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Rapid Communication

Host switch during evolution of a genetically distinct hantavirus in the American shrew mole (*Neurotrichus gibbsii*)

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ABSTRACT

A genetically distinct hantavirus, designated Oxbow virus (OXBV), was detected in tissues of an American shrew mole (*Neurotrichus gibbsii*), captured in Gresham, Oregon, in September 2003. Pairwise analysis of full-length S- and M- and partial L-segment nucleotide and amino acid sequences of OXBV indicated low sequence similarity with rodent-borne hantaviruses. Phylogenetic analyses using maximum-likelihood and Bayesian methods, and host–parasite evolutionary comparisons, showed that OXBV and Asama virus, a hantavirus recently identified from the Japanese shrew mole (*Urotrichus talpoides*), were related to soricine shrew-borne hantaviruses from North America and Eurasia, respectively, suggesting parallel evolution associated with cross-species transmission.

Introduction

Members of the genus *Hantavirus* (Family Bunyaviridae) are harbored by specific rodent species with which they share long-standing virus–host relationships (Monroe et al., 1999; Morzunov et al., 1998). Recently, the previously well-accepted notion of co-divergence has been opposed in favor of preferential host switching and local host-specific adaptation (Ramsden et al., 2009). Nevertheless, growing evidence suggests an additional congruent pattern of co-evolution of hantaviruses harbored by shrews. Analysis of the full genome of Thottapalayam virus (TPMV), a hantavirus isolated from the Asian house shrew (*Suncus murinus*) (Carey et al. 1971), demonstrates a separate phylogenetic clade consistent with an early evolutionary divergence from rodent-borne hantaviruses (Song et al., 2007a; Yadav et al., 2007). Moreover, long-ignored reports of serologic and antigenic evidence of hantavirus infection in shrews (Gavrilovskaya et al., 1983; Tkachenko et al., 1983) have been validated by the identification of genetically distinct hantaviruses in the Eurasian common shrew (*Sorex araneus*) from Switzerland (Song et al., 2007b), Chinese mole shrew (*Anourosorex squamipes*) from Vietnam (Song et al., 2007c), and northern short-tailed shrew (*Blarina brevicauda*), masked shrew (*Sorex cinereus*) and dusky shrew (*Sorex monticolus*) from the United States (Arai et al., 2007, 2008a). Hantavirus RNAs have also been detected in the Therese's shrew (*Crocidura theresae*) from Guinea (Klempa et al., 2007), Ussuri white-toothed shrew (*Crocidura lasiura*) from Korea (Song et al., in press), vagrant shrew (*Sorex vagrans*), Trowbridge's shrew (*Sorex trowbridgii*) and American water shrew (*Sorex palustris*) from the United States, and flat-skulled shrew (*Sorex roboratus*) and Laxmann's shrew (*Sorex caecutiens*) from Russia (H.J. Kang and R. Yanagihara, unpublished observations).

Although their evolutionary origins remain obscure, the discovery of hantaviruses in shrews (Order Soricomorpha, Family Soricidae) from widely separated geographic regions, spanning four continents, challenges the long-held view that rodents are the principal and primordial reservoir hosts. In addition, these findings raise the possibility that other soricomorphs, particularly moles (Family Talpidae), may harbor hantaviruses. Recent identification of a novel hantavirus, designated Asama virus (ASAV), in the Japanese shrew mole (*Urotrichus talpoides*) (Arai et al., 2008b) prompted us to intensively analyze tissues from the American shrew mole (*Neurotrichus gibbsii*). We now report on a phylogenetically distinct hantavirus, called Oxbow virus (OXBV), which provides additional support for the emerging concept that ancestral soricomorphs, rather than rodents, may have served as the original mammalian hosts of hantaviruses. Moreover, cross-species transmission events

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among soricomorphs on separate continents probably influenced the early evolution of hantaviruses.

Results and discussion

RT-PCR and sequence analysis

Novel hantavirus RNAs were detected by RT-PCR in lung, kidney, heart, salivary gland, adrenal gland, stomach, large intestine and terminal colon of an American shrew mole, captured in Oxbow Regional Park, in Gresham, Oregon, in September 2003 (Fig. 1). Amplified products from the above-listed tissues showed no sequence variation in a 370- and 450-nucleotide region of the OXBV S and L segments, respectively. The widespread tissue distribution of OXBV RNA was not unlike that found in hantavirus-infected rodent reservoir hosts. Also, the detection of OXBV RNA in salivary gland, terminal colon and kidney suggests modes of virus transmission similar to that observed in rodents (Lee et al., 1981; Yanagihara et al., 1985). Future longitudinal investigations, using a capture-recapture strategy, are warranted to clarify the intraspecies transmission dynamics of OXBV (Clay et al., 2009), including the level of hantavirus shedding in secretions and excretions, as well as the frequency, duration and heterogeneity of contacts among American shrew moles.

Earlier attempts to amplify hantavirus RNAs in the same tissues had been repeatedly unsuccessful, using oligonucleotide primers based on rodent-borne hantaviruses. Newly designed primers, based on the most highly conserved regions of recently acquired genomes of shrew-borne hantaviruses, were ultimately effective and allowed amplification of the full-length S- and M-genomic segments and partial L segment of OXBV.

Pairwise alignment and comparison of the OXBV genome with representative rodent-borne hantaviruses showed low nucleotide sequence similarity in the S segment, ranging from 59.7% to 62.6% (Table 1). OXBV sequences were even more divergent from TPMV and Imjin virus (MJNV), two crocidurine shrew-borne hantaviruses: approximately 50% in the S and M segments and 60% in the L segment. In contrast, much higher nucleotide sequence similarity of approximately 70% to 73% was found in the S, M and L segments of OXBV and Jemez Springs virus (JMSV) harbored by the dusky shrew (Arai et al., 2008a), whereas ASAV was more closely related to Seewis virus (SWSV), a hantavirus recently identified in the Eurasian common shrew (Song et al., 2007b). OXBV and ASAV genomic sequences were distinct, differing by approximately 30% at the nucleotide level (Table 1).

Table 1
Sequence similarities (%) of the coding regions of the full-length S and M and partial L segments of OXBV strain Ng1453 and representative hantaviruses harbored by rodents and soricomorphs.

Virus strain	S segment		M segment		L segment	
	1287 nt	428 aa	3426 nt	1141 aa	4358 nt	1452 aa
HTNV 76-118	62.1	64.6	62.2	60.3	68.1	74.2
SEOV 80-39	62.6	61.6	60.9	57.8	68.2	72.8
SOOV SOO-1	62.4	64.9	61.7	59.0	68.5	73.4
DOBV Greece	62.2	62.1	60.6	58.6	67.6	73.2
PUUV Sotkamo	61.9	59.7	58.6	52.2	66.3	68.2
TULV 5302v	61.3	59.5	57.3	52.4	66.1	67.8
PHV PH-1	59.8	57.8	56.7	52.4	64.9	67.9
SNV NMH10	59.7	58.0	57.1	53.9	65.1	67.8
ANDV Chile9717869	61.3	60.8	56.7	52.4	65.9	67.3
ARRV MSB73418	68.1	75.4	–	–	71.5	79.4
CBNV CBN-3	68.5	74.5	66.6	67.2	72.1	80.4
JMSV MSB144475	75.0	84.1	69.7	71.5	72.5	81.5
SWSV mp70	63.6	67.8	72.6	78.3	70.8	77.0
RPLV MSB89863	–	–	67.4	64.2	70.8	75.8
MJNV Cl05-11	53.2	46.8	51.0	41.6	61.5	60.0
TPMV VRC66412	53.2	47.5	52.0	42.0	61.1	60.0
ASAV N10	67.1	69.9	66.4	65.9	72.4	79.6

Abbreviations: ANDV, Andes virus; ARRV, Ash River virus; ASAV, Asama virus; CBNV, Cao Bang virus; DOBV, Dobrava virus; HTNV, Hantaan virus; JMSV, Jemez Spring virus; MJNV, Imjin virus; OXBV, Oxbow 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.

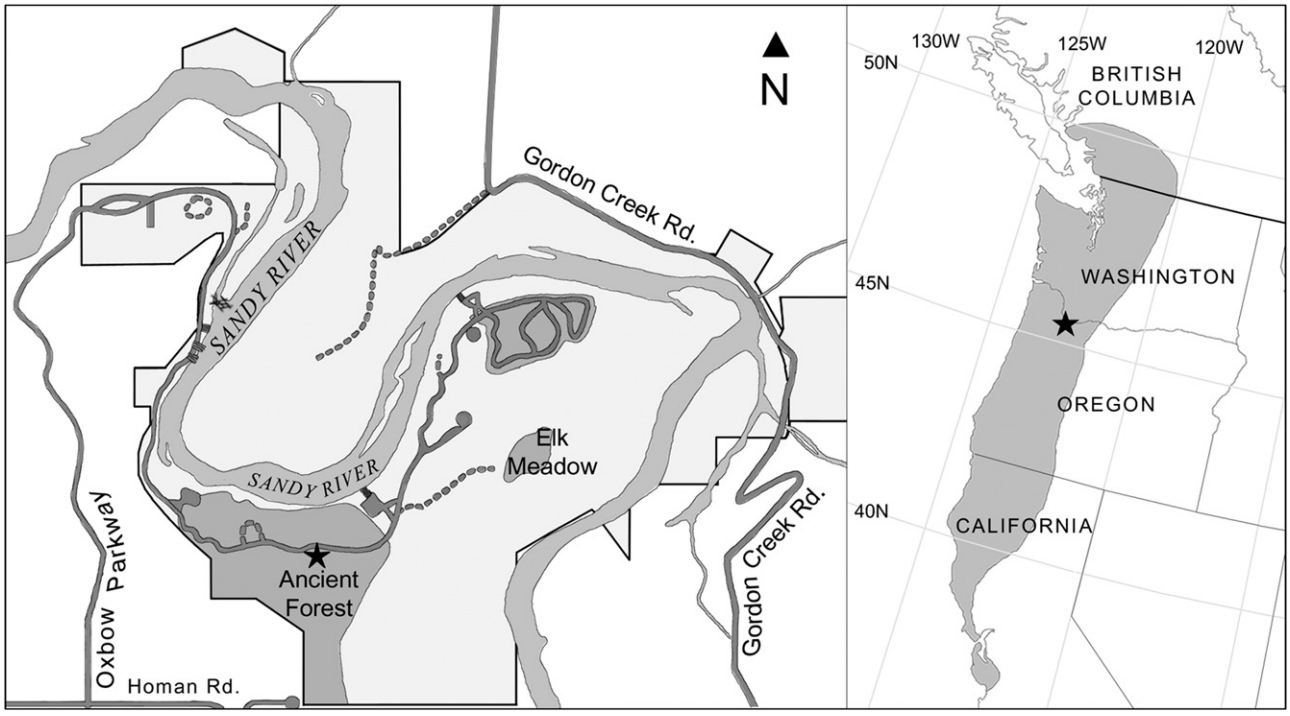


Fig. 1. Map of Oxbow Regional Park, in Gresham, Oregon, where an American shrew mole (*Neurotrichus gibbsii*) infected with a genetically distinct hantavirus was captured (star) in September 2003. Inset shows the geographic distribution of the American shrew mole (shaded area), which is restricted to the western regions of North America from Santa Cruz County, California, and northerly through western Oregon and Washington, and in southwestern British Columbia.

The full-length 1705-nucleotide S-genomic segment of OXBV contained a single open reading frame (ORF), encoding a predicted nucleocapsid (N) protein of 428 amino acids (nucleotide positions 39 to 1325), and a 380-nucleotide 3′-noncoding region (NCR), which exhibited significant variability from previously identified hantaviruses. The hypothetical NSs ORF was absent, as in murine rodent- and soricine and crocidurine shrew-borne hantaviruses. As determined by various prediction software available in the NPS@ structure server (Combet et al., 2000), the overall secondary structure of the OXBV N protein resembled that of other hantaviruses. Specifically, the predicted secondary structure of the OXBV N protein was composed of 53.5% α-helices and 6.5% β-sheets, with the characteristic coiled-coil domain in the N-terminal region (residues 1 to 31 and 48–68) (Fig. 2A). β-pleated sheets were present at the presumed RNA-binding domain region (residues 175–217) (Xu et al., 2002).

The full-length 3645-nucleotide M-genomic segment of OXBV encoded a predicted glycoprotein of 1141 amino acids (nucleotide positions 42 to 3467). In scanning the OXBV glycoprotein, the highly conserved WAASA amino acid motif cleavage site was found at positions 650 to 654. Also, the OXBV glycoprotein precursor had seven potential N-linked glycosylation sites (six in Gn at amino acid positions 138, 353, 405, 524, 617 and 623; and one in Gc at position 934), of which five are found among all hantavirus glycoproteins (Fig. 2B). Both the Gn and Gc of OXBV showed predicted transmembrane helices (Fig. 2C) serving as hydrophobic anchor domains at the C-terminal region, as determined by TMHMM, a program for predicting transmembrane helices based on a hidden Markov model (Krogh et al., 2001; Möller et al., 2001).

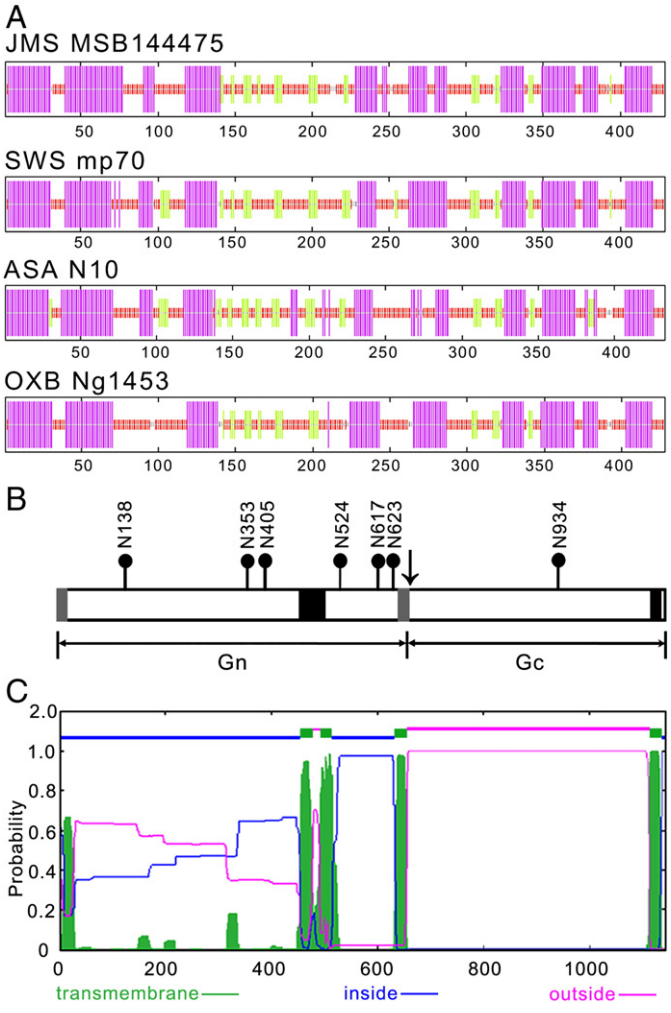


Fig. 2. Schematic diagram of (A) consensus secondary structure of the N protein and (B) potential N-linked glycosylation and (C) the four predicted transmembrane helices of the glycoprotein of OXBV. N protein structures of OXBV and representative hantaviruses were predicted using several methods, including HNN, DSC, PHD, PREDATOR and MLRC, available at the NPS@ structure server (Combet et al., 2000). Alpha helices are represented by purple bars, beta-strand by yellow-green bars, and random coil and unclassified structure by red and gray bars, respectively. For the GPC, the predicted transmembrane domains are indicated by the black box and signal peptide is shown by the gray box. The arrow indicates the WAASA amino acid motif. The predicted sites of N-linked glycosylation are represented by sticks with circle tag.

The partial 4396-nucleotide L-genomic segment of OXBV encoded an incomplete RNA-dependent RNA polymerase of 1452 amino acids (nucleotide positions 39 to 4396). In the amino acid sequences of the RNA-dependent RNA polymerases of all rodent-borne hantaviruses, major conserved regions have been reported for the polymerase function, which are designated as motifs A, B, C, D, E and premotif A or F. Motifs A, B and D have conserved aspartate, glycine and lysine, respectively. In motif C are two conserved aspartic acid residues. The XDD motif is essential for catalytic activity and motif E contains the E (F/Y)XS site. Premotif A has a conserved lysine and two arginine residues (Kukkonen et al., 2005). The five major motifs for viral RNA polymerases were also present in OXBV.

The coding regions of the full-length S- and M- and partial L-segment sequences of OXBV were analyzed extensively for recombination using multiple recombination-detection methods, including GENECONV, Bootscan, Chimaera, 3SEQ, RDP, SiScan, MaxChi and HyPhy Single Recombinant Breakpoint (Pond et al., 2005). The vast majority of these programs failed to disclose any evidence of recombination. Although separate regions of potential recombination were found in a few instances, there was no consistency or concordance between the detection methods, calling into question the validity of the identified sequences or the biological significance of recombination versus general heterogeneity in sequence evolutionary rates.

Phylogenetic analysis

Phylogenetic analysis of the OXBV genome, using the maximum-likelihood (ML) and Markov Chain Monte Carlo tree-sampling methods, with the GTR + I + Γ model of evolution, indicated high bootstrap support for close phylogenetic relationships between OXBV and ASAV with hantaviruses harbored by soricine shrews in the New and Old World, respectively (Fig. 3), consistent with cross-species transmission, occurring independently in different geographic regions during the distant past and with subsequent host adaptation (Arai et al., 2008b). However, while host switching has been operative, such events alone do not adequately account for the co-existence and widespread geographic distribution of genetically distinct hantaviruses among host species in two divergent taxonomic Orders of small mammals. Importantly, the well-supported phylogenies of hantaviruses and their rodent and soricid reservoir hosts spanning across four continents suggest that hantaviruses have likely co-diverged with some of their hosts during part of their long evolutionary history (Fig. 3).

Despite their physical resemblance, American and Japanese shrew moles are evolutionarily distinct, diverging prior to the diversification of the Talpini and represent separate lineages (Sanchez-Villagra et al., 2006; Shinohara et al., 2004). Based on analysis of mtDNA and nuclear gene sequences, the split between Old World and New World *Sorex* has been estimated at 13.9 MYA (95% CI: 10.2–17.5 MYA) (Dubey et al., 2007). As supported by the fossil record, three independent lineages of Soricinae shrews colonized North America during the middle Miocene. Accordingly, OXBV would have emerged during this period, long after ASAV. That is, since ASAV is in a clade that includes hantaviruses identified in both *Sorex* (M-segment) and *Anourosorex* (S-segment) (Fig. 3), one must hypothesize that it is older (95% CI:

14.0–20.5 MYA), as it would predate the Old World-New World dichotomy in *Sorex*.

Particularly during irruptions in rodent reservoir populations, hantavirus spillover into sympatric and syntopic hosts are more likely to occur (Gavrilovskaya et al., 1983; Mills et al., 1998). Such repeated or recurring cross-species transmission events could then result in long-term virus-host adaptation. A frequently cited example of host switching during hantavirus evolution is Topografov virus, which is genetically very similar to Khabarovsk virus but is found in the Siberian lemming (*Lemmus sibirica*), instead of the reed vole (*Microtus fortis*) (Vapalahti et al., 1999) or Maximowicz's vole (*Microtus maximowiczii*). However, while belonging to different genera, these rodent species are members of the same Family (Cricetidae) and Subfamily (Arvicolinae). Similarly, all other known examples of so-called host switching of hantaviruses have occurred across rodent species within the same Family or Subfamily. By contrast, in the case of OXBV, reported here, and ASAV reported previously (Arai et al., 2008b), their phylogenetic positions are consistent with cross-species virus transmission involving mammalian hosts of two separate and distinct families (Talpidae and Soricidae) within the Order Soricomorpha. These findings would seem to strengthen the conjecture that soricomorphs may have been the primordial mammalian hosts of ancestral hantaviruses. In this regard, to what extent greater flexibility or non-specificity in host selection was a primitive characteristic that was later reduced or lost in hantaviral evolution is unknown.

Co-phylogeny mapping

Phylogenetic trees were reconstructed for co-phylogeny mapping from a virus tree into a host tree in TreeMap 2.0β (Charleston and Page, 1998) in order to ascertain the co-evolutionary history of hantaviruses and their hosts. Similar topologies with high bootstrap support for each genomic segment (at both the nucleotide and amino acid levels) were found for the segregation of hantaviruses, according to the Subfamily of their rodent and shrew reservoir hosts (Fig. 3). Specifically, hantaviruses and their rodent and soricid hosts showed congruent topologies in their phylogenetic relationships—with the exception of OXBV and ASAV. Overall, these data support the contention that hantaviruses have generally co-diverged with and adapted to their reservoir hosts over many millions of years (Monroe et al., 1999; Morzunov et al., 1998). That is, for part of their evolutionary history, hantaviruses have co-diverged with their hosts, namely in the divergence of shrews and their viruses from a still-unknown shared common ancestor, and to some extent during the subsequent diversification of shrews. For OXBV and ASAV, cross-species transmission of hantaviruses from hosts of one Family to another (Soricidae to Talpidae), with subsequent adaptation, appears to have occurred at separate times on different continents. Again, this observation suggests that the primordial or ancestral hantaviruses may have been comparatively more broad in their host selection than the more well-known host specificities of present-day rodent-borne hantaviruses. Intensive attempts to identify hantaviruses in other talpid species and sympatric soricid species, now underway, will help to clarify the evolutionary history of hantaviruses.

The largest incongruity between hantavirus and host co-divergence remains the divergent position of two hantaviruses identified in crocidurine shrews, namely TPMV in the Asian house shrew (*S. murinus*) (Carey et al., 1971) and MJNV in the Ussuri white-toothed shrew (*C. lasiura*) (Song et al., in press). These viruses remain deeply divergent with respect to other hantaviruses, including the more recently identified hantaviruses reported here and elsewhere. Better resolution of the phylogeny of the soricid hosts is necessary, using multiple genetic loci. However, in unrooted trees based on each hantavirus genomic segment, the branch lengths leading to TPMV and MJNV were far longer than the other branches (data not shown), indicating greater evolutionary change and higher likelihood of older age. Thus, the deep divergences among crocidurine shrew-borne hantaviruses also suggest that soricomorphs were the original mammalian hosts. In this scenario, rodents would have acquired hantaviruses more recently from shrews (and moles).

Natural history of shrew moles

Confirmation of the identity of the American shrew mole, in which OXBV was detected, was achieved by phylogenetic analysis of the 1140-nucleotide mtDNA cytochrome *b* gene. Phylogenetic trees showed that rodents, shrews and moles segregated into three separate clades along two distinct lineages, one consisting of soricids and talpids, and the other of rodents (Fig. 3). Shrew moles occupy similar ecological niches as shrews (Campbell and Hochachka, 2000) and share physical features of both soricids (small body size, posteriorly directed pelage, paired ampullary glands and long and pointed noses) and talpids (heavy insectivorous dentition, large head and enlarged forefeet). Importantly, the geographical ranges of the American and Japanese shrew moles do not overlap. The Japanese shrew mole is limited strictly to Japan, while the distribution of the American shrew mole (Subfamily Talpinae, Tribe Neurotrichini), the only member of the genus *Neurotrichus* and the smallest and most primitive mole in North America, is restricted to an area approximately the size of Japan in the western regions of North America (Fig. 1), extending from southwestern British Columbia in Canada to central California in the United States, where sympatric and syntopic shrew and rodent hosts of hantaviruses (such as the vagrant shrew and Trowbridge's shrew and the deer mouse) are also found.

Shrew moles are active at all hours throughout the day and night, with intermittent periods of rest and/or sleep (Dalquest and Orcutt, 1942). They construct networks of runways or underground burrows, generally at two levels, one being shallow and the other deeper. Being almost completely blind, they rely on their prehensile nose to locate prey, which consists largely of worms and insects. Unlike most shrew species that are solitary and reclusive, shrew moles tend to be gregarious, often traveling in groups of more than 10 individuals. How these and other life history characteristics of the American shrew mole contribute to the transmission dynamics of OXBV remains open to future investigation.

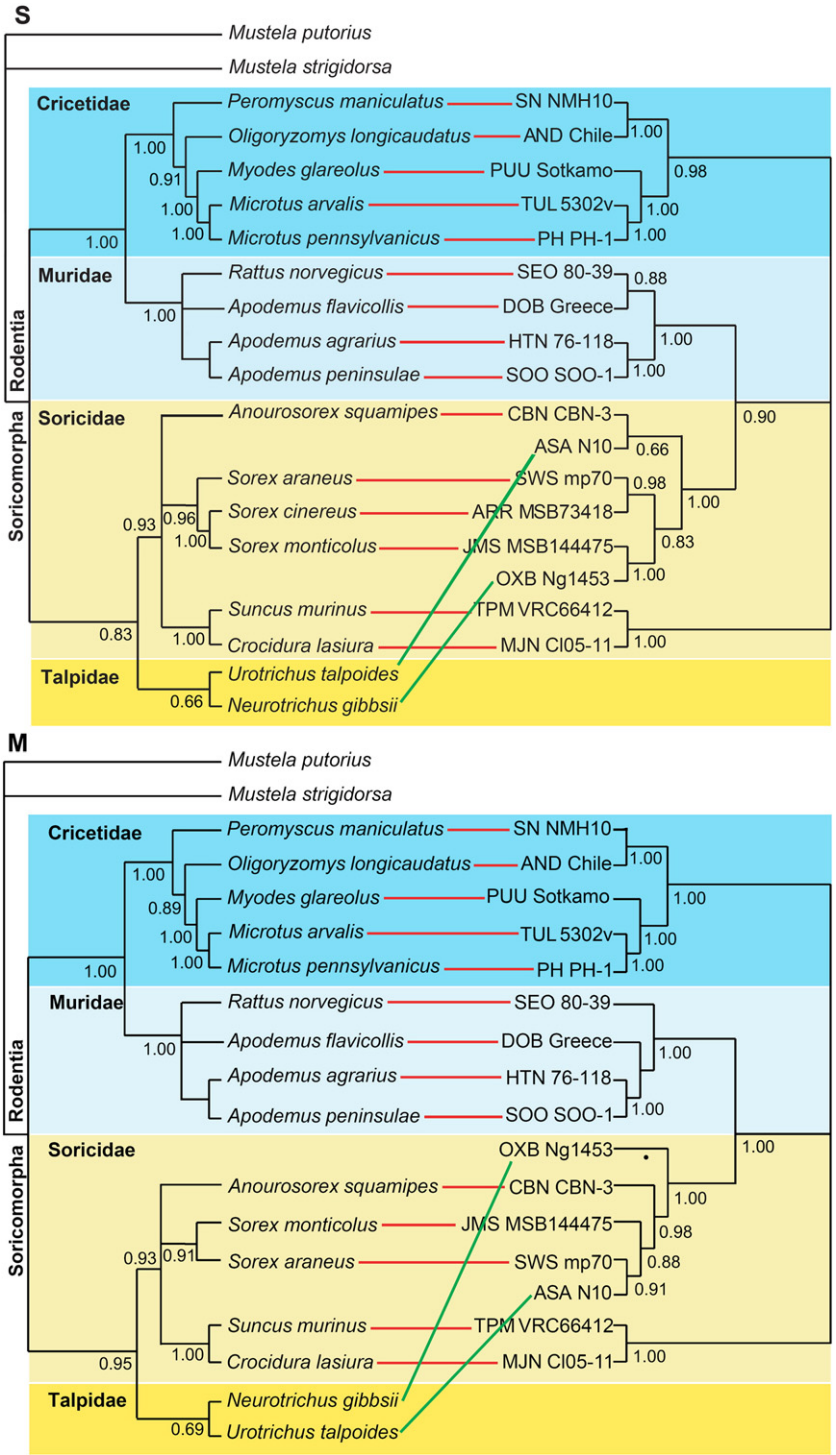
Materials and methods

Tissues, RNA extraction and cDNA synthesis

In our opportunistic search for non-rodent-borne hantaviruses, we were fortunate in gaining access to frozen tissues from shrew moles trapped coincidentally, as part of an epizootiological study of Sin Nombre virus infection in deer mice (*Peromyscus maniculatus*) in Oregon (Dizney and Ruedas, in press). Since sera from shrew moles were not collected and because appropriate immunological reagents for detecting talpid-borne hantaviruses are unavailable, we relied on RT-PCR. Total RNA was extracted, using the PureLink Micro-to-Midi total RNA purification kit (Invitrogen, San Diego, CA, USA), from tissues obtained from 10 American shrew mole (*N. gibbsii*), captured between July and October 2003 in Oregon: five from Oxbow Regional Park (45.4879°N, 122.2970°W), a 1200-acre natural area park located within the Sandy River Gorge in Gresham (Fig. 1); one from Tryon Creek State Park (45.4337°N, 122.6690°W) in Multnomah County; and four from Tualatin River National Wildlife Refuge (45.3957°N, 122.8305°W) in Washington County. cDNA was prepared at 65 °C for 3 min, 42 °C for 20 min, 50 °C for 50 min and 70 °C for 3 min, using the SuperScript III First-Strand Synthesis System (Invitrogen) and primer (OSM55: 5′-TAGTAGTAGACTCC-3′) designed from the conserved 5′-end of the S, M and L segments of hantaviruses.

RT-PCR and DNA sequencing

PCR was performed as described previously, with each 20-μL reaction containing 250 μM dNTP, 2 mM $MgCl_2$, 1 U of AmpliTaq polymerase (Roche, Basel, Switzerland) and 0.25 μM of oligonucleotide primers, which were designed from highly conserved regions of the hantavirus genome. Considerable trial-and-error testing was required to devise suitable primers and refine cycling conditions. Eventually, the following nested PCR primers sets provided the initial sequences of the newfound hantavirus: S segment (OSM55, 5′-TAGTAGTAGACTCC-3′ and HTN-S6, 5′-AGCTCNGGATCCATNTCATC-3′; and Han-S604F, 5′-GCHGADGARHTN ACACCNGG-3′ and Han-S974R, 5′-TCNGGNGCHCHNGCAAANAHCCA-3′); M segment (OSM55, 5′-TAGTAGTAGACTCC-3′ and TM-2957R, 5′-GAACCCCADGCCC CNTCYAT-3′ and HTN-M1190F, 5′-GGNCCNGGDGCWNVHTGTGA-3′; and HTN-M2020R, 5′-CCATGDGCAKTRTCANTCCA-3′); and L segment (Han-L1880F, 5′-CARAAR ATGAARNTNTGTGC-3′and Han-L3470R, 5′-TTRAACATNSNYTTCCACATHTC-3′; and Han-L2520F, 5′-ATNWGHYT-DAARGGNATGTCNGG-3′and Han-L2970R, 5′-CCNGGNGA CCAYTTNGTDGCATC-3′). Initial denaturation at 94 °C for 5 min was followed by 2 cycles each of denaturation at 94 °C for 40 s, two-degree step-down annealing from 48 °C to 38 °C for 40 s, and elongation at 72 °C for 1 min, then 32 cycles of denaturation at 94 °C for 40 s, annealing at 42 °C for 40 s, and elongation at 72 °C for 1 min, in a



GeneAmp PCR 9700 thermal cycler (Perkin-Elmer, Waltham, MA). Amplicons were separated by electrophoresis on 1.5% agarose gels and purified using the QIAQuick Gel Extraction Kit (Qiagen, Hilden, Germany). DNA was sequenced directly using an ABI Prism 377XL Genetic Analyzer (Applied Biosystems, Foster City, CA).

Protein analysis and secondary structure prediction

To predict secondary structures of nucleocapsid protein and glycoprotein, the whole amino acid sequences were submitted to NPS@ structure server (Combet et al., 2000). Glycosylation and transmembrane sites were predicted at the NetNlyc 1.0 and Predictprotein (Gavel and von Heijne, 1990) and TMHMM version 2.0 (Krogh et al., 2001) respectively. The program COILS (Lupas et al., 1991) was used to scan of N protein for expect of coiled-coil region.

Phylogenetic analysis

Newly acquired, full-length S- and M- and partial L-segment sequences of OXBV, amplified from an American shrew mole, were aligned and compared with publically available hantavirus sequences, using the ClustalW method, implemented in Lasergene program version 5 (DNASTAR, Inc., Madison, WI) (Thompson et al., 1994) and transAlign (for coding sequences) (Bininda-Emonds, 2005). Phylogenetic trees were estimated using the ML method implemented in PAUP* (Phylogenetic Analysis Using Parsimony, 4.0b10), RAxML Blackbox web-server (Stamatakis et al., 2008) and MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). The optimal evolutionary model was estimated under the GTR + I + Γ model of evolution, as selected by using ModelTest v.3.7 (Posada and Crandall, 1998). Parameters were re-estimated during successive rounds of ML heuristic searches using the TBR and SPR algorithms implemented in PAUP*. Respective individual parameter estimates for S-, M- and L-segment sequence alignments were as follows for base frequencies: A (0.3406, 0.3446, 0.3729); C (0.1940, 0.1680, 0.1388); G (0.2269, 0.1877, 0.1861); T (0.2385, 0.2997, 0.3023); substitution rate matrices: A–C (3.3442, 4.0319, 9.0177); A–G (5.1156, 5.8287, 10.6997); A–T (2.4196, 1.8545, 1.9089); C–G (1.5578, 2.9970, 5.4793); C–T (7.7701, 9.6963, 29.0711); G–T (1.0000, 1.0000, 1.0000); proportions of invariable sites (0.2050, 0.1440, 0.2210); among-site rate heterogeneity gamma distribution shape parameters (0.9840, 0.7660, 0.5560).

Topologies were evaluated by bootstrap analysis of 1000 iterations, using neighbor-joining trees in PAUP*, posterior node probabilities based on 2 million generations and estimated sample sizes well over 100 (implemented in MrBayes) and 100 ML bootstrap replicates implemented in RAxML. With a robust phylogeny of shrew- and rodent-borne hantaviruses (as defined by bootstrap support of >70% or 0.70), we readdressed the co-evolutionary relationship between hantaviruses and their hosts that forms the basis of our predictive paradigm for hantavirus discovery. We employed host-parasite phylogenetic comparisons to detect co-divergence or host switch in TreeMap 2.0β (Charleston and Page, 1998).

mtDNA sequence analysis

To verify the identity of the hantavirus-infected mole and to study its phylogenetic relationship to other reservoir hosts, genomic DNA was extracted from frozen kidney tissue using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The entire 1140-nucleotide region of the cytochrome *b* gene of mtDNA was amplified by PCR, using well-tested primers (forward: 5′-CGAAGCTTGATA-TGAAAAACCATCGTTG-3′; and reverse: 5′-CTGGTTTACAAGACC-AGAGTAAT-3′). PCR was performed in 50-μL reaction mixtures, containing 200 μM dNTP and 1 U of AmpliTaq polymerase (Roche, Basel, Switzerland). Cycling conditions consisted of 8 cycles at 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min, followed by 30 cycles at 94 °C for 40 s, 50 °C for 40 s and 72 °C for 1 min, and 1 cycle at 72 °C for 7 min. Amplified DNA was purified and then submitted for automated fluorescent sequencing. Host phylogenies based on mtDNA cytochrome *b* sequences were generated, using ML and Bayesian methods.

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Fig. 3. Phylogenetic comparisons, showing concordance (red line) and discordance (green line) for each virus–host relationship. Virus phylogenetic trees were generated by maximum-likelihood (ML) and Bayesian methods, using the GTR + I + Γ model of evolution, based on the coding regions of the full-length (S) 1287-nucleotide S- and (M) 3426-nucleotide M-genomic segments of OXBV. Similar topologies were also found for the partial 4358-nucleotide L segment and for the deduced amino acid sequences of each genomic segment (data not shown). The average standard deviations of split frequencies between 8 MCMC chains based on 2 million generations sampled at a frequency of 100 in MrBayes were 0.007536 (S tree), 0.001170 (M tree) and 0.000887 (L tree). In these unrooted ML trees, the phylogenetic positions of OXBV are shown in relation to representative murine rodent-borne hantaviruses, including Hantaan virus (HTNV 76–118, NC_005218, Y00386), Soochong virus (SOOV SOO-1, AY675349, AY675353), Dobrava virus (DOBV Greece, NC_005233, NC_005234) and Seoul virus (SEOV HR80-39, NC_005236, NC_005237); arvicoline rodent-borne hantaviruses, including Tula virus (TULV M5302v, NC_005227, NC_005228), Puumala virus (PUUV Sotkamo, NC_005224, NC_005223) and Prospect Hill virus (PHV PH-1, Z49098, × 55129); and a sigmodontine and neotomine rodent-borne hantavirus, Andes virus (ANDV Chile-9717869, NC_003466, NC_003467) and Sin Nombre virus (SNV NMH10, NC_005216, NC_005215). Also shown are Thottapalayam virus (TPMV VRC66412, AY526097, EU001329) from the Asian house shrew (*Suncus murinus*); Imjin virus (MJNV Cl05–11, EF641804, EF641798) 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 MSB73418, EF650086) from the masked shrew (*Sorex cinereus*); Jemez Springs virus (JMSV MSB144475, FJ593499, FJ593500) from the dusky shrew (*Sorex monticolus*); Seewis virus (SWSV mp70, EF636024, EF636025) from the Eurasian common shrew (*Sorex araneus*); and Asama virus (ASAV N10, EU929072, EU929075) from the Japanese shrew mole (*Urotrichus talpoides*). GenBank accession numbers for OXBV S, M and L segments are FJ539166, FJ539167 and FJ593497, respectively. Host phylogenetic tree were based on the full-length 1140-nucleotide cytochrome *b* gene of mitochondrial DNA of representative members of the Orders Rodentia and Soricomorpha. The phylogenetic position of *Neurotrichus gibbsii* (FJ595237) is shown in relationship to another talpid mole, *Urotrichus talpoides* (EU918371), as well as other murine rodents, including *Apodemus agrarius* (AB303225), *Apodemus peninsulae* (AB073811), *Apodemus flavicollis* (AJ605652) and *Rattus norvegicus* (AB355903); arvicoline rodents, including *Microtus arvalis* (EU439459), *Myodes glareolus* (AF119272) and *Microtus pennsylvanicus* (AF119279); neotomine and sigmodontine rodents, *Peromyscus maniculatus* (AF119261) and *Oligoryzomys longicaudatus* (AF346566), respectively; soricine shrews, including *Anourosorex squamipes* (AB175090), *Sorex cinereus* (FJ667512), *Sorex monticolus* (FJ667514) and *Sorex araneus* (FJ667524); and crocidurine shrews, including *Suncus murinus* (AB175075) and *Crocidura lasiura* (AB077071). For the outgroup, *Mustela putorius* (EF987746) and *Mustela strigidorsa* (EF987748) were used. Color was used to show grouping of hosts into their respective taxonomic Family. The numbers at each node are posterior node probabilities based on 30,000 trees: two replicate Markov Chain Monte Carlo runs consisting of four chains of 2 million generations each sampled every 100 generations with a burn-in of 5000 (25%). Bootstrap values, expressed as a percentage of 100 replicates in ML analysis, implemented in RAxML Blackbox web-server (Stamatakis et al., 2008), were similar.

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