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Divergent ancestral lineages of newfound hantaviruses harbored by phylogenetically related crocidurine shrew species in Korea

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ABSTRACT

Spurred by the recent isolation of a novel hantavirus, named Imjin virus (MJNV), from the Ussuri white-toothed shrew (*Crocidura lasiura*), targeted trapping was conducted for the phylogenetically related Asian lesser white-toothed shrew (*Crocidura shantungensis*). Pair-wise alignment and comparison of the S, M and L segments of a newfound hantavirus, designated Jeju virus (JJUV), indicated remarkably low nucleotide and amino acid sequence similarity with MJNV. Phylogenetic analyses, using maximum likelihood and Bayesian methods, showed divergent ancestral lineages for JJUV and MJNV, despite the close phylogenetic relationship of their reservoir soricid hosts. Also, no evidence of host switching was apparent in tanglegrams, generated by TreeMap 2.0β.

Introduction

For more than four decades, the only known non-rodent-associated hantavirus was Thottapalayam virus (TPMV), isolated from an Asian house shrew (*Suncus murinus*), captured in 1964 near Vellore in India (Carey et al., 1971). Recently, the discovery of genetically distinct hantaviruses in crocidurine and soricine shrews (Order Soricomorpha, Family Soricidae), including Tanganya virus (TGNV) in the Therese's shrew (*Crocidura theresae*) (Klempa et al., 2007), Azagny virus (AZGV) in the West African pygmy shrew (*Crocidura obscurior*) (Kang et al., 2011b), Camp Ripley virus (RPLV) in the northern short-tailed shrew (*Blarina brevicauda*) (Arai et al., 2007), Cao Bang virus (CBNV) in the Chinese mole shrew (*Anourosorex squamipes*) (Song et al., 2007b), Seewis virus (SWSV) in the Eurasian common shrew (*Sorex araneus*) (Song et al., 2007a), Ash River virus (ARRV) in the masked shrew (*Sorex cinereus*) and Jemez Spring virus (JMSV) in the dusky shrew (*Sorex monticolus*) (Arai et al., 2008a), and Kenkeme virus (KKMV) in the flat-skulled shrew (*Sorex roboratus*) (Kang et al., 2010), has challenged the conventional view that rodents are the principal reservoir hosts. That genetically divergent hantaviruses are also harbored by moles (Family Talpidae), including Asama virus (ASAV) in the Japanese shrew mole (*Urotrichus talpoides*) (Arai et al., 2008b), Oxbow virus (OXBV) in the America shrew mole (*Neurotrichus gibbsii*) (Kang et al., 2009b), Nova virus (NVAV) in the European common mole (*Talpa europaea*) (Kang et al., 2009c) and Rockport virus (RKPV) in the eastern mole (*Scalopus aquaticus*) (Kang et al., 2011a), has spurred the search for other soricomorph-borne hantaviruses, in hopes of clarifying their evolutionary history.

Beginning with the seminal identification of Hantaan virus (HTNV) (Lee et al., 1978), as the prototype virus of hemorrhagic fever with renal syndrome in the striped field mouse (*Apodemus agrarius*), the Republic of Korea has been the epicenter of hantavirus discovery. In fact, Seoul virus (SEOV) in the brown rat (*Rattus norvegicus*) (Lee et al., 1982), Soochong virus (SOOV) in the Korean field mouse (*Apodemus peninsulae*) (Baek et al., 2006), and Muju virus (MUJV) in the royal vole (*Myodes regulus*) (K.J. Song et al., 2007) were first reported in Korea.

Recently, the isolation of Imjin virus (MJNV) from the Ussuri white-toothed shrew (*Crocidura lasiura*) (Song et al., 2009), captured

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near the Imjin River in the Republic of Korea, prompted us to search for a hantavirus in the Asian lesser white-toothed shrew (*Crocidura shantungensis*). Because of the close phylogenetic relationship between *C. lasiura* and *C. shantungensis* and the separate lineages of Asian and African Crocidura shrews, we conjectured that a hantavirus harbored by *C. shantungensis* would be closely related to MJNV. Furthermore, we posited that the more distant phylogenetic relationship between *C. shantungensis* in Asia and *C. theresae* and *C. obscurior* in Africa (Dubey et al., 2008) would be similarly reflected by the divergence in their hantaviruses. Genetic and phylogenetic analyses, however, indicated that a newfound hantavirus, designated Jeju virus (JJUV), in *C. shantungensis* captured on Jeju Island, off the southern coast of Korea, differed significantly from MJNV, and shared a common ancestry with TGNV and AZGV, hantaviruses hosted by crocidurine shrews in sub-Saharan Africa. That is, despite the close phylogenetic relationship between their reservoir soricid hosts, JJUV and MJNV exhibited divergent ancestral lineages. Intensified efforts to detect JJUV in *C. shantungensis* on mainland Korea and phylogeographic studies of JJUV throughout the vast geographic range of the Asian lesser white-toothed shrew should provide greater insights into the complex evolutionary history of crocidurine shrew-borne hantaviruses.

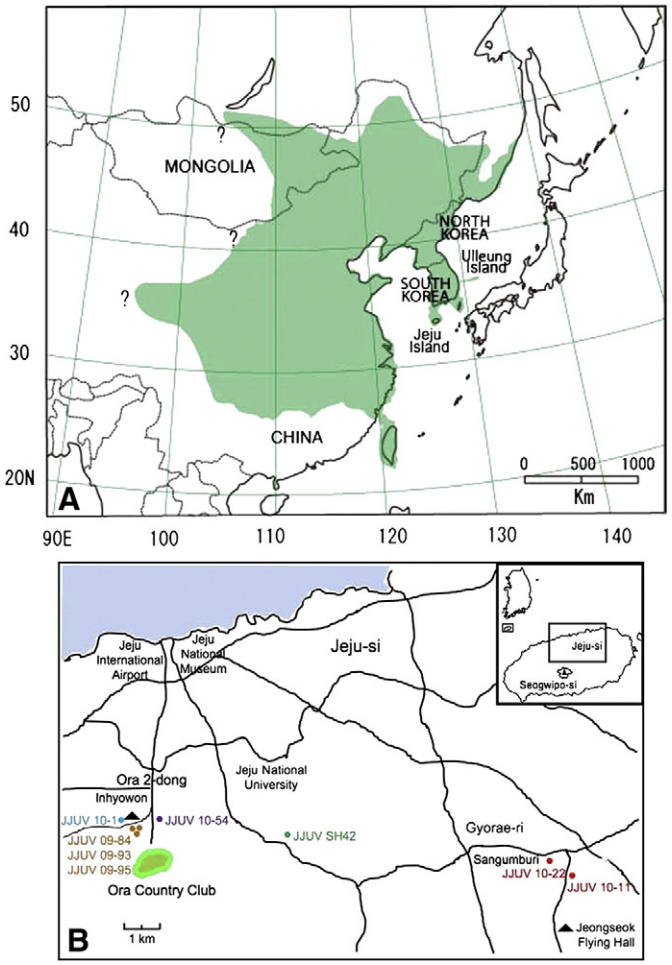

Fig. 1. (A) Geographic distribution, shaded in green (Bannikova et al., 2009; Ohdachi et al., 2004), of the Asian lesser white-toothed shrew, showing the locations of Jeju and Ulleung Islands. (B) Map of the Republic of Korea, showing locations of trap sites on Jeju Island, where hantavirus-infected shrews were captured.

Results and discussion

Detection of hantavirus RNA

Hantavirus RNA, designated JJUV, was detected by reverse transcription polymerase chain reaction (RT-PCR) in eight of 51 Asian lesser white-toothed shrews, captured on Jeju Island (Fig. 1B), whereas none of 63 *C. shantungensis* captured on Ulleung Island were infected (Table 1). Also, JJUV RNA was not found in 28 Asian lesser white-toothed shrews from locales on mainland Korea, 200 or more km from Jeju Island. The failure to detect JJUV infection in Asian lesser white-toothed shrews on the mainland may be due to the small sample size. On the other hand, the inability to find JJUV on Ulleung Island is reminiscent of the absence of HTNV-infected striped field mice on Jeju Island, compared to mainland Korea (Lee et al., 1981).

Recently, in a capture-mark-recapture study of Asian lesser white-toothed shrews in Taiwan, both females and males were found to mate with multiple partners, as evidenced by parentage analyses of 8 microsatellites in litters (Lin et al., 2009). As such, monogamous mating behaviors are unlikely to be responsible for the differential prevalence of JJUV carriage. Instead, founder groups of Asian lesser white-toothed shrews, naturally occurring on Jeju Island before the geological separation from the Korean peninsula approximately 12,000 to 16,000 years before present (Ohshima, 1990), might have already been infected. Alternatively, humans may have unintentionally transported JJUV-infected shrews to Jeju Island in the more recent past. Studies are underway to clarify the evolutionary history and phylogeography of JJUV, particularly in coastal regions on mainland Korea near the late Pleistocene land bridges.

Genetic analysis

Genome amplification of the newfound JJUV was accomplished from tissues of Asian white-toothed shrews captured in Ara-dong, Ora-dong and Gyorae-ri (Table 2), using oligonucleotide primers based on conserved regions. Pair-wise alignment and comparison with TPMV and MJNV, two crocidurine shrew-borne hantaviruses from Asia, showed low nucleotide similarity in the S, M and L segments, ranging from 50.8% to 63.1%. By contrast, much higher nucleotide sequence similarities of 62.2–79.1% were found between JJUV and hantaviruses harbored by soricine shrews from Africa. The deduced-amino acid sequences of the JJUV S and M segment products diverged by more than 15% from that of all known rodent- and soricomorph-borne hantaviruses, indicating that JJUV was a distinct hantavirus species (Fauquet et al., 2005; Maes et al., 2009).

Amplification of the S-genomic segment was achieved for two of the eight JJUV strains. The full-length 1754-nucleotide S-genomic segment of JJUV strains 10–11 and SH42 contained a single open

Table 1
Prevalence of hantavirus infection in *Crocidura shantungensis* captured on Jeju and Ulleung islands.

Island	Trap site	Month year	No. tested	JJUV RNA
Jeju	Ora-dong	May 2009	1	0
		Sep 2009	7	1
		Oct 2009	7	2
		Mar 2010	1	1
		Apr 2010	3	0
		Dec 2010	2	1
	Gosan-ri, Hangyeong-myeon	Apr 2009	1	0
	Sinheung-ri, Namwon-eup	May 2009	4	0
	Ara-dong	Nov 2007	4	1
		Oct 2009	5	0
	Gyorae-ri, Jocheon-eup	Apr 2010	8	1
		Oct 2010	8	1
Ulleung	Nari-ri, Buk-myeon	Sep 2009	48	0
	Sadong-ri, Ulleung-eup	Sep 2009	15	0

Table 2
Summary of JJUV strains detected in tissues of *Crocidura shantungensis* captured on Jeju Island, Korea.

			Nucleotides and GenBank accession numbers		
JJUV strain	Capture site	Capture date	S segment	M segment	L segment
SH42	Ara-dong	15-Nov-2007	1736 nt (1–1736) HQ663933	2312 nt (1259–3570) HQ663934	6588 nt (1–6588) HQ663935
09-84	Ora-dong	16-Sep-2009		2311 nt (1260–3570) HQ834699	1390 nt (1–1390) HQ834705
09-93	Ora-dong	15-Oct-2009		2311 nt (1260–3570) HQ834700	1390 nt (1–1390) HQ834706
09-95	Ora-dong	15-Oct-2009		2158 nt (1413–3570) HQ834701	1390 nt (1–1390) HQ834707
10-1	Ora-dong	31-Mar-2010		2311 nt (1260–3570) HQ824702	1390 nt (1–1390) HQ834708
10-11	Gyorae-ri	29-Apr-2010	1754 nt (1–1754) HQ834695	3570 nt (1–3570) HQ834696	6588 nt (1–6588) HQ834697
10-22	Gyorae-ri	14-Oct-2010		812 nt (1419–2230) HQ834703	
10-54	Ora-dong	10-Dec-2010		597 nt (1410–2006) HQ834704	

reading frame, encoding a predicted N protein of 428 amino acids (nucleotide positions 37 to 1320), and 36- and 431-nucleotide 5′- and 3′-noncoding region (NCR), respectively. The sequence variation in the entire S-genomic segment between the JJUV strains was 2.5% and 0.3% at the nucleotide and amino acid levels, respectively.

The predicted secondary structure of the JJUV N protein, as determined by various software available in the @NPS structure server, was composed of 45.79% α-helices, 8.18% extend strands and 46.03% random coils (data not shown), and comprised two major α-helical domains packed against a central β-pleated sheet, resembling that of other rodent- and soricomorph-borne hantaviruses.

Partial to full-length sequences of the M-genomic segment, exhibiting up to 11.1% nucleotide sequence variation, were available for all eight JJUV strains. The 3570-nucleotide full-length M segment of JJUV strain 10–11 encoded an entire glycoprotein of 1143 amino acids, with 38- and 100-nucleotide 5′- and 3′-NCR, respectively. Four potential N-linked glycosylation sites in Gn and Gc at positions 142, 355, 407, 936 and 1058 were found in JJUV, as determined by the NetNGlyc 1.0 server. In addition, the highly conserved WAASA amino-acid motif was present at amino acid positions 652–656.

The full-length 6588-nucleotide L-genomic segment of JJUV strains 10–11 and SH42 contained a single open reading frame, encoding a predicted RNA-dependent RNA polymerase of 2157 amino acids, with a 35- and 79-nucleotide 5′- and 3′-NCR, respectively. Analysis of the JJUV L protein revealed the five conserved motifs (A, B, C, D and E), previously identified for hantavirus RNA polymerases (data not shown) (Kang et al., 2009c; Yadav et al., 2007). The

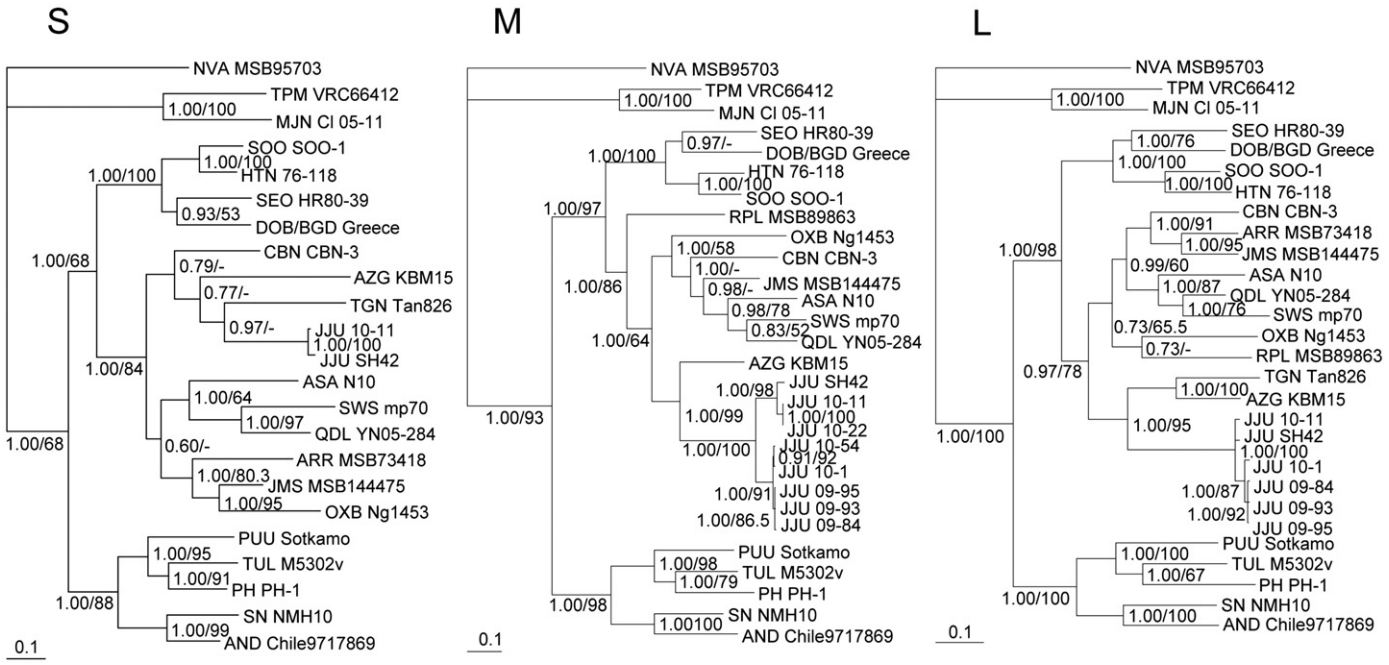

Fig. 2. Phylogenetic trees generated by the maximum-likelihood method, using the GTR + I+Γ model of evolution as estimated from the data, based on the alignment of the coding regions of the entire (S) 1287-nucleotide S segment, (M) 3432-nucleotide M segment and (L) 6474-nucleotide L genomic segment of JJUV strain 10–11 (S: HQ834695, M: HQ834696, L: HQ834697). The phylogenetic positions of JJUV strain 10–11 are shown in relationship to representative *Murinae* rodent-borne hantaviruses, including Hantaan virus (HTNV 76-118, S: NC_005218, M: NC_005219, L: NC_005222), Soochong virus (SOOV SOO-1, S: AY675349, M: AY675353, L: DQ056292), Dobrava–Belgrade virus (DOB/BGDV Greece, S: NC_005233, M: NC_005234, L: NC_005235) and Seoul virus (SEOV HR80-39, S: NC_005236, M: NC_005237, L: NC_005238); *Arvicolinae* rodent-borne hantaviruses, including Tula virus (TULV M5302v, S: NC_005227, M: NC_005228, L: NC_005226), Puumala virus (PUUV Sotkamo, S: NC_005224, M: NC_005223, L: NC_005225) and Prospect Hill virus (PHV PH-1, S: Z49098, M: X55129, L: EF646763); and *Neotominae* rodent-borne hantaviruses, Sin Nombre virus (SNV NMH10, S: NC_005216, M: NC_005215, L: NC_005217) and Andes virus (ANDV Chile9717869, S: AF291702, M: AF291703, L: AF291704). Also shown are Thottapalayam virus (TPMV VRC66412, S: AY526097, M: EU001329, L: EU001330), Imjin virus (MJNV CI05-11, S: EF641804, M: EF641798, L: EF641806), Cao Bang virus (CBNV CBN-3, S: EF543524, M: EF543526, L: EF543525), Ash River virus (ARRV MSB 73418, S: EF650086, L: EF619961), Jemez Springs virus (JMSV MSB144475, S: FJ593499, M: FJ593500, L: FJ593501), Seewis virus (SWSV mp70, S: EF636024, M: EF636025, L: EF636026), Qiandao Lake virus (QDLV YN05-284, S: GU566023, M: GU566022, L: GU566021), Oxbow virus (OXBV Ng1453, S: FJ5339166, M: FJ539167, L: FJ593497), Tanganya virus (TGNV Tan826, S: EF050455, L: EF050454), Nova virus (NVAV MSB95703, S: FJ539168, M: HQ840957, L: FJ593498), Asama virus (ASAV N10, S: EU929072, M: EU929075, L: EU929078), Azagny virus (AZGV KBM15, S: JF276226, M: JF276227, L: JF276228) and JJUV strain SH42 (S: HQ663933, M: HQ663934, L: HQ663935). The numbers at each node are posterior node probabilities based on 150,000 trees (left) and bootstrap values of 100 ML replicates executed on the RAxML BlackBox web server (right), respectively. The scale bars indicate nucleotide substitutions per site.

overall high sequence similarity of the L segment between JJUV and rodent- and soricid-borne hantaviruses was consistent with the functional constraints on the RNA-dependent RNA polymerase. Partial L-segment sequences, available from four additional JJUV strains, exhibited 2.0–3.4% nucleotide variation.

Phylogenetic analyses

Phylogenetic analyses based on nucleotide sequences of the entire S-, M- and L-genomic segments, generated by the maximum-likelihood method, indicated that JJUV was distinct from MJNV and other hantaviruses with high bootstrap values and high posterior node probabilities based on 150,000 trees (Fig. 2). The average standard deviations of split frequencies of S, M and L segments Markov Chain Monte Carlo generations were 0.000538, 0.000647 and 0.004236, respectively. Phylogenetic trees were derived, using the Bayesian Metropolis–Hastings Markov Chain Monte Carlo tree-sampling method. Since full genomes were unavailable for several viruses, such as TGNV and AZGV, phylogenetic tree based on each genomic segment consisted of different taxa or combinations of taxa (Fig. 2). Despite this, the most strikingly consistent topological feature was the phylogenetic position of JJUV with soricine shrew-borne hantaviruses. Also, JJUV shared a common ancestry with TGNV and AZGV, two crocidurine shrew-borne hantaviruses from Africa, rather than forming a monophyletic group with MJNV and TPMV, both from Asian crocidurine shrews. That is, two distinct, phylogenetically divergent lineages were found.

In applying a predictive paradigm based on phylogenetic relationships of known reservoir hosts, we have previously discovered novel hantaviruses (Baek et al., 2006; K.J. Song et al., 2007; Song et al., 2009). The recent isolation of MJNV and the incongruent phylogenies of MJNV and TGNV, two genetically distinct hantaviruses found in crocidurine shrew species in Korea and Guinea, respectively, prompted us to target the discovery of a hantavirus in the Asian lesser white-toothed shrew. Instead, analyses of JJUV strains detected in eight Asian lesser white-toothed shrews showed consistently higher genetic and phylogenetic similarity with TGNV, rather than MJNV or TPMV.

Despite the unexpected phylogenetic positions of JJUV and MJNV, JJUV strains from three capture sites on Jeju Island exhibited phylogenetic segregation according to the geographic origins of the reservoir host species. Thus, JJUV displayed geographic-specific clustering akin to that reported for rodent-borne hantaviruses and recently demonstrated for SWSV in *S. araneus* (Kang et al., 2009a; Yashina et al., 2010), MJNV in *C. lasiura* (Gu et al., 2011) and TPMV in *S. murinus* (Kang et al., 2011c).

Preferential host switching with local host-specific adaptation, instead of virus–host co-divergence, has been proposed recently to account for the congruent phylogenies of hantaviruses and their reservoir hosts (Ramsden et al., 2009). However, phylogenetic trees reconstructed for co-phylogeny mapping, generated by TreeMap 2.0β, using consensus ML topologies based on the amino acid sequences of the nucleocapsid protein, Gn and Gc glycoproteins and RNA-dependent RNA-polymerase, exhibited segregation of hantaviruses according to the subfamily of their soricomorph reservoir hosts, with no evidence of host switching for JJUV (Fig. 3). The phylogenetic position of JJUV relative to related viruses TGNV and AZGV is congruent with the phylogenetic relationships of their *Crocidura* hosts. However, the discovery and phylogenetic position of JJUV has thrown evolutionary events for MJNV into question. MJNV is clearly distant from the other viruses that infect *Crocidura*, occupying a divergent and basal position in the phylogeny of hantaviruses and suggesting that it may have switched into its *Crocidura lasiura* host, although from where is unclear. More sampling to resolve MJNV's evolutionary history is required. Other incongruencies between host and virus phylogenies were ASAV and OXBV, two hantaviruses harbored by moles that none-the-less group with shrew-borne hantaviruses (Arai et al., 2008b; Kang et al., 2009b).

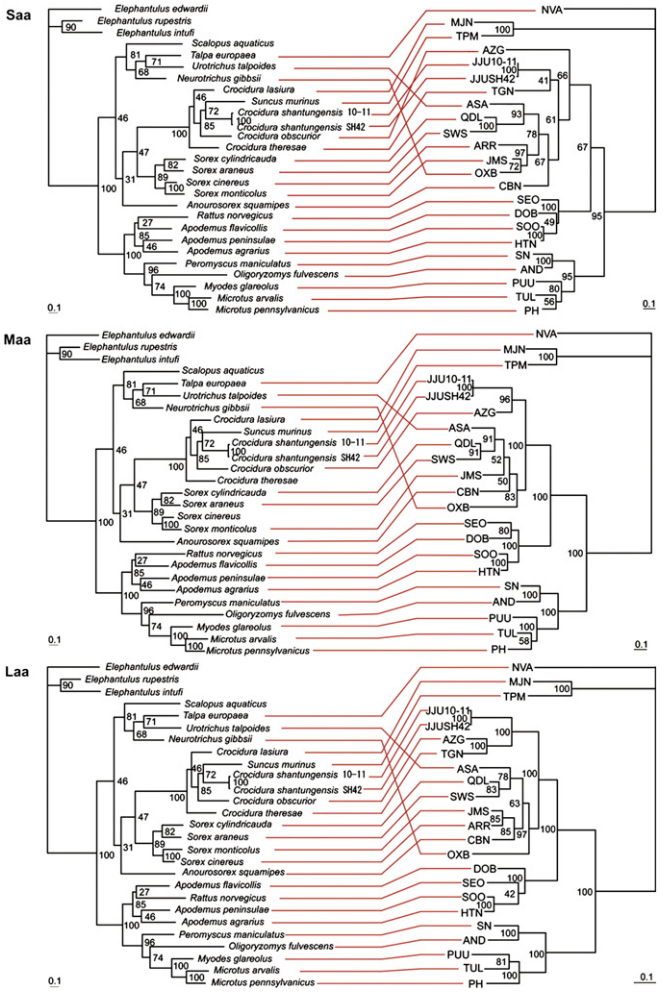

Fig. 3. Tanglegrams, generated by TreeMap 2.0β, using consensus ML topologies based on the amino acid sequences of the nucleocapsid protein (labeled Saa), Gn and Gc glycoproteins (labeled Maa) and viral RNA-dependent RNA-polymerase (labeled Laa) of JJUV 10–11 and JJUV SH42, and representative rodent-, shrew- and mole-borne hantaviruses and cytochrome *b* mtDNA sequences of the respective reservoir host species. Node support was derived from 100 ML bootstrap replicates executed on the RAxML BlackBox web server. Virus and host names are provided in the legend to Fig. 2.

mtDNA analysis and biogeographic inferences

Taxonomic classification of the hantavirus-infected Asian lesser white-toothed shrews was achieved by amplification and sequencing of the 1140-nucleotide mtDNA cytochrome *b* gene (SH42: HQ663932; 10-11: HQ834698). Phylogenetic analysis showed distinct grouping of JJUV-infected *C. shantungensis* from this study with *C. shantungensis* sequences available in GenBank (Fig. 4). Moreover, rodents and soricomorphs formed distinct lineages, well supported by bootstrap analysis. Also, as previously reported (Dubey et al., 2008), *C. shantungensis* and *C. lasiura* were well-differentiated species distinct from *C. theresae* and *C. obscurior* (Fig. 4).

Distributed widely across Europe, Africa and Asia, members of the genus *Crocidura* are among the most speciose of mammals, but their biogeographic origin and radiation are unclear (Dubey et al., 2008). As determined by in-depth analysis of karyologic, paleontologic and genetic data, a Palaearctic–Oriental origin has been proposed for *Crocidura* dating back to the Upper Miocene (6.8 million years before present) (Dubey et al., 2008). The molecular phylogeny of shrews belonging to the genus *Crocidura* is generally consistent with the biogeographic origins of species into an Afrotropical clade, an Asian clade and an Old World clade, which includes Afrotropical, East Palaearctic–Oriental and West Palaearctic species (Dubey et al.,

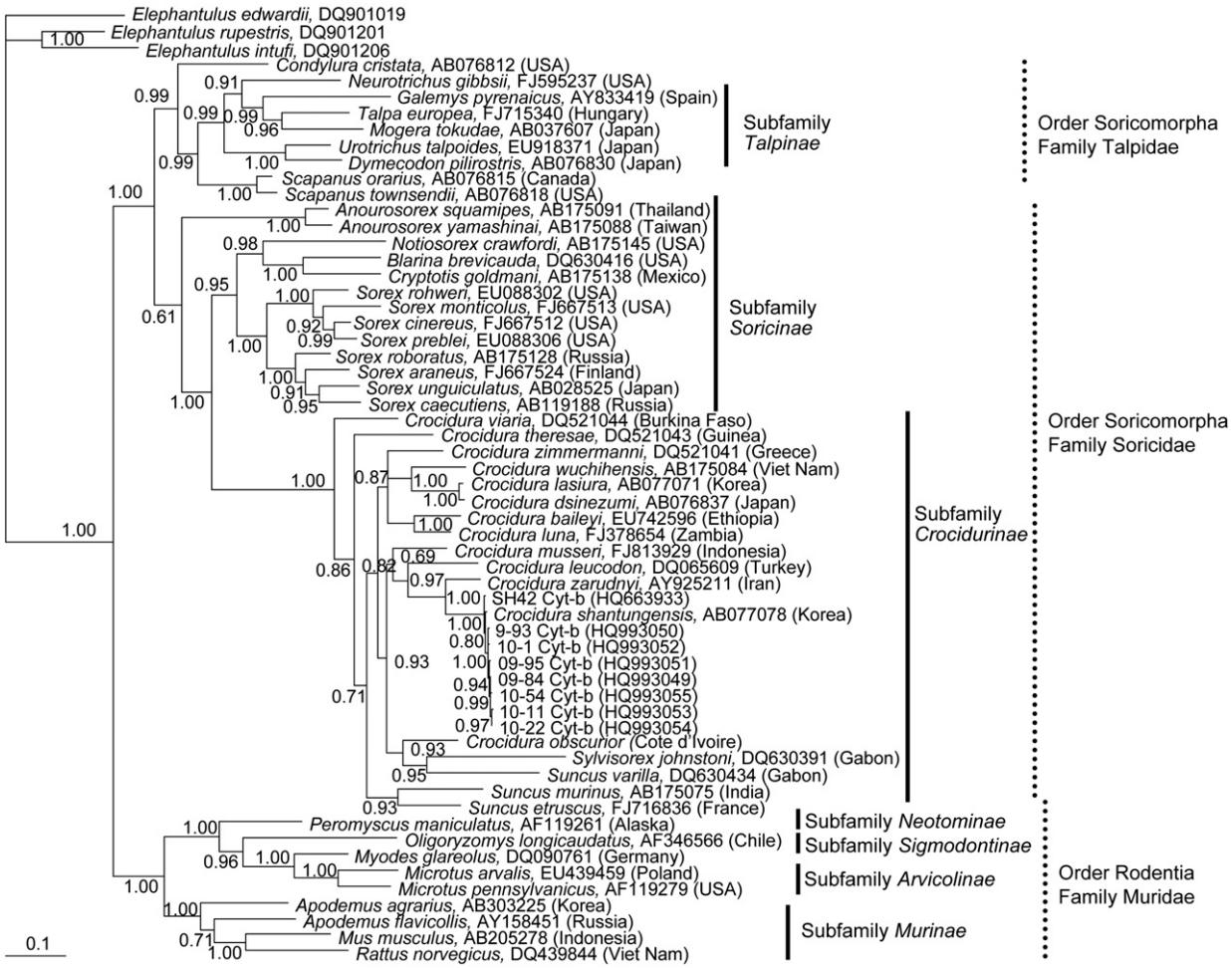

Fig. 4. Bayesian phylogenetic tree, based on the 1140-nucleotide cytochrome *b* region of mtDNA of small mammals within the Order Soricomorpha, Family Talpidae and Soricidae and the Order Rodentia, Family Muridae and Cricetidae. The tree was rooted using *Elephantulus* (Order Macroscelidea, GenBank accession numbers DQ901019, DQ901206 and DQ901201) as the outgroup. Numbers at nodes indicate posterior probability values.

2008). The geographic and genetic mismatching of the Old World clade is presumed to have resulted from migration and colonization of each species over hundreds of thousands of years or more (Dubey et al., 2008). However, *C. shantungensis* and *C. lasiura* group unambiguously into the Asian clade, while *C. theresae* groups in the Afrotropical clade.

The three scenarios, proposed for the molecular phylogeny of crocidurine shrews, presume a colonization of Africa during the Middle Miocene and two independent origins of the *Crocidura* genus lineages (Dubey et al., 2008). Even less well understood are the evolutionary histories of hantaviruses harbored by crocidurine shrews. The shared common ancestry between JJUV and both TGNV and AZGV would be consistent with replacement of an ancestral *Crocidura* lineage in Asia with a divergent lineage emerging from Africa.

Future efforts to analyze JJUV sequences from *C. shantungensis* captured in Taiwan, Buryatia and/or Mongolia (Bannikova et al., 2009), and efforts to detect JJUV-related hantaviruses in the *C. dsinezumi*, which has been recently introduced into Jeju Island from western Japan (Ohdachi et al., 2004), might yield additional important insights into the biogeography and evolutionary history of crocidurine shrew-borne hantaviruses.

Materials and methods

Trapping and specimen processing

Asian lesser white-toothed shrews were captured, using Sherman traps (H.B. Sherman, Tallahassee, FL), at several sites on Jeju Island (33°24′ N, 126°32′ E) during 2007, 2009 and 2010 and on Ulleung Island (37°30′ N, 130°50′ E), a small, sparsely populated island located in the East Sea, approximately 600 km from Jeju Island (Table 1 and Fig. 1A). In addition, 28 Asian lesser white-toothed shrews, from the mainland (15 from Haenam, Jeonllanam province and 13 from Sesan, Chungnam province), were captured. Shrews were sacrificed by cervical dislocation. Lung and liver tissues were dissected using separate instruments, and were stored at −80 °C until processed for RT-PCR and mtDNA analysis. All trapping and experimental procedures on animals were approved by the Korea University Institutional Animal Care and Use Committee and the Korean Ministry of Environment.

RNA extraction, cDNA synthesis and RT-PCR amplification

Total RNA was extracted from 20 to 50 mg of each tissue, using the PureLink Micro-to-Midi total RNA purification kit (Invitrogen, San Diego, CA). cDNA, synthesized using the SuperScript III First-Strand Synthesis Systems (Invitrogen), were analyzed for hantavirus RNA by RT-PCR, using primers designed from highly conserved regions of hantavirus genomes (Arai et al., 2007, 2008a, 2008b; Kang et al., 2009b, 2009c, 2010; Song et al., 2007a, 2007b, 2009).

First- and second-round PCR were performed in 20-μL reaction mixtures, containing 250 μM dNTP, 2.5 mM $MgCl_2$, 1 U of Takara LA Taq polymerase (Takara, Shiga, Japan) and 0.25 μM of each primer. Initial denaturation at 94 °C for 2 min was followed by two cycles each of denaturation at 94 °C for 30 s, two-degree step-down annealing from 46 °C to 38 °C for 40 s, and elongation at 72 °C for 1 min,

then 30 cycles of denaturation at 94 °C for 30 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) (Arai et al., 2008b). PCR products were separated, using MobiSpin S-400 spin columns (MoBiTec, Goettingen, Germany), and amplicons were sequenced directly using an ABI Prism 3130 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 Clustal W2 (Thompson et al., 1994) with publicly available hantavirus sequences. For phylogenetic analysis, we implemented two alternative methods based on maximum-likelihood (ML) and Bayesian inference: ML trees with ML bootstrap support were generated in RAxML Blackbox web server under the GTR + I+Γ model of evolution with codon partitioning (Stamatakis et al., 2008); consensus trees were generated by the Bayesian Metropolis–Hastings Markov Chain Monte Carlo (MCMC) tree-sampling methods using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) also under the GTR + I+Γ model of evolution with codon partitioning. Two replicate MCMC runs each consisted of six chains of 10 million generations sampled every 100 generations with a burn-in of 25,000 (25%), resulting in 150,000 trees overall. Model of evolution was selected by hierarchical likelihood-ratio test in MrModeltest v2.3 (Posada and Crandall, 1998) and jModelTest version 0.1 (Posada, 2008). We performed host–parasite phylogenetic comparisons to detect co-divergence or host-switching events using TreeMap2.0β (Charleston, 1998).

Secondary structures

To predict the secondary structures of the JJUV nucleocapsid (N) protein and envelope glycoproteins, sequences were submitted to the NPS@ server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html) and the CBS prediction server (http://www.cbs.dtu.dk/services/). For the N protein, the DSC method (King and Sternberg, 1996) was used.

mtDNA analysis

To confirm the taxonomic classification of the host species, total DNA was extracted from liver, lung or spleen tissues using the QIAamp Tissue Kit (QIAGEN). The entire 1140-nucleotide cytochrome *b* gene of mtDNA was amplified by PCR, using previously described universal primers (Irwin et al., 1991). PCR was performed in 50-μL reaction mixtures, containing 250 μM dNTP and 1 U of Expand Long Template polymerase (Roche Applied Science, Basel, Switzerland). Cycling conditions consisted of an initial denaturation at 95 °C for 4 min followed by 35 cycles with denaturation at 94 °C for 40 s, annealing at 57 °C for 1 min, and elongation at 72 °C for 2 min in a GeneAmp PCR9700 thermal cycler.

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