Haemorrhagic fever with renal syndrome (HFRS) is an important human disease and 60 000–100 000 hospitalized patients are reported annually worldwide, with the bulk of these cases occurring in China (Johnson, 1999). During the past 10 years, 25 000–60 000 HFRS cases were reported in these countries. These data suggest that HFRS is distributed widely in China, and is one of the predominant rodent species in forest areas in north-eastern China (Zhang et al., 1997). HFRS occurs in the far east of Russia and in South Korea (Baek et al., 2006). Characterization of these viruses indicated that hantaviruses carried by A. peninsulai in the far east of Russia and in South Korea are antigenically and genetically distinct from HTNV, which is usually carried by A. agrarius. This led to the suggestion that hantaviruses carried by A. peninsulai in China and from HFRS patients in Russia. However, the viruses detected in A. peninsulai in China are genetically different from those detected in A. agrarius in other countries. These data suggest that A. peninsulai is also a natural host for HTNV in north-eastern China.

Isolation and characterization of hantavirus carried by *Apodemus peninsulai* in Jilin, China

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To provide a better understanding of hantavirus epidemiology in China, Korean field mice (*Apodemus peninsulai*) and striped field mice (*Apodemus agrarius*) were captured in Jilin province, China, where haemorrhagic fever with renal syndrome (HFRS) is endemic. Hantavirus antigens were detected in eight of the 130 *A. peninsulai* individuals and in four of the 193 *A. agrarius* individuals by using an immunofluorescence assay. Partial S and M segments were amplified from all of the antigen-positive samples. Furthermore, two hantaviruses (CJAp89 and CJAp93) were isolated successfully in cell culture and the entire S and M segments were amplified from one of them (CJAp93). Phylogenetic analysis of these sequences (partial or complete) showed that hantaviruses carried by *A. peninsulai* and *A. agrarius* form two distinct lineages, although viruses carried by *A. peninsulai* are similar to those isolated previously from *A. agrarius* in China and from HFRS patients in Russia. However, the viruses detected in *A. peninsulai* in China are genetically different from those detected in *A. agrarius* in other countries. These data suggest that *A. peninsulai* is also a natural host for HTNV in north-eastern China.
Hunchun city, Jilin province, China. Only *A. peninsulae* (*n*=130) and *A. agrarius* (*n*=193) individuals were selected for this study. Lung tissues were obtained from trapped animals and stored immediately at −196 °C before being transported to the laboratory for processing. Viral antigens in the lung tissue (frozen sections) were detected by using an indirect immunofluorescence assay (IFA) as described by Lee et al. (1978). Lung tissues from BALB/c mice mock-infected or infected with HTNV strain 76-118 (Lee et al., 1978) were used as negative and positive controls, respectively. Hantavirus antigen was detected in eight of 130 (6 %) *A. peninsulae* mice (samples CJAp177, CJAp267, CJAp318, CJAp89, CJAp93, CJAp595, CJAp705 and CJAp787) and in four of 193 (2 %) *A. agrarius* mice (samples CJAa142, CJAa594, CJAa716 and CJAa1109).

To characterize these hantaviruses genetically, RNA was extracted from the antigen-positive lung tissues with TRizol reagent according to the manufacturer’s instructions (Gibco BRL). Primer P14 (Schmaljohn et al., 1986) was used for reverse transcription of both the S and M segments from total RNA by using avian myeloblastosis virus reverse transcriptase (Promega). Partial S segment sequences were amplified by using primer pair S1 and S2 (Puthavathana et al., 1992) for initial PCR, and primer pair S3 and S4 (Sun et al., 2005) for the second round of amplification. Partial M segment sequences were amplified by using primer pair HV-MFO/HV-MRO (Wang et al., 2002) for initial PCR, and primer pair HMF/HMR (Wang et al., 2000) for the second round of amplification. PCR products were purified from gel slices by using an agarose gel DNA purification kit (TaKaRa) and cloned into the pMD18-T vector (TaKaRa). Direct sequencing was performed with an ABI Prism Dye Terminator sequencing kit. The PHYLIP program package (v. 3.65) was used to construct phylogenetic trees by using the maximum-likelihood method with 1000 bootstrap replicates. Nucleotide or amino acid identities were calculated by using DNAStar (v. 5.01).

Phylogenetic analysis of the partial S segment sequences (nt 514–1026) indicated that all of the viruses from *A. peninsulae* were related closely to each other (94.2–99.6 % nucleotide identity; Fig. 1a). The sequences derived from *A. agrarius* were related closely to each other with 99.2–99.8 % nucleotide identity, except for CJAa142. This hantavirus, detected in *A. agrarius*, showed a diversity of 11.8–12.7 % from other viruses detected in *A. agrarius*, but was related closely to viruses from *A. peninsulae* (94.2–99.6 % nucleotide identity; Fig. 1a). Genetic analysis of partial M segment sequences (nt 2001–2301) showed similar results (Fig. 1b). Sequence identities were between 96.7 and 99.3 % among the viruses from *A. peninsulae*. Likewise, a
Characterization of hantavirus in Chinese Apodemus mice

A high degree of nucleotide identity (>99%) was also detected among the viruses from *A. agrarius*, except for CJAa142, which shares a high degree of similarity at the nucleotide level (99–99.8%) with viruses from *A. peninsulae*. In addition, sequence comparison of both S and M segments showed that viruses carried by either *A. peninsulae* or *A. agrarius* have a higher percentage similarity to HTNV (83.2–99.6% for S and 82.0–99.3% for M) than to SEOV (69.8–71.7%/70.0–72.0%), DOBV (68.6–70.8%/71.0–73.7%), PUUV (60.9–63.1%/55.9–61.5%) or SNV (61.4–62.7%/59–63.3%).

When compared with the partial S and M segments from other hantaviruses, all of the viruses derived from *A. peninsulae*, as well as CJAa142, in our study could be classified as a lineage that has high bootstrap support values with HTNV (Lee et al., 2001), Bao9, Bao10, Bao14, Jiang13 (Wang et al., 2000), AA1028 (GenBank accession no. AF427318) and AA2499 (GenBank no. AF427320). These viruses were isolated from *A. agrarius* in China or detected from *A. agrarius* or humans in the far-east region of Russia (Fig. 1). On the other hand, the other viruses derived from *A. agrarius* could be classified into another lineage together with viruses 76-118 (Lee et al., 1978), LR1 (Yao et al., 2001a), CFC 94-2 (Lee et al., 1997) and Maaji-2 (GenBank accession no. AF321095), isolated previously from *A. agrarius* in China or from *A. agrarius* or humans in South Korea (Fig. 1). These two lineages are related more closely to each other than to any other lineages within HTNV. Interestingly, the viruses detected in *A. peninsulae* in China are distinct from the strains isolated from *A. peninsulae* in South Korea (SC1-4) (Baek et al., 2006) and in Russia (Solovey-AP61-1999 and Solovey-AP63-1999) (Lokugamage et al., 2004).

To characterize the viruses derived from *A. peninsulae* in China further, two hantavirus-positive lung tissues (samples CJAp89 and CJAp93) were homogenized and inoculated onto Vero-E6 cell monolayers as described by Lee (1999). At day 28 post-infection, hantavirus antigen-positive cells were detected by IFA in cells inoculated with each of the samples (data not shown). Cells infected with one of the viruses (CJAp93) were used to prepare RNA for amplification of the entire S and M segments. The S segment was amplified by using the primers S5 and S6 (Yao et al., 2001b) and the M segment was amplified as two overlapping fragments using two pairs of primers: M1 and HMF, and MHR and M4 (Shi et al., 1998; Yao et al., 2000).

The S segment was determined to be 1701 nt long with an open reading frame (ORF) (nt 36–1325) encoding the N protein. Comparison of both the nucleotide and amino acid sequences of the complete S segment with previously published sequences of other hantaviruses indicated that CJAp93 has a higher identity to HTNV (83.1–94.8% nucleotide and 96.3–99.3% amino acid) than to SEOV (74.9%/83.3%) or DOBV (73.7%/82.6%) (Table 1). Detailed comparison between CJAp93 and viruses within the HTNV group revealed that CJAp93 displayed a high degree of nucleotide identity (94.8%) with Bao14 (Wang et al., 2000) isolated from *A. agrarius* in Heilongjiang, China. Relatively lower identities were seen with other HTNV strains: 87.8% with 76-118 isolated from *A. agrarius* in South Korea (Lee et al., 1978), 83.4–83.5% with B78, Liu and H5, which were isolated from humans in China (Liang et al., 1994), and 83.1–84.5% with SC 1-4 isolated from *A. peninsulae* in South Korea (Baek et al., 2006).

The entire M segment of the CJAp93 strain was determined to be 3616 nt in length with an ORF (nt 41–3448) encoding a predicted glycoprotein precursor (GPC). Comparison of the M segment from CJAp93 with previously published M segment sequences of other hantaviruses revealed that CJAp93 has 80.5–97.4% and 91.2–99.3% identity at the nucleotide and amino acid levels, respectively, to other HTNV strains (Table 1). Like the S segment, the M segment sequence from the CJAp93 virus displays a high degree of nucleotide identity to Bao14 (97.4%) (Wang et al., 2000). Low nucleotide identities were seen with HTNV strains 76-118 (87.5%) (Lee et al., 1978) and CUMC-B11 (87.5%) (GenBank accession no. U38177). Even lower nucleotide sequence identities were observed with other HTNV virus strains isolated from *A. peninsulae* and humans: 80.0–80.4% with B78, Liu and H5 (isolated from humans in China; Liang et al., 1994) and 80.1–80.6% with SC 1-4 (isolated from *A. peninsulae* in South Korea; Baek et al., 2006). On the other hand, the M segment sequence of CJAp93 only shares about 70% similarity with SEOV or DOBV. Phylogenetic analysis of both the entire S and M segment resulted in topologies similar to that constructed from partial S and M segment sequences (Fig. 2).

*A. peninsulae* has long been considered a carrier of pathogenic hantavirus in north-eastern China (Li et al., 1983). However, no virus has been isolated or characterized. This study is, to our knowledge, the first attempt to characterize genetically hantaviruses carried by *A. peninsulae* in China. Our studies indicate that hantaviruses carried by *A. peninsulae* in China share a higher degree of similarity in both nucleotide and amino acid sequence with HTNV than with any other hantavirus group (DOBV, SEOV, PUUV, SNV etc.). Further analysis showed that all of the viruses derived from *A. peninsulae* in China form a lineage together with viruses isolated previously from *A. agrarius* in China [CJAa142] and in Russia [AA1028 (GenBank accession no. AF427318) and AA2499 (GenBank no. AF427320)], as well as from humans in Russia [HTN/Far East/3829 (GenBank accession no. AF175694), 4029 (GenBank no. AF175695), 4226 (GenBank no. AF175706) and 4290 (GenBank no. AF175690)]. In fact, viruses detected from *A. peninsulae* in this study also share a high degree of similarity with one of the viruses (CJAa142) from *A. agrarius*. Interestingly, viruses isolated from *A. peninsulae* in China are genetically distinct from those from the same species in Korea (Baek et al., 2006) and Russia (Lokugamage et al., 2004). Hantaviruses in
Table 1. Entire S and M segment nucleotide and amino acid sequence identities of CIAp93 from A. peninsulae with those of other hantaviruses

Percentage identities for nucleotide (above the diagonal) and amino acid (below the diagonal) sequences are presented.

<table>
<thead>
<tr>
<th>Strain</th>
<th>S segment/N protein Identity (%) with strain:</th>
<th>M segment/GPC Identity (%) with strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CJAp93</td>
<td>94.8</td>
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</tr>
<tr>
<td>Bao14</td>
<td>99.3</td>
<td>88.9</td>
</tr>
<tr>
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<td>98.8</td>
</tr>
<tr>
<td>LR1</td>
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<td>98.1</td>
</tr>
<tr>
<td>CUMC-B11</td>
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<td>98.4</td>
</tr>
<tr>
<td>A9</td>
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<td>97.4</td>
</tr>
<tr>
<td>A16</td>
<td>97.2</td>
<td>97.7</td>
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<td>Z10</td>
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</tr>
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<td>97.2</td>
</tr>
<tr>
<td>H5</td>
<td>97.0</td>
<td>97.4</td>
</tr>
<tr>
<td>Liu</td>
<td>97.0</td>
<td>97.4</td>
</tr>
<tr>
<td>SC-1</td>
<td>96.7</td>
<td>97.2</td>
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<td>83.0</td>
</tr>
<tr>
<td>DOBV</td>
<td>82.6</td>
<td>83.0</td>
</tr>
</tbody>
</table>

this lineage have been isolated from patients in China (Liang et al., 1994; Wang et al., 2000) and one of them (H5) was isolated from Heilongjiang province, which neighbours Jilin (Liang et al., 1994). It is not known why this lineage of hantavirus was not detected in A. peninsulae in this study and thus further studies are warranted.

Although hantaviruses have co-evolved with their respective hosts, a particular hantavirus species is also associated with several closely related host species (Plyusnin & Morzunov, 2001). For example, SEOV has been associated with Rattus rattus, R. norvegicus, Rattus losea and Rattus flavipectus (Lee & Johnson, 1982; Sun et al., 2003); Tula virus with Microtus arvalis and Microtus rossiaemeridionalis (Plyusnin et al., 1994); SNV with Peromyscus maniculatus, Peromyscus leucopus, Peromyscus boylii and Peromyscus truei (Childs et al., 1994; Mills et al., 1997; Monroe et al., 1999; Morzunov et al., 1998; Ottesen et al., 1996); PUUV with Clethrionomys glareolus and Clethrionomys rufocanus (Brummer-Korvenkontio et al., 1982; Kariwa et al., 1999); and DOBV with Apodemus favioliss and A. agrarius (Avsic-Zupanc et al., 2000; Klempa et al., 2003, 2005; Nemirov et al., 2001).
et al., 1999; Plyusnin et al., 1997). In the present study, sequence analysis of both the S and M segments (partial or complete) indicates that the viruses derived from A. peninsulae could be classified as a lineage with viruses previously isolated or detected from A. agrarius in China (Sun et al., 2001; Wang et al., 2000) and Russia [AA1028 (GenBank accession no. AF427318) and AA2499 (GenBank no. AF427320)]. One of the viruses (CJAa142) carried by A. agrarius from the same locality belongs to this lineage as well. The rest of the viruses detected in A. agrarius in the present study could be classified into another lineage with viruses detected previously from A. agrarius (Yao et al., 2001a) in China and South Korea (Lee et al., 1978). These two lineages are related closely to each other within the HTNV group. A. agrarius has been considered the natural host for HTNV (Chen et al., 1986; Lee et al., 1978; Plyusnin & Morzunov, 2001; Song et al., 1983; Wang et al., 2000), as most of the HTNV viruses (irrespective of lineage) have been isolated from A. agrarius. Previously, viruses in the AMR lineage within HTNV were detected in A. peninsulae in Korea (Baek et al., 2006) and Russia (Lokugamage et al., 2004). Viruses in this lineage have been detected in human patients in China (Liang et al., 1994; Wang et al., 2000), although they have yet not been detected there in A. peninsulae. In the present study, A. peninsulae in China was found to harbour HTNV belonging to a different lineage that is commonly found in A. agrarius in North-East Asia (Sun et al., 2001; Wang et al., 2000). These studies, together with the detection of hantavirus antigens and antibodies in A. peninsulae since the 1980s (Chen et al., 1986, 1999; Li et al., 1983; Luo & Liu, 1989), strongly support the notion that A. peninsulae, in addition to A. agrarius, is also a natural host for HTNV (Lokugamage et al., 2002).

Based on further refined analysis and comprehensive characterization, some authors suggested that viruses carried by A. peninsulae could be classified as a new hantavirus group (Baek et al., 2006; Lokugamage et al., 2004). Phylogenetic analysis of both the S and M segments indicates that hantaviruses derived from A. peninsulae in the present study are different from those detected in the same rodent species in South Korea and the far east of Russia. Rather, viruses detected in our study could be classified into a lineage with viruses previously isolated from A. agrarius in China (Sun et al., 2001; Wang et al., 2000). However, the viruses carried by A. peninsulae in the far east of Russia (Lokugamage et al., 2004) and South Korea (Baek et al., 2006) are grouped into a lineage with the viruses isolated from patients in China (Liang et al., 1994), indicating that similar viruses exist in China. Interestingly, hantavirus strains A3 and A16, isolated from A. agrarius in China (Wang et al., 2000; Yao et al., 2001c), can also be grouped into this lineage when the M segment sequences are analysed (Figs 1b, 2b). These data indicate that all of these lineages of hantaviruses can also be detected in both A. agrarius and A. peninsulae. In addition, genetic analysis of

Fig. 2. Phylogenetic trees of hantaviruses based on: (a) entire S segment sequences; (b) entire M segment sequences. Numbers above the branches are bootstrap support values for 1000 replicates. GenBank accession nos for the sequences analysed are indicated. Sequences obtained in this study are shown in bold.
the viruses previously detected in *A. peninsularis* in relation to other HTNVs demonstrates <7% amino acid differences in the S segment sequences (Table 1). Together, these data suggest strongly that all hantaviruses isolated from *A. peninsularis* in China, Russia and Korea belong to the HTNV group and that both *A. agrarius* and *A. peninsularis* are natural hosts for HTNV.

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**References**


