 12.566   | 0.001  |
| ≤85g                       | 255               | 70               | 27.45             |          |        |

|      |     |     |       |
|------|-----|-----|-------|
| >85g | 252 | 107 | 42.46 |
|------|-----|-----|-------|

### 3.6 Multivariate Logistic Regression Analysis

Region, habitat, species, sex, body length, and body weight were included in a multivariate logistic regression analysis. Variables in the initial model were selected using the backward elimination method, and four variables (region, species, sex, and body length) were ultimately retained in the final regression model. The risk of *C. hepaticum* infection in small mammals from Tengchong City was 2.49-fold that in Longchuan County. Compared with *R. tanezumi*, *Suncus murinus* exhibited a 70% lower infection risk, while other non-dominant rodent species showed a 52% lower infection risk. Female small mammals had a 1.64-fold higher infection risk than males. Small mammals with a body length > 148 mm had a 1.68-fold higher infection risk than those with a body length ≤ 148 mm. The results are shown in detail in Table 4.

**Table 4. Multivariate Logistic Regression Analysis of Small Mammal Infection with *C. hepaticum***

| Variables                  | Detection samples | Positive samples | OR[95%CI]         | P[Wald test] | P[LR-test] |
|----------------------------|-------------------|------------------|-------------------|--------------|------------|
| Region                     |                   |                  |                   |              | <0.001     |
| Tengchong City             | 308               | 118              | Ref.              |              |            |
| Longchuan County           | 199               | 59               | 2.49[1.58 ~ 3.94] | <0.001       |            |
| Species                    |                   |                  |                   |              | 0.018      |
| <i>Rattus tanezumi</i>     | 225               | 96               | Ref.              |              |            |
| <i>Rattus andamanensis</i> | 61                | 27               | 0.75[0.41 ~ 1.38] | 0.357        |            |
| <i>Suncus murinus</i>      | 74                | 12               | 0.30[0.13 ~ 0.67] | 0.003        |            |
| Others                     | 147               | 42               | 0.48[0.27 ~ 0.88] | 0.017        |            |
| Sex                        |                   |                  |                   |              | 0.012      |
| Female                     | 253               | 100              | Ref.              |              |            |
| Male                       | 254               | 77               | 1.64[1.11 ~ 2.42] | 0.012        |            |
| Body length                |                   |                  |                   |              | 0.047      |
| ≤148mm                     | 260               | 67               | Ref.              |              |            |
| >148mm                     | 247               | 110              | 1.68[1.00 ~ 2.81] | 0.048        |            |

## 4 Discussion

*C. hepaticum*, a zoonotic parasite, exhibits a broad geographic distribution spanning multiple continents, including Asia, Africa, Europe, North America, South America, and Oceania[13]. It has a vast host range, with over 180 mammalian species recognized as hosts for *C. hepaticum*, including more than 80 rodent species confirmed to be susceptible to infection by this parasite[8, 17]. Among these hosts, small mammals consist of a group of relatively small-sized taxa within the class Mammalia, primarily including Rodentia, Eulipotyphla, Scandentia, and others[18]. They predominantly inhabit terrestrial, subterranean, and arboreal ecosystems. This habitat characteristic places them in proximity to human activities, often making them key players in the transmission and spread of *C. hepaticum*. Therefore, systematically investigating *C. hepaticum* infection status in small mammals across different regions is crucial for scientifically assessing the zoonotic risk of this parasite.

In this study, tissue pressing microscopy and conventional PCR were used to detect and compare *C. hepaticum* infection in small mammals from Tengchong City and Longchuan County along the China-Myanmar border in Yunnan Province. The results showed that the detection rate of tissue pressing microscopy was 15.19%, while that of conventional PCR was 34.91%, and all microscopy-positive samples were successfully amplified for the 18S rRNA gene fragment by conventional PCR. McNemar's test indicated that the detection rate of conventional PCR was superior to that of microscopy ( $\chi^2_{\text{McNemar}} = 100$ ,  $P < 0.001$ ). Furthermore, Cohen's kappa analysis revealed a moderate level of agreement between the two methods ( $\kappa = 0.501$ ,  $P < 0.001$ ). The main reasons for the discrepancy between the two methods are that, compared with microscopy, PCR technology has higher sensitivity and enables precise detection of trace amounts of pathogen nucleic acids, especially during early infection when the number of parasites is low [19]. In addition, PCR improves the accuracy of differential diagnosis when encountering morphologically similar parasite eggs [20]. Based on these findings, this study confirms that conventional PCR has higher sensitivity and better detection performance than microscopy for detecting *C. hepaticum* infection in small mammals, suggesting that infection rates obtained solely by microscopy may underestimate the actual infection levels. Regarding regional epidemiological characteristics, previously reported infection rates of *C. hepaticum* in small mammals from Hong Kong (China), Inner Mongolia, Jiangxi Province, Dali City (Yunnan Province), and the Honghe River Basin (Yunnan Province) were all obtained using tissue pressing microscopy. Comparison using the same method shows that the microscopy detection rate of 15.19% in this study is similar to those reported in Jiangxi Province (12.21%, 21/172) [21] and the Honghe River Basin (10.34%, 39/377) [22], slightly lower than those in Inner Mongolia (19.16%, 192/1002) [23] and Dali City (20.21%, 58/287) [24], and significantly lower than that in Hong Kong (36.65%, 81/221) [7]. However, the conventional PCR detection rate of 34.91% in this study is close to the microscopy-based result from Hong Kong and higher than the microscopy data from other inland regions. This discrepancy is not only due to differences in detection method sensitivity but is also closely related to the geographical climate and environmental characteristics of the study areas. Studies have shown that *C. hepaticum* eggs develop into infective eggs within approximately 5 ~ 8 weeks at an ambient temperature of 23 ~ 30°C[7]. Tengchong City and Longchuan County, located in western Yunnan Province, have a subtropical monsoon climate that is warm and humid, which is more suitable for the development of *C. hepaticum* eggs [25]. Moreover, as an

important region along the China-Myanmar border with long border lines and complex ecological environments, frequent cross-border animal movements and human–animal interactions may facilitate the transregional spread of eggs [26]. These factors may explain the higher infection rate of small mammals in this region compared with other inland areas. In summary, conventional PCR has higher sensitivity for detecting *C. hepaticum* infection, and it is recommended as the preferred detection method in future epidemiological surveys of small mammals to ensure accuracy and reliability of the results.

Based on the 18S rRNA gene, PCR was used to detect *C. hepaticum* in 507 small mammal liver samples. After sequencing and sequence alignment, 177 positive samples were successfully obtained. A phylogenetic tree constructed using the neighbor-joining method based on representative sequences showed that the positive samples in this study clustered in the same clade as sequences from *Rattus norvegicus* in India (LC425008, 2017) and Japan (OR841356, PQ241655, PQ764976, 2023 and 2024), with nucleotide similarities ranging from 99.5% to 100.0%. This finding indicates that *C. hepaticum* infecting small mammals across different geographical regions is highly conserved at the genetic level, suggesting a possible common evolutionary origin or transregional transmission routes. In terms of species composition, the genus *Rattus* (mainly including *R. tanezumi*, *R. andamanensis*, *R. nitidus*, and *R. norvegicus*) accounted for the highest proportion among all 15 genera, at 59.76% (303/507), with a *C. hepaticum* infection rate of 42.90% (130/303). Rodents of the genus *Rattus* are in close contact with human living environments, and the co-occurrence of their high composition ratio and high infection rate increases the potential risk of cross-species transmission of *C. hepaticum* from *Rattus* to humans. To substantiate this risk, this study further analyzed relevant published molecular data: the *C. hepaticum* sequence derived from *R. norvegicus* in the Wuhan section of the Yangtze River in China (2020) showed 98% nucleotide similarity with the Indian sequence LC425008 [27]; the *C. hepaticum* sequence isolated from a male patient in Israel (2021) exhibited 100% nucleotide similarity with LC425008 [13]. These molecular pieces of evidence confirm at the genetic level that *C. hepaticum* infecting humans shares high homology in the 18S rRNA gene with strains infecting rodents, fully demonstrating the capacity of *C. hepaticum* for cross-species transmission from rodents to humans. Therefore, given the high infection rate of the genus *Rattus* observed in this study and its close contact with humans, close attention should be paid to the potential transmission risk of this parasite in human populations. It is recommended to strengthen the surveillance and control of rodent-borne *C. hepaticum* in public health monitoring systems to reduce the likelihood of human exposure and infection.

This study employed PCR technology to analyze 507 small mammal liver samples using the 18S rRNA gene. Following sequencing and sequence alignment, 177 positive samples for *C. hepaticum* were successfully identified. The phylogenetic tree constructed using Neighbor-joining analysis of representative sequences revealed that the positive samples from this study clustered on the same branch as *Rattus norvegicus* from India (LC425008, 2017) and Japan (OR841356, PQ241655, PQ764976; 2023, 2024), which also clustered together, with nucleotide similarities ranging from 99.5% to 100.0%. This finding reveals that *C. hepaticum* infecting small mammals across different geographical regions exhibits high genetic conservation, suggesting a potential common evolutionary origin or the existence of cross-regional transmission pathways. Secondly, comparisons with findings from studies on the *Rattus*

*norvegicus* from the Wuhan section of China's Yangtze River in 2020 (98% nucleotide similarity to LC425008)[27] and a male patient in Israel in 2021 (100% nucleotide similarity to LC425008)[13] further support the conclusions of this study. Although the sequences from the latter two studies could not be incorporated into the phylogenetic tree construction of this study due to their absence from NCBI, they indirectly confirm the pivotal role of small mammals in the transmission of *C. hepaticum*. This further indicates that *C. hepaticum* infections in small mammals pose a risk of cross-species transmission to humans.

A total of 507 small mammals were captured in this study, identified as belonging to 27 species, including 17 species of Rodentia, 9 species of Eulipotyphla, and 1 species of Scandentia. These findings indicate a relatively rich species composition of small mammals in Tengchong City and Longchuan County. Research has confirmed that land use patterns, habitat suitability, and seasonality fundamentally influence the population size, species richness, diversity, evenness, and community composition of small mammals[28]. In terms of habitat suitability, Tengchong City and Longchuan County are situated in the China-Myanmar border region of Yunnan Province. Their complex topography encompasses diverse landforms, including mountains, hills, and river valleys, providing varied habitats for small mammals with different ecological requirements[29]. At the same time, the region boasts diverse vegetation types, ranging from evergreen broadleaf forests to shrublands and grasslands, providing ample food sources and hiding places for various small mammals. In terms of climate-related seasonal patterns and overall environmental stability, both regions are influenced by a subtropical monsoon climate, characterized by mild and humid conditions, small annual temperature variations, and relatively uniform precipitation distribution. This not only mitigates the adverse effects of extreme seasonal fluctuations on small mammals but also provides consistently favorable climatic conditions for their survival and reproduction. Moreover, the relatively well-balanced land use patterns within the survey area have also provided a stable habitat for local small mammals to some extent. These factors collectively contribute to the rich species composition of small mammals in both locations.

There exists a certain disparity in *C. hepaticum* infection rates among small mammals between Tengchong City and Longchuan County. The risk of *C. hepaticum* infection in small mammals in Tengchong City was 2.49-fold higher than that in Longchuan County (OR = 2.49, 95% CI: 1.58–3.94). This difference may be related to geographical and climatic factors. Tengchong City is situated in the southern section of the Hengduan Mountains. Its complex terrain of interlacing mountains and river valleys provides suitable habitats for numerous small mammal species. In this study, 23 small mammal species were captured in Tengchong City, with 17 species infected with *C. hepaticum*. In contrast, Longchuan County, characterized by plains and basins, exhibits a relatively monotonous ecological environment. Compared to Tengchong City, its small mammal species richness was slightly lower. This study recorded 13 species in Longchuan County, with 6 species infected by *C. hepaticum*. This suggests that small mammal species richness may influence the transmission of *C. hepaticum*. Additionally, Tengchong City features a subtropical plateau mountain monsoon climate with relatively high annual average temperatures and abundant precipitation. This environment not only supports the survival and reproduction of small mammals but also facilitates the development and survival of *C. hepaticum* eggs. In contrast, although Longchuan County is also

located in a subtropical region, its climate is relatively drier due to local climatic factors, with greater seasonal fluctuations in temperature. This to some extent inhibits the spread of *C. hepaticum*, resulting in a lower infection rate of *C. hepaticum* among small mammals in Longchuan County compared to Tengchong City.

In previous global studies, *R. norvegicus* and *R. tanezumii* have been identified as the primary hosts for *C. hepaticum*[30]. Similar studies in Hong Kong indicate that *R. norvegicus* is the dominant rat species locally, while *C. hepaticum* infections have been documented in *R. norvegicus*, *R. tanezumii*, and *R. andamanensis*. *R. norvegicus* exhibits a 3.48-fold higher risk of *C. hepaticum* infection compared to *R. tanezumii*(OR = 3.48, 95% CI 1.71–7.50)[7]. In this study, a total of 20 species were infected, with infection rates ranging from 15.79% to 100% across different species. Only two individuals of *R. norvegicus* were captured, one of which was infected with *C. hepaticum*. However, due to the small sample size, these data lacked representativeness for analysis. Consequently, only the dominant species (*R. tanezumii*, *R. andamanensis*, and *S. murinus*) and other species were included in the analysis.

Compared with *R. tanezumii*, *S. murinus* exhibited a 70%(OR = 0.30, 95% CI: 0.13–0.67) lower infection risk, while other non-dominant rodent species showed a 52%(OR = 0.48, 95% CI: 0.27–0.88) lower infection risk. This suggests that regional variations in small mammal species composition may influence the primary local hosts for *C. hepaticum* infection. Future epidemiological surveillance and control strategies must fully account for regional species composition characteristics, developing interventions targeting locally dominant hosts to effectively reduce *C. hepaticum* transmission risks. Female small mammals had a 1.64-fold higher risk of infection than males (OR = 1.64, 95% CI: 1.11–2.42), possibly because females, especially those in reproductive stages, engage in more frequent foraging and nesting activities due to the demands of bearing and rearing offspring[31], making them more likely to encounter food and soil contaminated with *C. hepaticum* eggs, thereby increasing their infection risk. Small mammals with a body length > 148 mm had a 1.68-fold higher infection risk than those with a body length ≤ 148 mm(OR = 1.68, 95% CI: 1.00-2.81), suggesting that infection with *C. hepaticum* in small mammals is associated with body size. Larger small mammals may have broader foraging and habitat ranges, increasing their exposure to environments contaminated with *C. hepaticum* eggs and thereby raising infection probability. Additionally, larger individuals consuming infected smaller prey through cannibalism would also face heightened infection risk.

This study also has certain limitations. First, environmental factors such as temperature and humidity can influence the development of *C. hepaticum* eggs; however, this study did not investigate seasonal variations in *C. hepaticum* infections among small mammals. Secondly, this study covered only two of the 18 counties and cities along the China-Myanmar border in Yunnan Province. Given the variations in geography, vegetation types, and species distribution among different counties and cities along the border, the current findings may not fully represent the infection status of *C. hepaticum* in small mammals across the entire China-Myanmar border region. Therefore, further research is needed to clarify the primary hosts of *C. hepaticum* across different regions by expanding the study areas and sample sizes, thereby gaining deeper insights into its transmission mechanisms and infection status within small mammal populations.

## 5 Conclusion

A total of 20 species of small mammals infected with *C. hepaticum* were identified in Tengchong City and Longchuan County along the China–Myanmar border in Yunnan Province, with an overall infection rate of 34.91%. The genus *Rattus* had the highest composition ratio in the small mammal community and exhibited a *C. hepaticum* infection rate as high as 42.90%. This co-occurrence of high composition ratio and high infection rate increases the potential risk of cross-species transmission of *C. hepaticum* from *Rattus* to humans. The detection rate of conventional PCR was superior to that of tissue pressing microscopy, and it is recommended that conventional PCR be used as the preferred detection method in relevant epidemiological surveys. The infection status of *C. hepaticum* in small mammals is influenced by geographical region, species, sex, and body length. Therefore, monitoring the infection status of *C. hepaticum* in small mammals across different regions should be conducted to timely understand the disease transmission patterns and provide data support for the formulation of prevention and control strategies. Furthermore, strict standardization of animal carcass disposal is needed to reduce potential transmission routes at the source and further lower the risk of zoonotic transmission. Establishing an integrated "human-animal-environment" collaborative prevention and control system and fully implementing the One Health concept are of great significance for safeguarding human and animal health and maintaining public health security.

## Abbreviations

*Calodium hepaticum* (*C. hepaticum*); polymerase chain reaction (PCR); *Rattus tanezum* (*R. tanezum*), *Rattus andamanensis* (*R. andamanensis*), *Rattus nitidus* (*R. nitidus*), and *Rattus norvegicus* (*R. norvegicus*).

## Declarations

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### Author contributions

Y.J.X: Conceptualization, funding acquisition, project administration, resources, supervision, software, writing-review and editing. L.P.G: Data curation, formal analysis, investigation, methodology, software, validation, visualization, writing-original draft. L.Y and G.S.L: Data curation, formal analysis, investigation, methodology, validation. L.Y.Y: Data curation, formal analysis, investigation. W.H, L.X.D, and Z.X.Y: Data curation, investigation. S.Z.T: Investigation, sample collection. All authors reviewed the manuscript.

## **Ethics statement**

The experimental protocol for this study has been approved by the Medical Ethics Committee of Dali University (No. MECDU-201901-3) and complies with medical research ethics principles and relevant national regulations on the welfare of animals in medical research.

## **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the first and corresponding author upon reasonable request.

## **Consent to publish declaration**

Not applicable.

## **Declaration of competing interest**

The authors declare that they have no conflict of interest.

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Figures

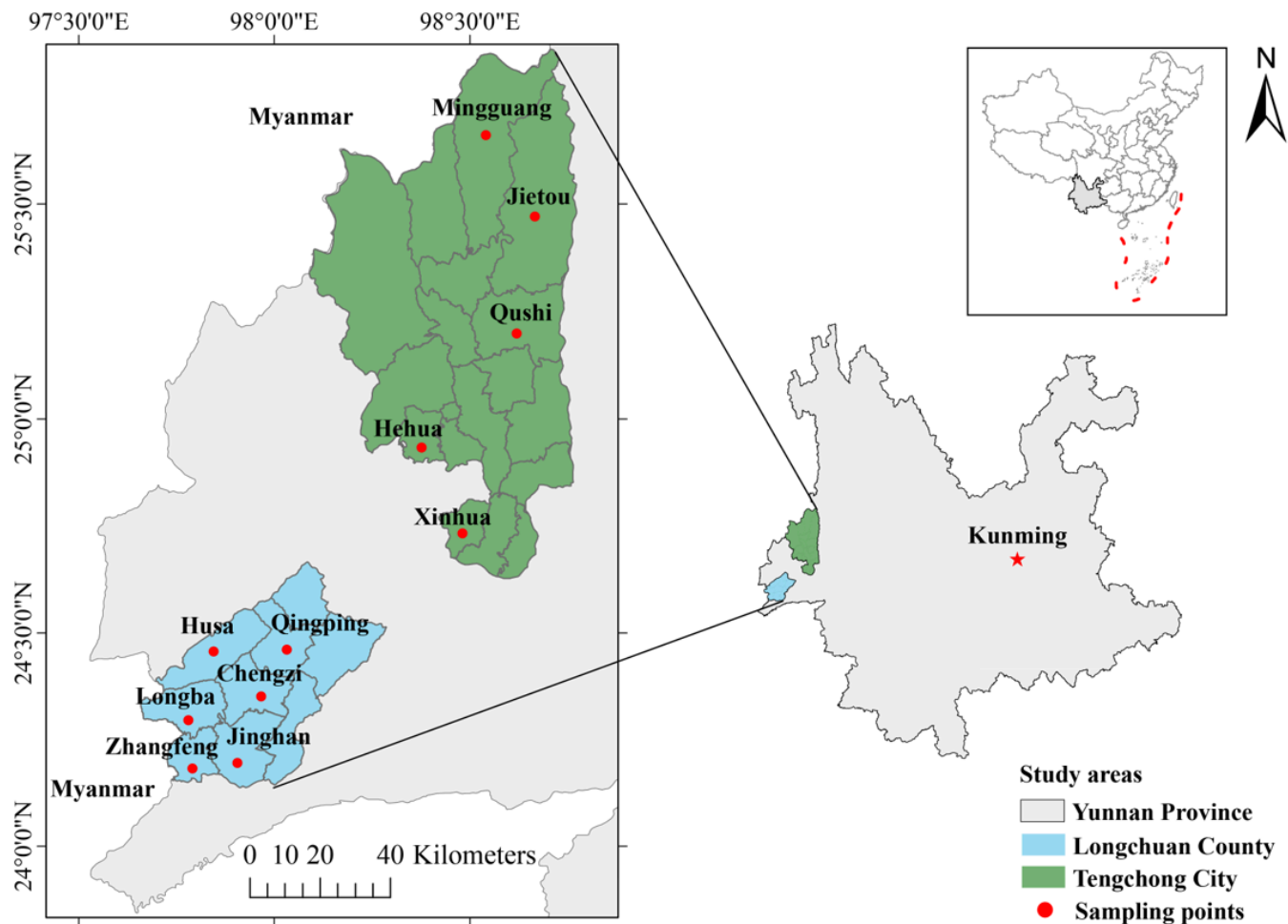


Figure 1  
Sampling Areas for Small Mammal Capture.

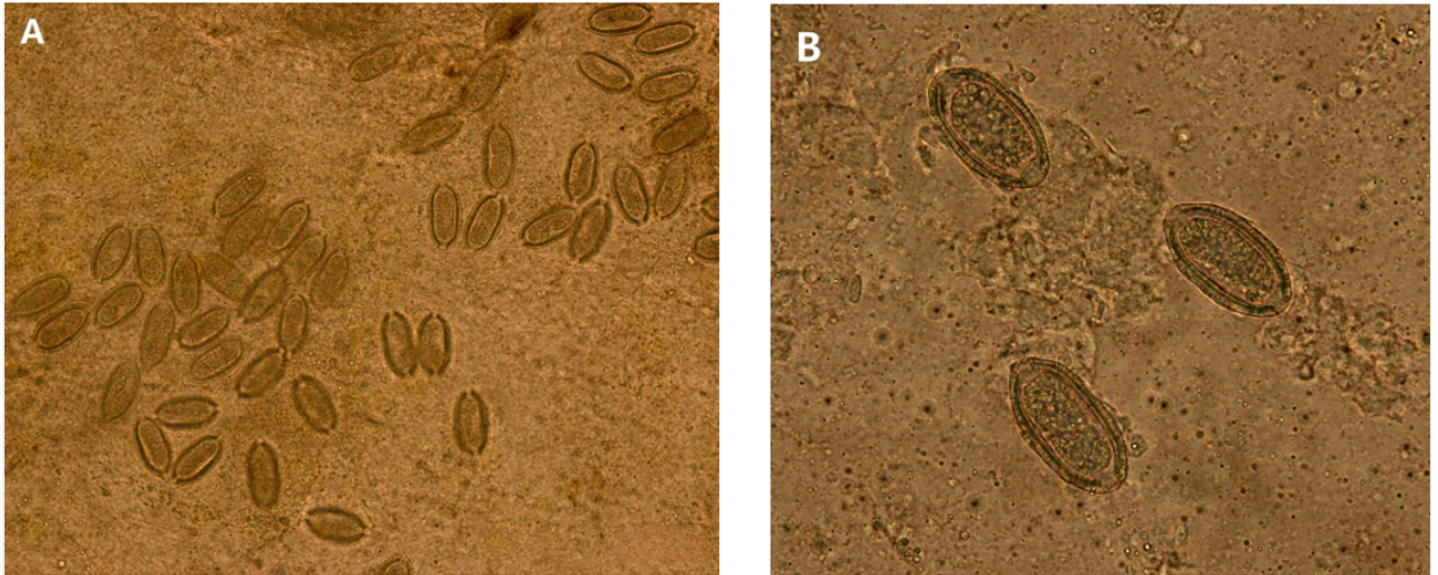


**Figure 2**

Tissue pressing microscopy image of *C. hepaticum*

Note: A, field of view under 10× objective; B, field of view under 40× objective.

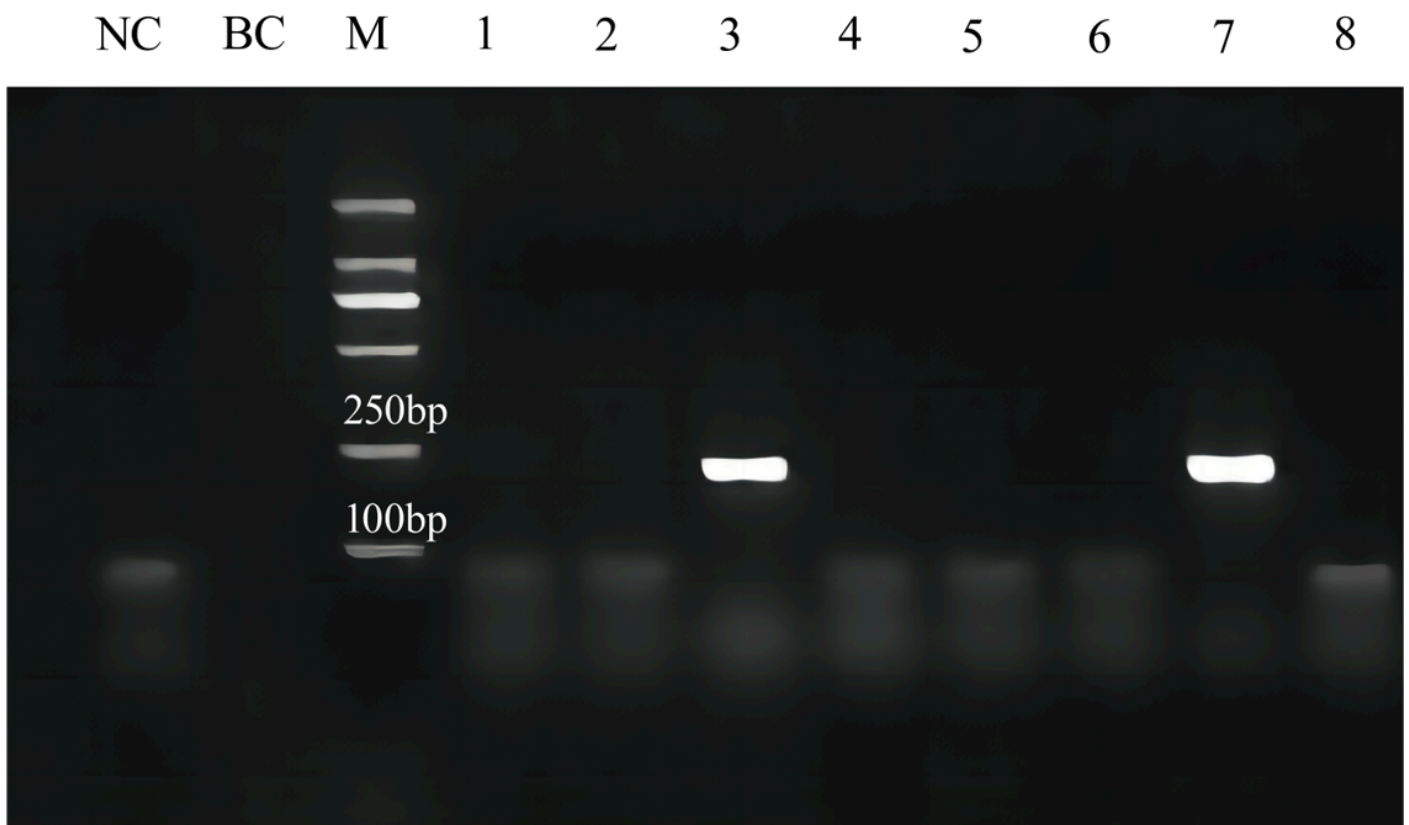


**Figure 3**

PCR Amplification Results for *C. hepaticum*.

Note: NC represents the negative control; BC represents the blank control; M represents the DNA marker; 1-8 represent the amplification products, and 3 and 7 represent the positive ones.

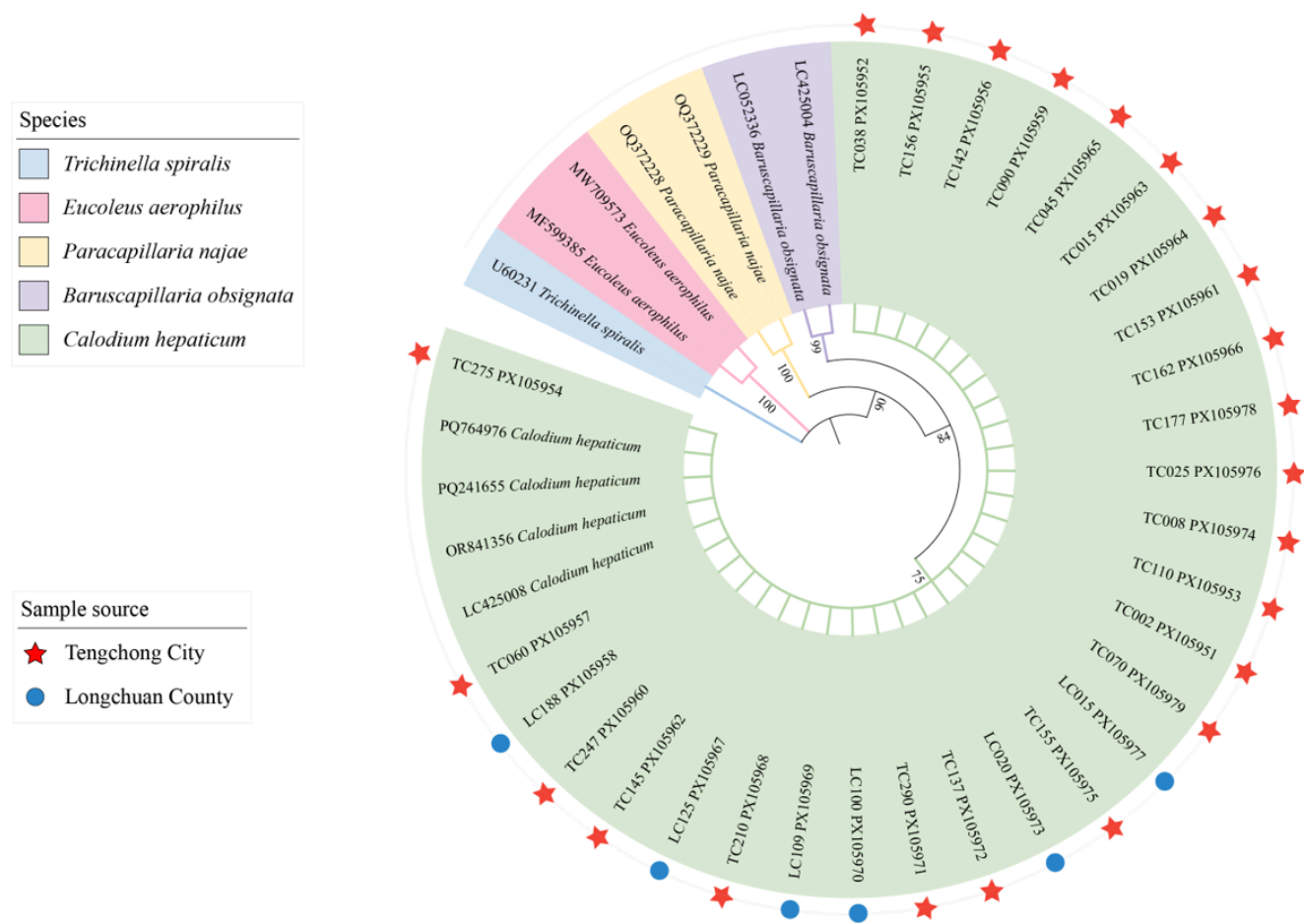


Figure 4

Phylogenetic Analysis of *C. hepaticum* 18S rRNA.

## Supplementary Files

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