INTRODUCTION

Anaplasma phagocytophilum is a Gram-negative obligate intracellular bacterium, which encompasses former Ehrlichia phagocytophila, E. equi, and the human granulocytic ehrlichiosis (HGE) agent based on phylogenetic analyses. This bacterium has long been recognized as a veterinary agent. Since HGE (now called human granulocytic anaplasmosis), the human infection, was first reported in the United States in 1994,2,3 A. phagocytophilum has been considered as an emerging pathogen of public health importance.4 The disease usually presents as an acute febrile illness characterized by headache, chill, myalgias, arthralgia, malaise, and hematologic abnormalities, such as thrombocytopenia, leukopenia, and elevated hepatic aminotransferase levels.5

A. phagocytophilum is thought to be naturally maintained in a tick–rodent cycle, with humans being involved only as incidental “dead-end” hosts.6–10 The agent is usually associated with genus Ixodes ticks, including I. scapularis, I. pacificus, and I. spinipalpis in the United States,8,11,12 I. trianguliceps in the United Kingdom,6,13 I. ricinus in mainland Europe,9,14–18 and Africa,10 and I. persulcatus in eastern Europe20 and Asia.21–24 Wild rodents have been implicated as natural reservoirs for A. phagocytophilum in the United States, the United Kingdom, and mainland Europe.6,7,9,10

A. phagocytophilum was only documented in I. persulcatus ticks from northeastern China where Lyme disease is endemic.21,22 However, the existence of the agent has not been established in Jilin Province, although its ecological features are similar to the previously mentioned areas. Furthermore, no information is available concerning animal reservoirs of A. phagocytophilum in Asia. Lack of such knowledge has inhibited us to understand the ecology, epidemiology, and potential threats of the pathogens to human health. The objectives of this study were to investigate the presence of A. phagocytophilum in Jilin Province of China, and to determine if rodents, the common hosts of immature stages of ticks, are naturally infected by the agent.

MATERIALS AND METHODS

Study site. Rodents and actively questing ticks were collected in May 2005 in the hinterland of Changbai Mountains situated at 42°45′ north latitude and 130°35′ east longitude within Hunchun County, Jilin Province. The terrain consists of forested rolling hills with an average elevation of 825 m. The annual precipitation is about 800 mm, and the relative humidity is around 70%. The temperature ranges from −41.4°C to 33.6°C, with the average of 4.0°C.

Sample collection. Host-seeking ticks were collected by flagging vegetation. Ticks were identified to the species level and the developmental stage, and were stored alive until use. Rodents were captured using box traps with peanuts as baits. Attached feeding ticks were removed by using sterile forceps, identified by species and life stage, and stored in 1.5-mL microcentrifuge tubes in the refrigerator until DNA extraction. After identification of species and sex, the spleen was removed from each rodent and stored at −20°C until tested.

DNA extraction. DNA extraction from ticks was performed as previously described.22 Briefly, each tick was placed into a microtube and mechanically disrupted with sterile scissors in 50 μL DNA extraction buffer (10 mM Tris pH 8.0, 2 mM EDTA, 0.1% sodium dodecyl sulfate, and 500 μg of proteinase K per ml). The sample was incubated for 2 hours at 56°C, and then boiled at 100°C for 10 minutes to inactivate proteinase K. After centrifugation, the supernatant was transferred to fresh sterile microtube and purified by extracting twice with an equal volume of phenol-chloroform before use. A small piece of spleen (about 500 mg) from individual rodent was used for DNA extraction. Briefly, each spleen specimen was crushed with Trizol (Invitrogen, Carlsband, CA, USA) to separate DNA from RNA after centrifugation. The precipitated DNA were obtained after washing twice in a solution containing 0.1 M sodium citrate in 10% ethanol, then the DNA pellet was suspend in 75% ethanol and kept at room temperature for 10–20 minutes. After centrifuging at 2000 g at 2–8°C for 5 minutes, the DNA was dissolved in weak base by adding a certain quantity of 8 mM NaOH and centrifuged to
remove insoluble material. Then the supernatant containing the DNA was transferred to a new tube, adjusted with HEPES to pH 7–8, and stored at 4°C for PCR use.

**Polymerase chain reaction amplification.** A nested PCR was performed with primers designed to amplify the partial 16S rRNA gene of *A. phagocytophilum.*

Primers GE9f and GE10r, previously described by Chen and colleagues, were applied for the primary amplification. In nested PCR, Ehr521g and GE10r were used as primers, and expected to yield a 441-bp product. Both primary and nested PCR amplifications were performed in a volume of 30 μL in a Perkin-Elmer model 2400 thermal cycler. Cycling conditions involved an initial 3-minute denaturation at 95°C followed by 35 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 15 s, and a final extension at 72°C for 5 minutes. In parallel with each amplification, a positive control (a plasmid containing the 16S rRNA gene of HGE agent, kindly provided by Dr. J. Stephen Dumler at Department of Pathology, The Johns Hopkins Medical Institutions) and a negative control (distilled water) were included.

To further identify the agent in the samples positive for the *A. phagocytophilum* 16S rRNA gene, another nested PCR was performed to amplify partial sequence of the citrate synthase gene (gltA) of *A. phagocytophilum.* The primers W1 (5’-TGTTTTGAGTGTTGAGAC-3’) and W2 (5’-GGTGACCAATCTCAGCAA-3’) for the initial amplifications, and the primers N1 (5’-ATATAGAAATCTGATCGG-3’) and N2 (5’-CTCTAAGTTTGCAGCAG-3’) for the nested reactions were designed, and expected to produce a 357-bp fragment. PCR amplifications were conducted in a volume of 30 μL in a Perkin-Elmer model 2400 thermal cycler. The cycling conditions were identical for primary and nested amplifications, which involved 3-minute denaturation at 95°C, followed by 35 cycles of 94°C for 15 seconds, 50°C for 20 seconds, and 70°C for 20 seconds, and a final extension at 70°C for 5 minutes.

All the PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. To minimize contamination, DNA extraction, the reagent setup, amplification, and agarose gel electrophoresis were performed in separate rooms.

**Sequencing and phylogenetic analyses.** All samples positive for both 16S rRNA and gltA were re-amplified with the primer set GE9f and GE10r and the primer set Ehr521g and 317, which could yield 919-bp and 964-bp products, respectively. Consequently, a fragment of 1442-bp of *A. phagocytophilum* 16S rRNA gene was obtained from each sample, based on overlapping region of sequence.

The PCR products of both 1442-bp 16S rRNA gene and 357-bp gltA of *A. phagocytophilum* were collected and purified. The nucleotide sequences of positive samples were determined by a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (ABI PRISM 377, Perkin-Elmer, Inc.). The sequences obtained at present study were compared with the previously published sequences deposited in GenBank using BLAST program from the National Center for Biotechnology Information web site.

Multiple sequence alignment was conducted with the Clustal X, version 1.83 (http://www.bio-software.com/soft/mat.html). The phylogenetic analyses were performed with PHYLIP (version 3.63). The distance matrices for the aligned sequences were calculated by the Kimura 2-parameter method; phylogenetic trees were constructed using the neighbor-joining method. The stability of the tree obtained was estimated by bootstrap analysis with 1,000 replications. Tree figures were generated with the TreeView program (version 1.66).

**Statistical analysis.** *x*² test or Fisher’s exact test (whenever necessary) was used to compare the proportions. A *P* value < 0.05 was considered statistically significant.

**Nucleotide sequencing accession number.** The partial nucleotide sequences of *A. phagocytophilum* 16S rRNA gene and gltA from representative *I. persulcatus* and *Dermacentor silvarum* ticks, *Apodemus peninsulae*, *A. agrarius*, and *Tamias sibiricus* rodents in this study were deposited in GenBank under the accession numbers DQ449947, DQ449948, DQ449945, DQ432324, DQ449946, DQ160228, DQ449952, DQ449950, DQ449949, and DQ449951, respectively.

**RESULTS**

*A. phagocytophilum* was first detected by a nested PCR specifically targeting the 16S rRNA gene. A total of 386 unfed, host-seeking ticks, including 100 adult *I. persulcatus* and 286 *D. silvarum*, were collected on vegetation and individually examined. Infection rates of ticks in light of species, sex, and stage are shown in Table 1. The positive rate of *A. phagocytophilum* in *I. persulcatus* (4.0%) was significantly higher than that in *D. silvarum* (0.7%) (Fisher’s exact test, *P* = 0.041). The male *I. persulcatus* ticks had significant higher infection rate than females did (Fisher’s exact test, *P* = 0.011). There was no significant difference in positive rates among male, female, and nymphal *D. silvarum* ticks (Fisher’s exact test, *P* = 0.398).

A total of 102 rodents from 7 species were captured and detected for the presence of *A. phagocytophilum*. Nine rodents from 3 species, including Japanese field mouse (*A. agrarius*), black-striped field mouse (*A. agrarius*), and Siberian chipmunk (*T. sibiricus*), were found naturally infected, with an overall positive rate of 8.8% (Table 2). The difference in infection rates among species was not statistically significant (*x*² = 9.82, df = 6, *P* = 0.132).

Only *I. persulcatus* ticks were found to be “parasiting” on the captured rodents. Among the 102 rodents, 25 (24.5%) hosted 71 *I. persulcatus* ticks (see Table 2), including 56 larvae and 15 nymphs. Only 2 nymphs were positive for *A. phagocytophilum* with an overall infection rate of 2.8%. The 2 infected nymphs were respectively from a positive *A. peninsulae* and a negative *A. agrarius* (see Table 2). The samples positive for the *A. phagocytophilum* 16S rRNA gene were further tested by another nested-PCR assay targeting gltA, and all showed positive results.

**Table 1**

<table>
<thead>
<tr>
<th>Tick species</th>
<th>Sex and stage</th>
<th>No. tested</th>
<th>No. (%) positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>I. persulcatus</em></td>
<td>Male</td>
<td>34</td>
<td>4 (11.8)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>66</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td><em>D. silvarum</em></td>
<td>Male</td>
<td>137</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>111</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td></td>
<td>Nymph</td>
<td>38</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>386</td>
<td>6 (1.6)</td>
</tr>
</tbody>
</table>
All the PCR-positive specimens (i.e., 6 questing ticks, 9 rodents, and 2 parasitizing *I. persulcatus*) were re-amplified, and a 1442-bp fragment of *A. phagocytophilum* 16S rRNA gene was obtained from each specimen. The nucleotide sequences amplified from the above positive specimens were identical to each other. The phylogenetic tree based on the alignments of the 16S rRNA gene sequences showed that the agent detected in this study was distinct from the previously reported *A. phagocytophilum* (Figure 1), although they were in the same clade.

The sequence analysis of 357-bp partial *gltA* revealed the nucleotide sequences amplified from the positive ticks and rodents had 100% homology, and varied from all known *A. phagocytophilum* sequences deposited in GenBank. The *gltA*-based phylogenetic tree (Figure 2) showed more distance than the tree derived from analysis of the 16S rRNA gene. The agent identified in the study appeared to be most closely related to *A. phagocytophilum* detected in *I. persulcatus* ticks from Russia (GenBank accession no. AY339602), but genetically far away from *A. phagocytophilum* agents found in other countries.

### DISCUSSION

*A. phagocytophilum* is believed to be naturally maintained in a tick–rodent cycle with *Ixodes* ticks as vectors. Considering the fact that both *A. phagocytophilum* and *Borrelia burgdorferi* agents share the similar ecological system,18,20–22 an investigation on presence of *A. phagocytophilum* was carried out in a forest area of Jilin Province of northeastern China, where Lyme disease is highly endemic.28 As a result, *A. phagocytophilum* was firstly identified in the province. The infection rate of the agent in *I. persulcatus* ticks was 4.0%, which is in agreement with the prevalence of 4.6% in the same tick species previously collected in Inner Mongolia Autonomous Region and Heilongjiang Province, China.22 The infection rate in male *I. persulcatus* ticks seems higher than that in the female. These diversities could be attributable to geographical and seasonal variations of infected ticks or to different sampling approaches. Further studies using a greater number of tick samples in different seasons, locations, and habitats are required to understand the infection levels. Discrepant infection rates of *A. phagocytophilum* in ticks have been observed around the world. The prevalence of *A. phagocytophilum* in *I. persulcatus* ticks from Korea23 was obviously lower than that in *I. persulcatus* and *I. ovatus* ticks from Japan.24 The infection rate could be <1% in adult *I. pacificus* from California,29 and in *I. ricinus* from the United Kingdom15 and Switzerland.17 Higher prevalences were reported in *I. scapularis* in the United States8 and *I. ricinus* in the Netherlands18 and Sweden.30

![Diagram 1](image1.png)

DIAGRAM 1. Phylogenetic tree based on 1442-bp of the 16S RNA gene using PHYLIP software (version 3.63).25 The distance matrix was calculated by using Kimura 2-parameter method. The tree was obtained by the neighbor-joining method. The numbers at the nodes are the proportions of 1000 bootstrap resamplings that support the topology shown.

![Diagram 2](image2.png)

DIAGRAM 2. Phylogenetic tree based on 357-bp of *gltA* using PHYLIP software (version 3.63).25 The distance matrix was calculated by using Kimura 2-parameter method. The tree was obtained by the neighbor-joining method. The numbers at the nodes are the proportions of 1000 bootstrap resamplings that support the topology shown.

<table>
<thead>
<tr>
<th>Rodent species</th>
<th>No. of rodents tested</th>
<th>No. of rodents positive (%)</th>
<th>No. of rodents parasitized with ticks</th>
<th>No. of parasitizing ticks tested</th>
<th>No. of ticks positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japanese field mouse (<em>Apodemus peninsulae</em>)</td>
<td>43</td>
<td>3 (7.0)</td>
<td>12</td>
<td>40</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>Black-striped field mouse (<em>A. agrarius</em>)</td>
<td>24</td>
<td>5 (20.8)</td>
<td>5</td>
<td>7</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Grey red-backed vole (<em>Clethrionomys rufocanus</em>)</td>
<td>23</td>
<td>0 (0.0)</td>
<td>7</td>
<td>15</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Great long-tailed hamster (<em>Tseherska triton</em>)</td>
<td>4</td>
<td>0 (0.0)</td>
<td>1</td>
<td>9</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Siberian chipmunk (<em>Tamias sibirica</em>)</td>
<td>3</td>
<td>1 (33.3)</td>
<td>0</td>
<td>0</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Small home rat (<em>Mus musculus</em>)</td>
<td>4</td>
<td>0 (0.0)</td>
<td>0</td>
<td>0</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Brown home rat (<em>Rattus norvegicus</em>)</td>
<td>1</td>
<td>0 (0.0)</td>
<td>0</td>
<td>0</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>102</strong></td>
<td><strong>9 (8.8)</strong></td>
<td><strong>25</strong></td>
<td><strong>71</strong></td>
<td><strong>2 (2.8)</strong></td>
</tr>
</tbody>
</table>

Table 2
Results of nested PCR for the detection of *A. phagocytophilum* in rodents and their parasitizing *I. persulcatus* ticks collected from Jilin, China
In the study, *A. phagocytophilum* was first detected in *D. silvarum* in the world, although the infection rate in this species (0.7%) was significantly lower than that in *I. persulcatus* (4.0%). *A. phagocytophilum* is well documented to be associated with *Ixodes* ticks that may act as vectors; however, the agent has also been reported in *Dermacentor* ticks such as *D. reticulatus* in Austria31 and *D. variabilis* in California.32 The presence in alternate ticks may be due to the existence of secondary maintenance cycles, in which *A. phagocytophilum* circulates between relatively host-specific, usually nonhuman-biting ticks and their hosts.6,33 The additional cycles would possibly buffer the agent from local extinction and assist to re-establish the primary cycles.33 *D. silvarum* is a 3-host tick well adapted to a broad range of habitats and infests a variety of domestic animals such as scaler, sheep, goat and horse, but has rarely been found to feed on humans in China. The finding of this study provides further evidence to the above. The competency of *D. silvarum* as a vector for *A. phagocytophilum* and its significance in veterinary medicine has yet to be demonstrated.

Among the 7 rodent species trapped in the study, *A. peninsulae*, *A. agrarius*, and *T. sibirica* were found positive for *A. phagocytophilum*. The overall infection rate of 102 rodents was 8.8%. As far as we know, this is the first observation of *A. phagocytophilum* infection in wild animals from China. In the forest areas of northeastern China, *Apodemus* mice and *Clethrionomys* voles are dominant rodents and important hosts for larval and nymphal *I. persulcatus* ticks. This was confirmed by our study. It is unknown if *A. phagocytophilum*, as some other tick-borne agents, is maintained through transovarial transmission in ticks.10 A 2-year longitudinal study carried out in a woodland area in northwest England indicated a seasonal variation in rodent infections, which appears to be associated with seasonal increases in the abundance of *I. trianguliceps* nymphs and adults, but not larvae.6 This finding further tests the transstadial, but not transovarial, maintenance of *A. phagocytophilum* by ixodid ticks. Although the *A. phagocytophilum* infection in rodents could be low because they would receive few bites from infected ticks, a rodent-tick cycle of infection was verified. In the United States, molecular and serological studies indicate that the white-footed mouse (*P. leucopus*) is the main host for immature *I. scapularis* ticks, and is apparently the reservoir for *A. phagocytophilum*.10,34,35 Antibodies against *A. phagocytophilum* were also detected in *Tamias striatus*, *Clethrionomys gapperi*,34 *Neotoma spp.*, and other *Peromyscus* spp.36

About a quarter of rodents (24.5%) in our study were being parasitized with ticks, all of which were *I. persulcatus*. Most of the ticks collected on rodents were larvae (78.9%), and the others were nymphs. Two nymphs were found positive, but no larvae were infected. The role of rodents in transmission and maintenance of *A. phagocytophilum* in China requires further investigations.

In the study, spleen specimens were used to identify the natural infection of *A. phagocytophilum* in rodents with reference to previously reported results. Experimental studies on monkeys infected with *A. phagocytophilum* showed that the spleen harbors the organism for the longest period of time and is the best source for the diagnosis of carrier state by PCR.35 In mice that were experimentally infected with the HGE agent, splenic infection was obvious and persistent.36 A field investigation indicates that spleens from wild small animals are most often infected over other samples such as blood, livers, and ears.7

The nucleotide sequences of partial 16S rRNA gene amplified from rodents as well as questing and parasitizing ticks were identical to each other and to those previously published in China.21,22 To further classify and determine the agents, DNA of gltA (357-bp) was amplified and sequenced. The gltA sequence analyses indicate that the agent detected in the study is closely related to *A. phagocytophilum* identified in Russian *I. persulcatus* (GenBank accession no.AY339602), with 2-bp difference (99.4% similarity) at nucleotide level and 1-position difference (99.1% similarity) at amino acid level. The nucleotide sequence of the agent has only 87–93% homology with other *A. phagocytophilum* strains. Sequences of gltA exhibit higher variation than the 16S rRNA gene, therefore allowing better discrimination among *Rickettsia* species.23 In conclusion, the agent discovered in this study is unique and worthy of studying its public health and veterinary significances.

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