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Title: **Molecular phylogeny of a newfound hantavirus in the Japanese shrew mole (*Urotrichus talpoides*)**

Running title: Hantavirus in the Japanese Shrew Mole

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HFRS, hemorrhagic fever with renal syndrome; HPS, hantavirus pulmonary syndrome; ASAV, Asama virus

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ASAV S segment (EU929070, EU929071, EU929072); ASAV M segment (EU929073, EU929074, EU929075); and ASAV L segment (EU929076, EU929077, EU929078).

Abstract

Recent molecular evidence of genetically distinct hantaviruses in shrews, captured in widely separated geographical regions, corroborates decades-old reports of hantavirus antigens in shrew tissues. Apart from challenging the conventional view that rodents are the principal reservoir hosts, the newly identified soricid-borne hantaviruses raise the possibility that other soricomorphs, notably talpids, similarly harbor hantaviruses. In analyzing RNA extracts from lung tissues of the Japanese shrew mole (*Urotrichus talpoides*), captured in Japan between February and April 2008, a novel hantavirus genome, designated Asama virus (ASAV), was detected by RT-PCR. Pair-wise alignment and comparison of the S-, M- and L-segment nucleotide and amino acid sequences indicated that ASAV was genetically more similar to hantaviruses harbored by soricine shrews than rodents. However, the predicted secondary structure of the ASAV nucleocapsid protein was similar to that of rodent- and shrew-borne hantaviruses, exhibiting the same coiled-coil helix at the amino terminus. Phylogenetic analyses, using the maximum-likelihood method and other algorithms, consistently placed ASAV with recently identified soricine shrew-borne hantaviruses, suggesting a possible host-switching event in the distant past. The discovery of a hantavirus in the Japanese shrew mole enlarges our concepts about the evolutionary history of hantaviruses.

Key Words: Hantavirus, Mole, Shrew, Phylogeny, Host Switching

Introduction

Dating from investigations conducted independently by Japanese and Russian medical scientists along opposite sides of the Amur River in the 1930s and 1940s, rodents have been suspected to harbor the etiological agent(s) of hemorrhagic fever with renal syndrome (HFRS) (1, 2). Following a several decades-long impasse, the striped field mouse (*Apodemus agrarius*) was identified as the reservoir host of Hantaan virus (3), the prototype virus of HFRS (4). This seminal discovery made possible the identification of genetically distinct hantaviruses in other murinae and arvicolinae rodent species (5-12). Also, a previously unrecognized, frequently fatal respiratory disease, called hantavirus pulmonary syndrome (HPS) (13), is now known to be caused by hantaviruses harbored by neotominae and sigmodontinae rodents in the Americas, the prototype being Sin Nombre virus (SNV) in the deer mouse (*Peromyscus maniculatus*) (14). Remarkably, each of these genetically distinct hantaviruses appears to share a long co-evolutionary history with a specific rodent host species. That is, based on phylogenetic analyses of full-length viral genomic and rodent mitochondrial DNA (mtDNA) sequences, these hantaviruses segregate into clades, which parallel the evolution of rodents in the murinae, arvicolinae, neotominae and sigmodontinae subfamilies (15, 16).

Until recently, the single exception to the strict rodent association of hantaviruses was Thottapalayam virus (TPMV), a long-unclassified virus originally isolated from the Asian house shrew (*Suncus murinus*) (17, 18). Analysis of the recently acquired full genome of TPMV strongly supports an ancient non-rodent host origin and an early evolutionary divergence from rodent-borne hantaviruses (19, 20). Employing reverse transcription polymerase chain reaction (RT-PCR) and oligonucleotide primers based on the TPMV genome, we have targeted the discovery of hantaviruses in shrew species from widely separated geographical regions, including the Chinese mole shrew (*Anourosorex squamipes*) from Vietnam (21), Eurasian common shrew (*Sorex araneus*) from Switzerland (22), northern short-tailed shrew (*Blarina brevicauda*), masked shrew (*Sorex*

cinereus) and dusky shrew (*Sorex monticolus*) from the United States (23, 24) and Ussuri white-toothed shrew (*Crocidura lasiura*) from Korea (J.-W. Song and R. Yanagihara, unpublished observations). Many more shrew-hantavirus associations undoubtedly exist, as evidenced by preliminary studies of *Sorex caecutiens* and *Sorex roboratus* from Russia (H. J. Kang, S. Arai and R. Yanagihara, unpublished observations) and *Sorex palustris, Sorex trowbridgii* and *Sorex vagrans* from North America (H. J. Kang and R. Yanagihara, unpublished observations).

In addition to challenging the view that rodents are the sole or principal reservoirs of hantaviruses, the discovery of soricid-borne hantaviruses predicts that other soricomorphs, notably talpids, might also harbor genetically distinct hantaviruses. In this regard, hantavirus antigens had been detected by enzyme immunoassay and fluorescence techniques in tissues of the European common mole (*Talpa europea*) captured in Russia (25) and Belgium (26), but no further studies have been published and no reports are available in the literature about hantavirus infection in shrew moles. Relying on oligonucleotide primers designed from our expanding sequence database of shrew-borne hantaviruses, we have identified a novel hantavirus genome, designated Asama virus (ASAV), in the Japanese shrew mole (*Urotrichus talpoides*). Genetic and phylogenetic analyses indicate that ASAV is distinct but related to hantaviruses harbored by soricine shrews, suggesting a very ancient evolutionary history, probably involving multiple host-switching events in the distant past.

Results

RT-PCR detection of hantavirus sequences. In using RT-PCR to analyze RNA extracts, from lung tissues of three Laxmann's shrew (*Sorex caecutiens*), five slender shrew (*Sorex gracillimus*), six long-clawed shrew (*Sorex unguiculatus*), one dsinezumi shrew (*Crocidura dsinezumi*) and six Japanese shrew mole (*Urotrichus talpoides*), hantavirus sequences were not detected in shrew tissues, but were found in one of two

and in two of three Japanese shrew moles, captured in Ohtani (34 28'14.0° N; 136 45'46.2° E) and near Asama River (34 28'12.79° N; 136 45'45.81° E), respectively, located approximately 1 km apart at 50-m elevation in Mie Prefecture, during February and April 2008. After the initial detection of hantavirus sequences, amplification of the S-, M- and L-genomic segments was accomplished using oligonucleotide primers based on conserved regions.

Nucleotide and amino acid sequence analysis. The S, M and L segments of ASAV, as amplified from tissues of three wild-caught Japanese shrew moles, indicated an overall genomic structure similar to that of other rodent- and soricid-borne hantaviruses. The nucleotide and deduced-amino acid sequences of each ASAV genomic segment were highly divergent from that of rodent-borne hantaviruses, differing by approximately 30-40% (Table 1).

The S-genomic segment of ASAV (1,801 nucleotides for strains H4 and N9 and 1,756-nucleotides for strain N10) encoded a predicted nucleocapsid (N) protein of 434 amino acids, starting at nucleotide position 39, and a 465-nucleotide 3'-noncoding region (NCR). The hypothetical NSs opening reading frame, typically found in the S segment of arvicolinae, neotominae and sigmodontinae rodent-borne hantaviruses, was not found in ASAV. The inter-strain variation of the S segment among the ASAV strains was negligible (1.1% at the nucleotide and 0% at the amino acid levels). In the hypervariable region of the N protein, between amino acid residues 244 and 269, ASAV differed by 18-20 and 20-22 amino acids from soricine shrew- and rodent-borne hantaviruses, respectively. Sequence similarity of the entire S-genomic segment of ASAV strains H4, N9 and N10 was higher with soricine shrew-borne hantaviruses than with hantaviruses harbored by rodents (Table 1).

The 3,646-nucleotide full-length M-genomic segment of ASAV (including 21-nucleotide end primers) encoded a predicted glycoprotein of 1,141 amino acids, starting at nucleotide position 41, and a 183-nucleotide 3'-NCR. Four potential N-linked glycosylation sites (three in Gn at amino acid positions 138, 352, 404, and one in Gc at position 933) were found in ASAV. In addition, the highly conserved WAASA amino-acid motif,

which in ASAV was WAVSA (amino acid positions 649-653), was present. An inter-strain variation of 0.1-0.7% and 0-0.4% at the nucleotide and amino acid levels, respectively, was found among ASAV strains H4, N9 and N10. The full-length Gn/Gc amino acid sequence of ASAV exhibited the highest similarity with Seewis virus (79.5%) from the Eurasian common shrew (Table 1).

Analysis of the nearly full-length 6,126-nucleotide (2,041-amino acid) L segment of ASAV revealed the five conserved motifs (A, B, C, D and E), previously identified among all hantavirus RNA polymerases. The overall high sequence similarity of the L segment among ASAV and rodent- and soricid-borne hantaviruses was consistent with the functional constraints on the RNA-dependent RNA polymerase (Table 1).

Secondary structure of N protein. Secondary structure analysis revealed striking similarities, as well as marked differences, among the N protein sequences of ASAV and 13 representative rodent- and soricid-hantaviruses. Each sequence appeared to adopt a two-domain, predominantly α-helical structure joined by a central β-pleated sheet. While the length of the N-terminal domain was mostly invariant, the length of the central β-pleated sheet and of the adjoining C-terminal α-helical domain showed systematic reciprocal structural changes according to the genetic relationship and evolutionary descent of the individual sequences.

The N-terminal α-helical domain, from residues 1 to approximately 140, was composed of four helices connected by large loops (representative viruses shown in Fig. 1). The C-terminal α-helical domain, from residues 210/230 to 430, contained seven to nine helices that were connected by tighter loops (Fig. 1). And the central β-pleated region, from residues 140 to 210/230, was composed of three to five possible anti-parallel strands. Interestingly, an increasing number of strands in this section were observed when the hantaviral sequences were arranged according to their positions in the phylogenetic tree. This resulted in a widening of the central β-pleated region with a concomitant shortening of the C-terminal α-helical domain while preserving the total length of the protein. The helix adjoining the central β-sheet progressively shortened in this

architectural change. These structural alterations were reversed in TPMV, which was evolutionarily more distant from the other sequences (Fig. 1).

Phylogenetic analysis. Exhaustive phylogenetic analyses based on nucleotide and deduced amino acid sequences of the S-, M- and L-genomic segments, generated by the maximum-likelihood (ML) method, indicated that ASAV was distinct from rodent-borne hantaviruses (with high posterior node probabilities based on 30,000 trees) (Fig. 2). Nearly identical topologies were consistently derived, using various algorithms and different taxa and combinations of taxa, suggesting an ancient evolutionary origin. The most strikingly consistent feature was the phylogenetic position of ASAV with soricine shrew-borne hantaviruses, rather than being placed as an outgroup beyond TPMV, the prototype crocidurine shrew-borne hantavirus. That is, the prediction that a shrew mole-associated hantavirus would be phylogenetically distant from hantaviruses harbored by shrews was not validated.

Sequence and phylogenetic analysis of mole mtDNA. Molecular confirmation of the taxonomic identification of the hantavirus-infected Japanese shrew moles based on morphological features was achieved by amplification and sequencing of the 1,140-nucleotide mtDNA cytochrome *b* gene. Phylogenetic analysis showed distinct grouping of hantavirus-infected *U. talpoides* from this study with other *U. talpoides* mtDNA sequences available in GenBank, rather than with soricids or rodents (Fig. 3).

Discussion

Newfound shrew mole-borne hantavirus. Despite reports of hantavirus antigens in tissues of the Eurasian common shrew (*Sorex araneus*), alpine shrew (*Sorex alpinus*), Eurasian water shrew (*Neomys fodiens*) and common mole (*Talpa europea*) (25-28), shrews and moles have been generally dismissed as being important in the transmission dynamics of hantaviruses. With the recent demonstration that TPMV and other

newly identified soricid-borne hantaviruses are genetically distinct and phylogenetically distant from rodent-borne hantaviruses (19-24), the conventional view that rodents are the principal or primordial reservoir hosts of hantaviruses is being challenged. In its wake, a compelling conceptual framework, or paradigm shift, is emerging that supports an ancient origin of hantaviruses in soricomorphs (or insectivores). To this emerging concept must now be added a newfound hantavirus, designated ASAV, in the Japanese shrew mole (family *Talpidae*, subfamily *Talpinae*). The demonstration of ASAV sequences in this endemic shrew mole species captured in two separate locations in Mie Prefecture argues strongly against this being an isolated or coincidental event. Instead, these data suggest a well-established co-existence of this newfound hantavirus in the Japanese shrew mole and further solidifies the notion of a long-standing evolutionary association between soricomorphs and hantaviruses.

Shrew moles differ from typical or true moles in that they look like shrews and are much less specialized for burrowing. The greater Japanese shrew mole, which morphologically resembles semi-fossorial shrew moles in China (*Scaptonyx*) and North America (*Neurotrichus*), is widely distributed in the lowlands and peripheral islands of Japan, except Hokkaido, and is not found on mainland Asia (29, 30). Also endemic in Japan, the lesser Japanese shrew mole (*Dymecodon pilirostris*) is largely restricted to mountainous regions on Honshu, Shikoku and Kyushu and is considered the more ancestral species. As determined by cytochrome *b* mtDNA and nuclear recombination activating gene-1 (RAG1) sequence analyses, the greater and lesser Japanese shrew moles are closely related, but their evolutionary origins and biogeography remain unresolved (31, 32). The existence of two distinct chromosomal races of *U. talpoides*, geographically separated by the Fuji and Kurobe rivers in central Honshu (33, 34), provides an opportunity to further clarify the evolutionary origins of shrew mole-borne hantaviruses in Japan. Studies, now underway, will examine if ASAV is harbored by *U. talpoides* in locations east of Mie Prefecture, as well as ascertain if *D. pilirostris* also serves as a reservoir of ASAV-related hantaviruses.

Although our previous RT-PCR attempts have failed to detect hantavirus sequences in other talpid species, including the long-nosed mole (*Euroscaptor longirostris*) (21) and eastern mole (*Scalopus aquaticus*) (H. J. Kang and R. Yanagihara, unpublished observations), it may be because appropriate primers were not employed. That is, based on the vast genetic diversity of soricid-borne hantaviruses, talpid-associated hantaviruses may be even more highly divergent and would require designing very different primers for amplification.

Finally, as for shrew-borne hantaviruses, the importance of this newfound shrew mole-associated hantavirus to human health warrants careful inquiry. Virus isolation attempts have been unsuccessful to date. In the meantime, an ASAV recombinant N protein is being prepared for use in enzyme immunoassays. In this regard, as evidenced by the corresponding sequence of YIEVNGIRKP in the ASAV N protein, the monoclonal antibody E5/G6, which recognizes the epitope YEDVNGIRKP (with variations) in rodent-borne hantaviruses (35), might be useful as a capturing antibody. In addition, other sensitive technologies, including nucleic acid and protein microarrays, are being developed to establish if ASAV is pathogenic for humans.

Secondary structure of hantavirus N protein. The overall N protein secondary structure of ASAV and other hantaviruses was compatible with a putative bi-lobed three-dimensional protein architecture, which would allow the protein to clamp around the RNA as often observed in a variety of RNA-binding proteins. While the core elements of the central β-pleated sheet appeared also to be conserved, more evolutionary variability was seen in the number of constituent strands and in the adjoining connecting elements and helices. This variability may reflect the function of this region as a flexible spacer element that can determine the relative orientation and separation of the two main α-helical domains and can accommodate the conformational changes upon RNA binding. The connecting regions could act as hinges of variable size leading to opening of the nucleocapsid. The flexible domain linkage would allow the interaction with the differently sized virus-specific RNA structures and also may modulate the oligomerization or assembly of the N protein in an

evolutionarily and systematically changing fashion.

Phylogeny of hantaviruses. Just as the identification of novel hantaviruses in the Therese shrew (*Crocidura theresae*) (36) and the northern short-tailed shrew (*Blarina brevicauda*) (23) heralded the discovery of other soricid-borne hantaviruses (21, 22, 24), the detection of ASAV in the Japanese shrew mole forecasts the existence of other hantaviruses in talpids. Perhaps more importantly, these findings emphasize that the evolutionary history and transmission dynamics of hantaviruses are far more rich and complex than originally imagined. That is, instead of a single progenitor virus being introduced into the rodent lineage more than 50 million years ago, mounting evidence supports a more ancient virus lineage with parallel co-evolution of hantaviruses in crocidurine and soricine shrews. And given the sympatric and synchronistic co-existence of moles, shrews and rodents, through a long continuum dating from the distant past to the present time, it seems plausible that ongoing exchanges of hantaviruses continues to drive their evolution.

In this regard, several rodent species may occasionally serve as reservoir hosts for the same hantavirus. For example, Vladivostok virus (VLAV) may be found in its natural host, the reed vole (*Microtus fortis*) (37-39), as well as an ancillary host, the tundra or root vole (*Microtus oeconomus*) (40). Similarly, the Maximowiczi vole (*Microtus maximowiczii*) is the natural reservoir of Khabarovsk virus (KHAV), which may also be harbored by *Microtus fortis* (10, 39, 40). Moreover, a KHAV-related hantavirus, named Topografov virus (TOPV), has also been found in the Siberian lemming (*Lemmus sibiricus*) (41). This is a far more extreme situation in which a hantavirus has switched from its natural rodent reservoir host and become well established in a rodent host of a different genus. Such host-switching or species-jumping events may account for the extraordinarily close phylogenetic relationship between TOPV and KHAV (41). That is, whereas *Lemmus* and *Microtus* are very distantly related, TOPV and KBRV are monophyletic.

In much the same way, as evidenced by the polyphylogenetic relationship between ASAV and other soricid-associated hantaviruses, a hantavirus closely related to ASAV may have ‘jumped’ from its natural

soricine shrew host to establish itself in the Japanese shrew mole. That is, burrows and shallow tunnel systems excavated by Japanese shrew moles may be occasionally shared with sympatric species, including shrews, allowing opportunities for virus transmission through interspecies wounding or contaminated nesting materials. Such a host-switching event may have occurred in the distant past, possibly before the present-day Japanese shrew mole became endemic in Japan. Accordingly, intensive investigations of shrews in Japan and elsewhere in Far East Asia may uncover this progenitor hantavirus in its natural soricid reservoir.

Materials and Methods

Trapping. Sherman traps (H.B. Sherman, Tallahassee, FL) and pit-hole traps were used to capture shrews and shrew moles in Japan between October 2006 and April 2008. Traps were set at intervals of approximately 4 to 5 m during the evening hours of each day, over a four-day period, at sites in Hokkaido (Hamatonbetsu, Saruhutsu and Nopporo) and Honshu (Nara and Mie), where soricomorphs had previously been captured. Species, gender, weight, reproductive maturity and global positioning system (GPS) coordinates of each captured animal were recorded.

Specimen processing. Lung, liver, kidney and spleen tissues, dissected using separate instruments, were frozen in dry ice, then stored at -80^{o}C until used for testing. In some instances, portions of tissues were also placed in RNAlater RNA Stabilization Reagent (QIAGEN, Inc., Chatsworth, CA) and processed for RT-PCR, within 4 weeks of tissue collection.

RNA extraction and cDNA synthesis. Total RNA was extracted from tissues, using the PureLink Micro-to-Midi total RNA purification kit (Invitrogen, San Diego, CA), in a laboratory in which hantaviruses had never been handled. cDNA was then prepared using the SuperScript™ III RNase H- reverse transcriptase

kit (Invitrogen) with a primer based on the conserved 5'-terminus of the S, M and L segments of hantaviruses (5'-TAGTAGTAGACTCC-3').

RT-PCR. Touchdown-PCR was performed using oligonucleotide primers designed from TPMV and other hantaviruses: S (outer: 5'-TAGTAGTAGACTCCTTRAARAGC-3' and 5'-AGCTCIGGATCCATITCATC-3'; inner: 5'-AGYCCIGTIATGRGWGTIRTYGG-3' and 5'-AIGAYTGRTARAAIGAIGAYTTYT T-3'); M (outer: 5'-GGACCAGGTGCADCTTGTGAAGC-3' and 5'-GAACCCCADGCCCCITCYAT-3'; inner: 5'-TGTGTICCWGGITTYCATGGIT-3' and 5'-CATGAYATCTCCAGGGTCHCC-3'); and L (outer: 5'-ATGTAYGTBAGTGCWGATGC-3' and 5'-AACCADTCWGTYCCRTCATC-3'; inner: 5'-TGCWGATGCHACIAARTGGTC-3' and 5'-GCRTCRTCWGARTGRTGDGCAA-3').

First- and second-round PCR were performed in 20-μL reaction mixtures, containing 250 μM dNTP, 2.5 mM $MgCl_2$, 1 U of LA Taq polymerase (Takara, Shiga, Japan) and 0.25 μM of each primer (24). Initial denaturation at 94°C for 2 min was followed by two cycles each of denaturation at 94°C for 30 sec, two-degree step-down annealing from 46°C to 38°C for 40 sec, and elongation at 72°C for 1 min, then 30 cycles of denaturation at 94°C for 30 sec, annealing at 42°C for 40 sec, and elongation at 72°C for 1 min, in a GeneAmp PCR 9700 thermal cycler (Perkin-Elmer, Waltham, MA). PCR products were separated by agarose gel electrophoresis and purified using the Qiaex Gel Extraction Kit (Qiagen, Hilden, Germany). Amplified DNA was sequenced directly using an ABI Prism 3130 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA).

Genetic and phylogenetic analyses. Sequences were processed using the Genetyx version 9 software (Genetyx Corporation, Tokyo, Japan) and aligned using Clustal W and W2 (42). For phylogenetic analysis, ML consensus trees were generated by the Bayesian Metropolis–Hastings Markov Chain Monte Carlo (MCMC) tree-sampling methods as implemented by Mr. Bayes (43) using a GTR+I+G model of evolution, as

selected by hierarchical likelihood-ratio test (hLRT) in MrModeltest2.3 (http://www.abc.se/~nylander/mrmodeltest2/mrmodeltest2.html) (44), partitioned by codon position.

An initial ML estimate of the model of evolutionary change among aligned viruses was generated by MrModeltest2.3. ML tree estimation in PAUP (45) was conducted starting with a neighbor-joining (NJ) tree based on this initial ML model of evolution, and proceeding with successive rounds of heuristic tree-searches to select the single most likely ML tree. Support for topologies was generated by bootstrapping for 1,000 NJ replicates (under the ML model of evolution, implemented in PAUP) and for 100 ML replicates (data not shown). Phylogenetic relationships were further confirmed using amino acid sequences analyzed by Bayesian tree sampling, using the WAG model (46) implemented by Mr. Bayes (43).

Secondary structure prediction. Secondary structure prediction of the N protein was performed using the NPS@ structure server (47). To achieve 70-80% accuracy and to validate the prediction, five different methods were employed jointly: DSC (48), HNN (49), PHD (50), PREDATOR (51), and MLRC (49), which in turn were based on GOR4 (52), SIMPA96 (53) and SOPMA (54). The minimum number of conformational states was set to four (helix, sheet, turn and coil) for each analysis, and the results were combined into a consensus structure where the most prevalent predicted conformational state was reported for each residue. For convenience in visualization of the predicted structures, the NPS@ server also provided graphic outputs for the individual sequences which were subsequently combined into a multipart joint image.

PCR amplification of shrew mole mtDNA. Total DNA, extracted from liver tissues using the QIAamp Tissue Kit (QIAGEN), was used to verify the identity of the hantavirus-infected shrew moles. The 1,140-nucleotide mtDNA cytochrome *b* gene was amplified by PCR, using previously described universal primers (5'- CGAAGCTTGATATGAAAAACCATCGTTG-3'; 5'-AACTGCAGTCATCTCCGGTTTACAAGAC-3') (55). PCR was performed in 50-μL reaction mixtures, containing 200 μM dNTP and 1.25 U of rTaq polymerase (Takara). Cycling conditions consisted of an initial

denaturation at 95^{o}C for 4 min followed by 40 cycles with denaturation at 94^{o}C for 1 min, annealing at 57^{o}C for 1 min, and elongation at 72^{o}C for 1 min in a GeneAmp PCR9700 thermal cycler.

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References

1. Yanagihara R (1990) *Rev Infect Dis* 12:449-457.

2. Yanagihara R, Gajdusek DC (1988) in *CRC Handbook of Viral and Rickettsial Hemorrhagic Fevers*, ed Gear JHS (CRC Press, Boca Raton), pp 151-188.

3. Lee HW, Lee P-W, Johnson KM (1978) *J Infect Dis* 137:298-308.

4. Schmaljohn CS, Hasty SE, Harrison SA, Dalrymple JM (1983) *J Infect Dis* 148:1005-1012.

5. Brummer-Korvenkontio M, Vaheri A, Hovi T, von Bonsdorff CH, Vuorimies J, Manni T, Penttinen K, Oker-Blom N, Lähdevirta J (1980) *J Infect Dis* 141:131-134.

6. Lee HW, Baek LJ, Johnson KM (1982) *J Infect Dis* 146:638-644.

7. Lee P-W, Amyx HL, Yanagihara R, Gajdusek DC, Goldgaber D, Gibbs CJ Jr (1985) *J Infect Dis* 152:826-829.

8. Avsic-Zupanc T, Xiao SY, Stojanovic R, Gligic A, van der Groen G, LeDuc JW (1992) *J Med Virol* 38:132-137.

9. Plyusnin A, Vapalahti O, Lankinen H, Lehväslaiho H, Apekina N, Myasnikov Y, Kallio-Kokko H, Henttonen H, Lundkvist A, Brummer-Korvenkontio M, Gavrilovskaya I, Vaheri A (1994) *J Virol* 68:7833-7839.

10. Hörling J, Chizhikov V, Lundkvist A, Jonsson M, Ivanov L, Dekonenko A, Niklasson B, Dzagurova T, Peters CJ, Tkachenko E, Nichol S (1996) *J Gen Virol* 77:687-694.

11. Nemirov K, Vapalahti O, Lundkvist A, Vasilenko V, Golovljova I, Plyusnina A, Niemimaa J, Laakkonen J, Henttonen H, Vaheri A, Plyusnin A (1999) *J Gen Virol* 80:371-379.

12. Baek LJ, Kariwa H, Lokugamage K, Yoshimatsu K, Arikawa J, Takashima I, Chung SY, Lee EJ, Moon SS, Song K-J, Klein TA, Yanagihara R, Song J-W (2006) *J Med Virol* 78:290-297.

13. Duchin JS, Koster FT, Peters CJ, Simpson GL, Tempest B, Zaki SR, Ksiazek TG, Rollin PE, Nichol S, Umland ET, et al (1994) *N Engl J Med* 330:949-955.
14. Nichol ST, Spiropoulou CF, Morzunov S, Rollin PE, Ksiazek TG, Feldmann H, Sanchez A, Childs J, Zaki S, Peters CJ (1993) *Science* 262:914-917.
15. Plyusnin A, Vapalahti O, Vaheri A (1996) *J Gen Virol* 77:2677–2687.
16. Hughes AL, Friedman R (2000) *Mol Biol Evol* 17:1558-1568.
17. Carey DE, Reuben R, Panicker KN, Shope RE, Myers RM (1971) *Indian J Med Res* 59:1758-1760.
18. Zeller HG, Karabatsos N, Calisher CH, Digoutte J-P, Cropp CB, Murphy FA, Shope RE (1989) *Arch Virol* 108:211-227.
19. Song J-W, Baek LJ, Schmaljohn CS, Yanagihara R (2007) *Emerg Infect Dis* 13:980-985.
20. Yadav PD, Vincent MJ, Nichol ST (2007) *Virol J* 4:80.
21. Song J-W, Kang HJ, Song K-J, Truong TT, Bennett SN, Arai S, Truong NU, Yanagihara R (2007) *Emerg Infect Dis* 13:1784-1787.
22. Song J-W, Gu SH, Bennett SN, Arai S, Puorger M, Hilbe M, Yanagihara R (2007) *Virol J* 4:114.
23. Arai S, Song J-W, Sumibcay L, Bennett SN, Nerurkar VR, Parmenter C, Cook JA, Yates TL, Yanagihara R (2007) *Emerg Infect Dis* 13:1420-1423.
24. Arai S, Bennett SN, Sumibcay L, Cook JA, Song J-W, Hope A, Parmenter C, Nerurkar VR, Yates TL, Yanagihara R (2008) *Am J Trop Med Hyg* 78:348-351.
25. Tkachenko EA, Ivanov AP, Donets MA, Miasnikov YA, Ryltseva EV, Gaponova LK, Bashkirtsev VN, Okulova NM, Drozdov SG, Slonova RA, Somov GP, Astakhova TI (1983) *Ann Soc Belg Med Trop* 63:267-269.
26. Clement J, McKenna P, Leirs H, Verhagen R, Lefevre A, Song G, Tkachenko E, van der Groen G (1994) in *Virus Infections of Rodents and Lagomorphs*, ed Horzinek MC (Elsevier Science BV, Amsterdam), pp 295-

316.

27. Gavrilovskaya IN, Apekina NS, Myasnikov YuA, Bernshtein AD, Ryltseva EV, Gorbachkova EA, Chumakov MP (1983) *Arch Virol* 75:313-316.
28. Gligic A, Stojanovic R, Obradovic M, Hlaca D, Dimkovic N, Diglisic G, Lukac V, Ler Z, Bogdanovic R, Antonijevic B, Ropac D, Avsic T, LeDuc JW, Ksiazek T, Yanagihara R, Gajdusek DC (1992) *Eur J Epidemiol* 8:816-825.
29. Ishii N (1993) *J Mamm Soc Jpn* 18:87-98.
30. Yokohata Y (2005) *Mammal Study* 30:S25-S30.
31. Shinohara A, Campbell KL, Suzuki H (2003) *Mol Phylogenet Evol* 27:247-258.
32. Shinohara A, Campbell KL, Suzuki H (2005) *Mammal Study* 30:S19-S24.
33. Kawada S, Obara Y (1999) *Zoolog Sci* 16:167-174.
34. Harada M, Ando A, Tsuchiya K, Koyasu K (2001) *Zoolog Sci* 18:433-442.
35. Okumura M, Yoshimatsu K, Kumperasart S, Nakamura I, Ogino M, Taruishi M, Sungdee A, Pattamadilok S, Ibrahim IN, Erlina S, et al (2007) *Clin Vaccine Immunol* 14:173-181.
36. Klempa B, Fichet-Calvet E, Lecompte E, Auste B, Aniskin V, Meisel H, Barrier P, Koivogui L, ter Meulen J, Krüger DH (2007) *Emerg Infect Dis* 13:520-552.
37. Kariwa H, Yoshimatsu K, Sawabe J, Yokota E, Arikawa J, Takashima I, Fukushima H, Lundkvist Å, Shubin FN, Isachkova LM, Slonova RA, Leonova GN, Hashimoto N (1999) *Virus Res* 59:219-228.
38. Zou Y, Wang JB, Gaowa HS, Yao LS, Hu GW, Li MH, Chen HX, Plyusnin A, Shao R, Zhang YZ (2008) *J Med Virol* 80:680-688.
39. Zou Y, Xiao QY, Dong X, Lv W, Zhang SP, Li MH, Plyusnin A, Zhang YZ (2008) *Virus Res* (Epub ahead of print)

40. Plyusnina A, Laakkonen J, Niemimaa J, Nemirov K, Muruyeva G, Pohodiev B, Lundkvist Å, Vaheri A, Henttonen H, Vapalahti O, Plyusnin A (2008) *Virol J* 5:4do

41. Vapalahti O, Lundkvist Å, Fedorov V, Conroy CJ, Hirvonen S, Plyusnina A, Nemirov K, Fredga K, Cook JA, Niemimaa J, Kaikusalo A, Henttonen H, Vaheri A, Plyusnin A (1999) *J Virol* 73:5586-5592.

42. Thompson JD, Higgins DG, Gibson TJ (1994) *Nucl Acids Res* 22:4673-4680.

43. Ronquist F, Huelsenbeck JP (2003) *Bioinformatics* 19:1572-1574.

44. Posada D, Crandall KA (1998) *Bioinformatics* 14:817-818.

45. Swofford DL. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sunderland, Massachusetts: Sinauer Associates, 2003.

46. Whelan S, Goldman N (2001) *Mol Biol Evol* 18:691-699.

47. Combet C, Blanchet C, Geourjon C, Deléage G (2000) *Trends Biochem Sci* 25:147-150.

48. King RD, Sternberg MJ (1996) *Protein Sci* 5:2298-2310.

49. Guermeur Y, Geourjon C, Gallinari P, Deléage G (1999) *Bioinformatics* 15:413-421.

50. Rost B, Sander C (1993) *J Mol Biol* 232:584-599.

51. Frishman D, Argos P (1996) *Protein Eng* 9:133-142.

52. Garnier J, Gibrat J-F, Robson B (1996) *Methods Enzymol* 266:540-553.

53. Levin JM, B. Robson B, Garnier J (1986) *FEBS Lett* 205:303-308.

54. Geourjon C, Deléage G (1995) *Comput Appl Biosci* 11:681-684.

55. Irwin DM, Kocher TD, Wilson AC (1991) *J Mol Evol* 32:128-144.

Table 1. Nucleotide and amino acid sequence similarity (%) between ASAV strain N10 and representative rodent- and shrew-borne hantaviruses.

	S segment		M segment		L segment	
Virus strain	1710 nt	434 aa	3604 nt	1141 aa	6126 nt	2041aa
HTNV 76-118	58.5	62.7	62.7	59.4	70.3	74.6
SEOV 80-39	63.1	62.0	62.8	59.1	70.4	74.7
SOOV SOO-1	62.8	62.9	63.3	59.7	70.2	74.3
DOBV Greece	62.2	62.2	63.0	59.3	70.2	75.7
PUUV Sotkamo	59.3	59.3	59.6	52.2	68.1	68.0
TULV 5302v	61.5	59.4	60.5	52.6	68.3	67.9
PHV PH-1	60.7	59.3	59.3	51.9	66.4	67.1
SNV NMH10	60.9	58.9	59.0	54.1	68.2	68.8
RPLV MSB89866	-	-	68.8	63.5	75.2	83.2
CBNV CBN-3	67.7	70.4	68.2	71.0	76.0	84.7
ARRV MSB73418	65.7	66.6	70.9	77.0	73.8	83.5
JMSV MSB89332	66.2	66.9	-	-	74.3	82.6
SWSV mp70	63.8	69.9	75.2	79.5	75.0	83.2
ASAV H4	98.9	100	99.3	99.6	98.2	99.6
ASAV N9	100	100	99.9	100	100	100
MJNV 05-11	57.2	46.0	56.1	44.4	65.8	61.5
TPMV VRC	58.0	45.8	57.7	43.0	64.3	62.0

Abbreviations: ARRV, Ash River virus; ASAV, Asama virus; CBNV, Cao Bang virus; DOBV, Dobrava virus; HTNV, Hantaan virus; JMSV, Jemez Spring virus; MJNV, Imjin virus; PHV, Prospect Hill virus; PUUV,

Puumala virus; RPLV, Camp Ripley virus; SEOV, Seoul virus; SNV, Sin Nombre virus; SOOV, Soochong virus; SWSV, Seewis virus; TPMV, Thottapalayam virus; TULV, Tula virus. nt, nucleotides; aa, amino acids.

Figure Legends

Fig. 1. Japanese shrew mole (*Urotrichus talpoides*) (family *Talpidae*, subfamily Talpinae), one of two endemic shrew mole species found only in Japan.

Fig. 2. Consensus secondary structure of N protein of ASAV and representative rodent- and soricid-borne hantaviruses, predicted using a high-performance method implemented on the NPS@ structure server (47). As shown, the ASAV N protein was very similar to that of other hantaviruses, characterized by the same coiled-coil helix at the amino terminal end and similar secondary structure motifs at their carboxyl terminals. The predicted structures were represented by colored bars to visualize the schematic architecture: α-helix, blue; β-sheet, red; coil, magenta; unclassified, gray. For simplicity, turns and other less frequently occurring secondary structural elements were omitted. All sequences are numbered from Met-1.

Fig. 3. Phylogenetic trees generated by the ML method, using the GTR+I+G model of evolution as estimated from the data, based on the alignment of the coding regions of the full-length (A) 1,302-nucleotide S and (B) 3,423-nucleotide M segments and partial (C) 6,126-nucleotide L-genomic segment of ASAV. The phylogenetic positions of ASAV strains H4, N9 and N10 are shown in relationship to representative murinae rodent-borne hantaviruses, including Hantaan virus (HTNV 76-118, NC_005218, NC_005219, NC_005222), Soochong virus (SOOV SOO-1, AY675349, AY675353, DQ056292), Dobrava virus (DOBV AP99, NC_005233, NC_005234, NC_005235) and Seoul virus (SEOV HR80-39, NC_005236, NC_005237, NC_005238); arvicolinae rodent-borne hantaviruses, including Tula virus (TULV M5302v, NC_005227, NC_005228, NC_005226), Puumala virus (PUUV Sotkamo, NC_005224, NC_005223, NC_005225) and Prospect Hill virus (PHV PH-1, Z49098, X55129, EF646763); and a neotominae rodent-borne hantavirus, Sin Nombre virus (SNV NMH10, NC_00521, NC_005215, NC_005217). Also shown are Thottapalayam virus

(TPMV VRC, AY526097, EU001329, EU001330) from the Asian house shrew (*Suncus murinus*); Imjin virus (MJNV 05-11, EF641804, EF641798, EF641806) from the Ussuri white-toothed shrew (*Crocidura lasiura*); Cao Bang virus (CBNV CBN-3, EF543524, EF543526) from the Chinese mole shrew (*Anourosorex squamipes*); Ash River virus (ARRV MSB 73418, EF650086) from the masked shrew (*Sorex cinereus*); Jemez Springs virus (JMSV MSB89332, EF619962) from the dusky shrew (*Sorex monticolus*); and Seewis virus (SWSV mp70, EF636024) from the Eurasian common shrew (*Sorex araneus*). The numbers at each node are posterior node probabilities based on 30,000 trees: two replicate MCMC runs consisting of six chains of 3 million generations each sampled every 1,000 generations with a burn-in of 7,500 (25%). The scale bar indicates nucleotide substitutions per site. GenBank accession numbers: ASAV S segment (H4, EU929070; N9, EU929071; N10, EU929072); ASAV M segment (H4, EU929073; N9, EU929074; N10, EU929075); and ASAV L segment (H4, EU929076; N9, EU929077; N10, EU929078).

Fig. 4. Confirmation of host identification of ASAV-infected *Urotrichus talpoides* by mtDNA sequencing. Phylogenetic tree, based on the 1,140-nucleotide cytochrome b (cyt b) gene, was generated by the ML method. The phylogenetic positions of *Urotrichus talpoides* H4 (EU918369), N9 (EU918370) and N10 (EU918371) are shown in relationship to other *Urotrichus talpoides* cyt b sequences from GenBank (Ut76835: AB076835; Ut76834: AB076834; Ut76833: AB076833; Ut76832: AB076832), as well as other talpids, including *Desmana moschata* (AB076836), *Talpa altaica* (AB037602), *Talpa europea* (AB076829), *Mogera imaizumii* (AB037616), *Dymecodon pilirostris* (AB076830) and *Bos frontalis* (EF061237). Also shown are representative murinae rodents, including *Apodemus agrarius* (AB303226), *Apodemus peninsulae* (AY389003), *Apodemus flavicollis* (AB032853) and *Rattus norvegicus* (DQ439844); arvicolinae rodents, including *Microtus arvalis* (EU439459), *Myodes glareolus* (DQ090761) and *Microtus pennsylvanicus* (AF119279); and a neotominae rodent, *Peromyscus maniculatus* (AF119261), as well as crocidurinae shrews, including *Suncus murinus*

(DQ630386), *Crocidura lasiura* (AB077071) and *Crocidura dsinezumi* (AB076837); and soricinae shrews, including *Anourosorex squamipes* (AB175091), *Sorex cinereus* (EU088305), *Sorex monticolus* (AB100273), *Sorex araneus* (DQ417719), *Sorex caecutiens* (AB028563), *Sorex unguiculatus* (AB028525) and *Sorex gracillimus* (AB175131). The numbers at each node are posterior node probabilities based on 30,000 trees: two replicate MCMC runs consisting of six chains of 3 million generations each sampled every 1,000 generations with a burn-in of 7,500 (25%). The scale bar indicates nucleotide substitutions per site.

Fig. 1.

Fig. 2.

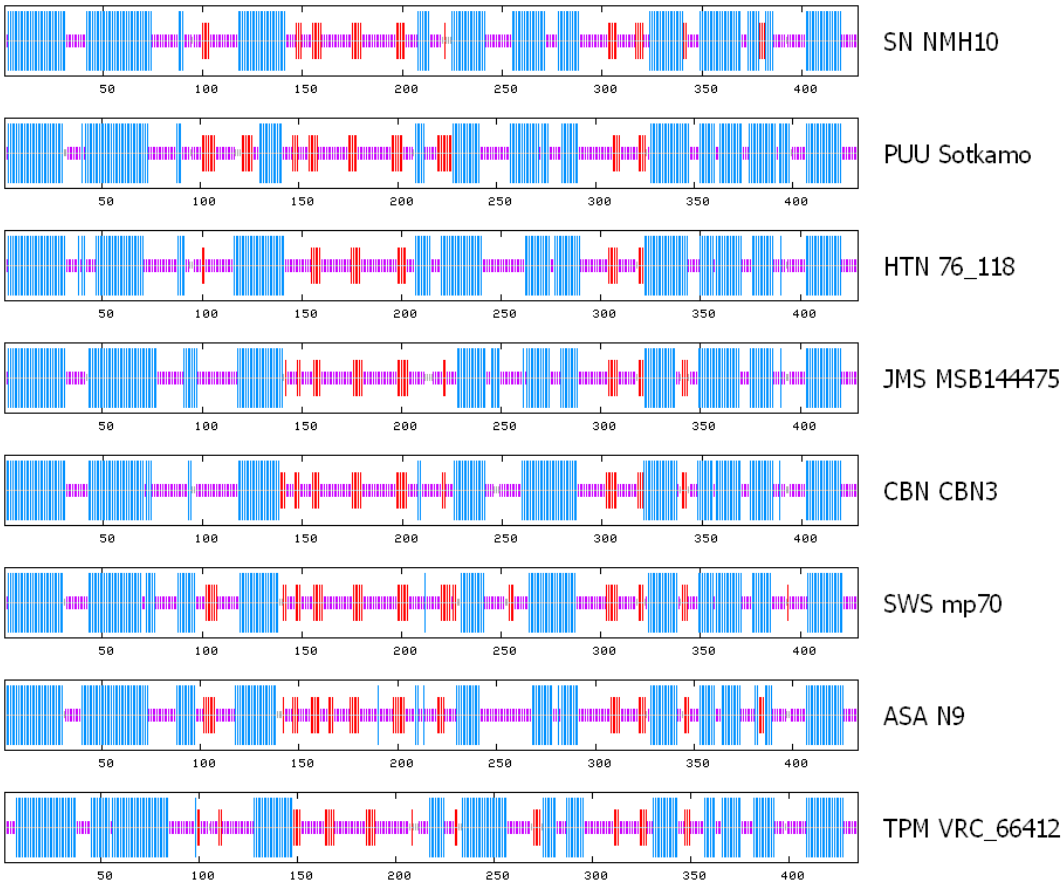
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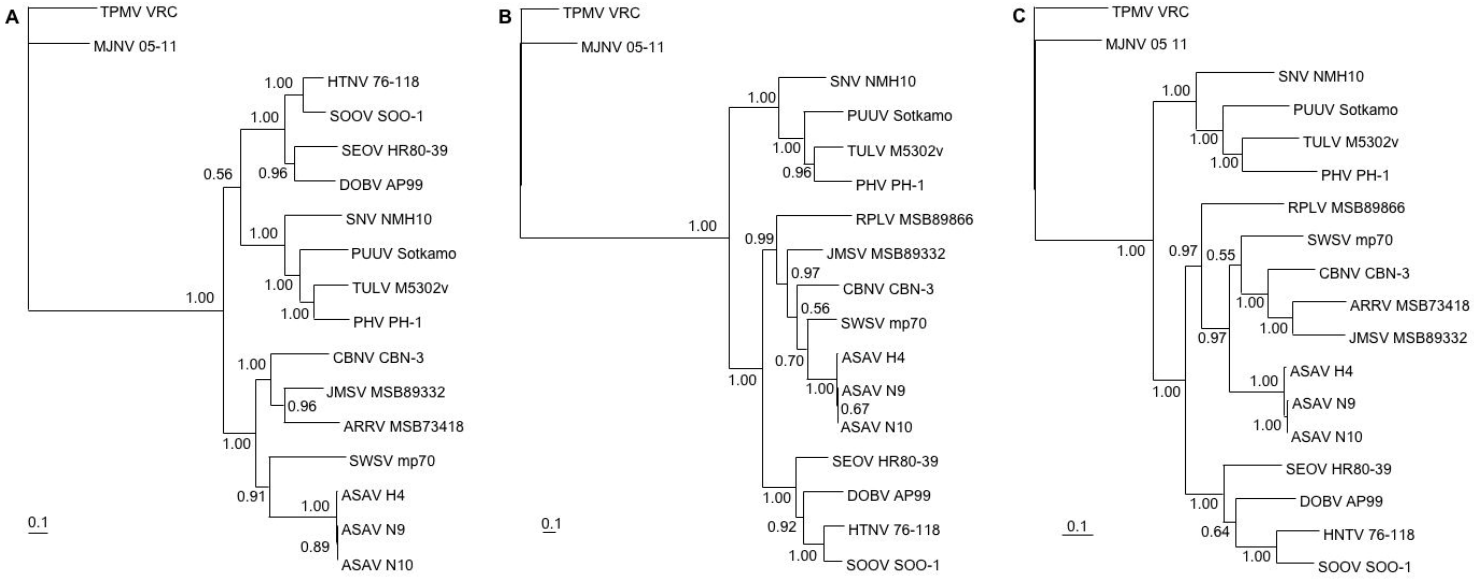
Fig. 3.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SEOV HR80-39
DOBV AP99
SNV NMH10
PUUV Sotkamo
TULV M5302v
PHV PH-1
CBNV CBN-3
JMSV MSB89332
ARRV MSB73418
SWSV mp70
ASAV H4
ASAV N9
ASAV N10
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MJNV 05-11
SNV NMH10
PUUV Sotkamo
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CBNV CBN-3
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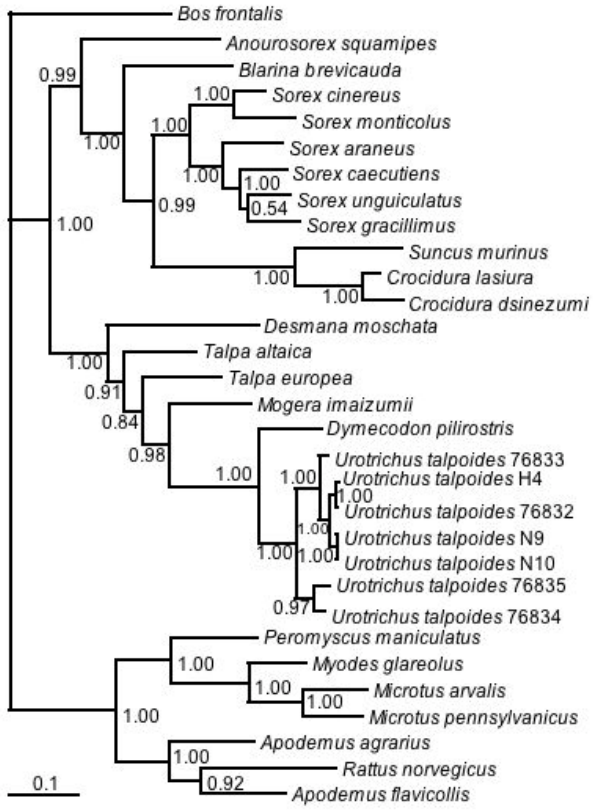
Fig. 4.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Blarina brevicauda
Sorex cinereus
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Sorex araneus
Sorex caecutiens
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Sorex gracillimus
Suncus murinus
Crocidura lasiura
Crocidura dsinezumi
Desmana moschata
Talpa altaica
Talpa europea
Mogera imaizumii
Dymecodon pilirostris
Urotrichus talpoides 76833
Urotrichus talpoides H4
Urotrichus talpoides 76832
Urotrichus talpoides N9
Urotrichus talpoides N10
Urotrichus talpoides 76835
Urotrichus talpoides 76834
Peromyscus maniculatus
Myodes glareolus
Microtus arvalis
Microtus pennsylvanicus
Apodemus agrarius
Rattus norvegicus
Apodemus flavicollis
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