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Genetic Identification and Characterization of Limestone Canyon Virus, a Unique *Peromyscus*-Borne Hantavirus

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Hantaviruses, family *Bunyaviridae,* are rodent-borne RNA viruses that can cause hantavirus pulmonary syndrome (HPS) in various regions of the Americas. A coevolutionary relationship exists between hantaviruses and their specific rodent reservoir hosts; the phylogeny of the viruses generally matches that of the rodents. There are several *Peromyscus*-borne hantaviruses, including Sin Nombre virus, the most common cause of HPS in North America. This report describes the genetic detection and characterization of a newly discovered *Peromyscus boylii*-borne virus, Limestone Canyon (LSC) virus, the most divergent member of the *Peromyscus*-borne hantaviruses to date. Analysis of a 1209-nucleotide region of the S segment of LSC virus showed it to be more closely related to hantaviruses found in harvest mice (*Reithrodontomys megalotis* and *R. mexicanus*) than to other *Peromyscus*-associated hantaviruses (Sin Nombre, New York, and Monongahela). Phylogenetic analysis of virtually the entire M genome segment (3489 nucleotides) of LSC virus revealed a similar picture in which LSC virus was found to be very distinct from other *Peromyscus*-associated viruses, but its exact relationship to the other *Peromyscus*-borne and the *Reithrodontomys*-borne viruses was not resolved. These results indicate that hantavirus host species-jumping events can occur by which a hantavirus may switch to, and become established in, a rodent host belonging to a different genus. *P. boylii* are present throughout the southwestern United States and central Mexico. More extensive screening of HPS patients by using RT–PCR assays will be necessary to determine if LSC virus can cause human disease.
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Key Words: hantavirus; *Peromyscus boylii;* coevolution; genetics; RNA virus.

INTRODUCTION

Hantaviruses make up one of five recognized genera within the family *Bunyaviridae.* These negative-stranded RNA viruses consist of a tripartite genome encoding four structural proteins: the nucleoprotein (N), two glycoproteins (G1 and G2), and a viral polymerase (Elliott *et al.,* 1991). The hantaviruses hosted by rodents in the *Murinae* and *Arvicolinae* subfamilies of the family *Muridae* (reviewed in Plyusnin *et al.,* 1996) have long been recognized as the cause of hemorrhagic fever with renal syndrome in the Old World.

Within the past decade, several New World hantaviruses have been identified as the cause of hantavirus pulmonary syndrome (HPS). The hosts of these hantaviruses are rodents of the subfamily *Sigmodontinae,* also within the family *Muridae.* Many different strains of hantaviruses have been discovered in humans and/or rodents throughout North, Central, and South America (Peters, 1998). HPS-associated hantaviruses of North America include Sin Nombre (SN), New York (NY), Black Creek Canal (BCC), Bayou (BAY), Monongahela (MGL), and Choclo viruses (Nichol *et al.,* 1993; Hjelle *et al.,* 1995b; Ravkov *et al.,* 1995; Morzunov *et al.,* 1995; Rhodes *et al.,* 2000; Vincent *et al.,* 2000). Other hantaviruses have been identified in *Sigmodontinae* rodents in North and Central America but are not known to be associated with human disease. These include Blue River (BR), Muleshoe (MUL), El Moro Canyon (ELMC), Rio Segundo (RIOS), and Calabazo (Morzunov *et al.,* 1998; Rawlings *et al.,* 1996; Hjelle *et al.,* 1994, 1995a; Vincent *et al.,* 2000).

Phylogenetic studies have established a coevolutionary relationship between hantaviruses and their specific rodent hosts, with the occurrence of occasional species-jumping events (Monroe *et al.,* 1999; Nichol, 1999; Plyusnin and Morzunov, 2000). Among the North American hantaviruses associated with *Peromyscus* spp., species-jumping has been observed (Morzunov *et al.,* 1998). While all of the known hantaviruses of *Peromyscus* species origin have been shown to be monophyletic (Johnson *et al.,* 1997, 1999; Morzunov *et al.,* 1998; Monroe *et al.,* 1999), NY virus, a *P. leucopus*-borne hantavirus, has been shown to be contained within the same monophyletic clade as viruses associated with *P. maniculatus* (SN

The nucleotide sequences of the Limestone Canyon virus S and M RNA segments reported in this article have been deposited with the GenBank Database under Accession Nos. AF307322 and AF307323, respectively.

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and MGL) rather than in a clade with BR virus, another *P. leucopus*-associated hantavirus (Morzunov *et al.,* 1998).

In an earlier study to analyze the patterns of association of hantaviruses with different rodent hosts and habitats throughout the southwestern United States, 20% of *Peromyscus boylii* (brush mice) were found to have been infected by hantaviruses based on serologic testing of the rodent blood specimens by using a SN virus-recombinant nucleoprotein antigen in an enzyme-linked immunosorbent assay (ELISA) (Mills *et al.,* 1997). At some sites, seropositive *P. boylii* were found in the absence of *P. maniculatus,* suggesting that these infections did not represent spillover infection with Sin Nombre virus from *P. maniculatus.* Subsequently, a mark–recapture rodent study in central Arizona reported the predominant species captured as *P. boylii* (70%), of which 20% had been infected with a hantavirus (Abbott *et al.,* 1999). In the current study, genetic material of a unique hantavirus, for which we propose the name Limestone Canyon (LSC) virus, was amplified from the blood of a representative sample of these same *P. boylii.* Phylogenetic analysis based on virus S segment sequences shows this newly identified virus to be more closely related to *Reithrodontomys*-borne hantaviruses than to the other *Peromyscus*-associated hantaviruses. Similar phylogenetic analysis of virus M segment sequences also shows the *P. boylii*-borne LSC virus to reside outside of the main *Peromyscus*-borne hantavirus clade. These results suggest that in addition to occasional species-jumping events among rodent members of the same genus, host-switching can also occur between more distantly related rodents.

RESULTS

Diagnostic PCR sequence analysis

Rodent blood samples were obtained from a mark–recapture trapping study over a 3-year period in Limestone Canyon, north of Prescott, Arizona (Abbott *et al.,* 1999). Each month rodents were captured in this study, they were tested by ELISA for the presence of antibodies reactive with SN virus recombinant nucleocapsid antigen. Due to the observation that *P. boylii* was the predominant rodent captured, as well as the predominantly seropositive rodent in this study, three *P. boylii* from each of two web-trapping sites (S-1 and S-2) were selected for genetic testing. Given the recapture element of the study, samples representing the first hantavirus-specific antibody-positive bleed from each of these rodents were chosen for genetic testing, based on the assumption that these rodents were recently infected and would be more likely to have virus present in the blood.

Primers previously designed to target partial N, G1, and G2 coding regions of *Sigmodontinae*-associated hantaviruses were used for reverse transcription–polymerase chain reaction (RT–PCR) and second-round PCR testing of RNA from the six selected seropositive rodent blood samples (Johnson *et al.,* 1997; Morzunov *et al.,* 1995; Fulhorst *et al.,* 1997). A PCR product for each of these three regions was obtained for all six rodents. After primers were trimmed away, a 394-nt fragment of N, a 259-nt fragment of G1, and a 139-nt fragment of G2 for each of the rodent samples were compared with one another. Nucleotide differences between the N sequences were less than 1% for the six rodents, while aa sequences were all identical. The G1 fragments had nt differences of 0 to 1.9% and no aa differences. The G2 nt and aa sequences were all identical.

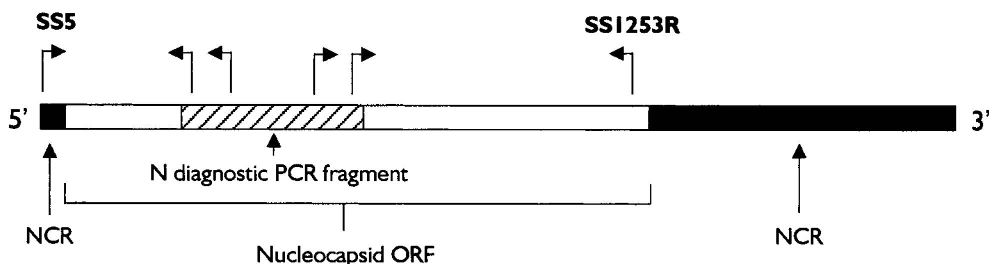
One of the rodents in this study (68273) had been recaptured and rebled several times. RT–PCR was used to detect viral RNA extracted from the first hantavirus antibody-positive blood sample (month 1) and the last collected blood sample (month 8). Virus N- and G1-encoding RNA was detected in both samples and possessed identical nt sequences.

S segment sequence

Since analysis of the diagnostic fragment sequences showed a high degree of identity between the six rodents analyzed, one rodent (68273) was chosen for further PCR and sequencing analysis. Primers SS5 and SS1253R (Fig. 1) were used in conjunction with specific primers designed within the diagnostic N-coding fragment to produce a longer sequence of 1209 nt [mostly N-coding region with 15 nt of noncoding region (NCR) before the N start codon]. Pairwise comparison of this sequence with the equivalent region of other known New World hantaviruses revealed that the closest relationship at the nt level was with ELMC virus (19.7% difference), followed by RIOS virus (22.0% difference), as shown in Table 1A. Amino acid comparisons showed the same pattern, with ELMC virus being the closest (only 9.1% difference in identity), again followed by RIOS (10.8% difference). Both ELMC and RIOS viruses are from *Reithrodontomys* spp. and are 8.0% different from one another at the aa level. Greater differences were seen with the aa sequence of the *P. boylii* virus (LSC) and those of other *Peromyscus*-borne hantaviruses (SN, MGL, and NY), ranging from 14.3 to 14.8%. This result contrasts with the 3.5 to 6.3% difference seen on comparison of the other *Peromyscus*-borne hantaviruses to one another. Even greater aa differences were seen when comparing the *P. boylii* LSC virus with the South American sigmodontine-associated hantaviruses [BAY, BCC, MUL, Caño Delgadito, Rio Mamore (RIOM), Laguna Negra (LN), and Andes (AND)]. Interestingly, LSC virus and ELMC virus shared 100% aa identity for the immunodominant linear N protein epitope previously characterized in SN virus (Yamada *et al.,* 1995).

Phylogenetic analysis of the 1209-nt S segment fragment of LSC virus and the corresponding region of other known hantaviruses was performed. Maximum parsi-

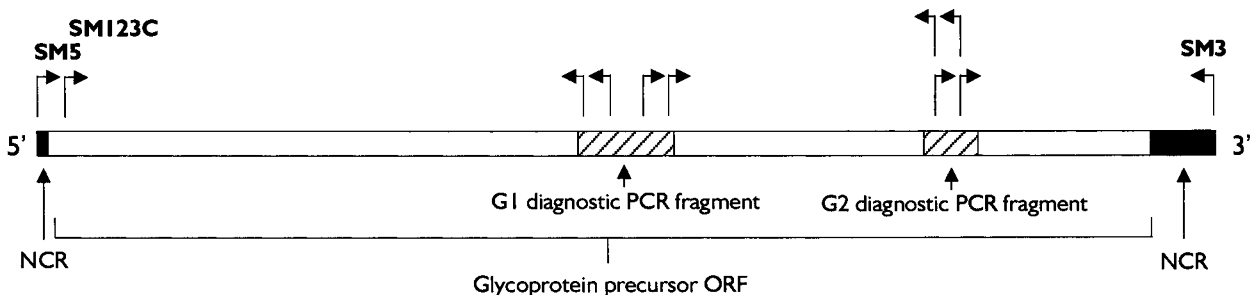
S segment



SS5 - TAGTAGTAGACTICITIA(GA)AAGCTACT

SS1253R - TCIGGITCCAT(GA)TCATC

M segment



SM5 - TAGTAGTAGACTCCGCAIGAAGAAGC

SM123C - ATGAI(CT)TIAAI(GA)TIGAATG(CT)CC

SM3 - TAGTAGTAIGCTCCGCIGGAACAAA

FIG. 1. Primers used to extend Limestone Canyon (LSC) virus diagnostic PCR fragments. Hantavirus segments are shown in cDNA sense. Primers were designed on the basis of multiple sequence alignments of *Sigmodontinae*-associated hantaviruses. Primers SS5, SM5, and SM3 target the termini of the viral segments. Primers SS1253R and SM123C target internal regions of viral segments; the number in the primer name denotes the position of the primer in the multiple sequence alignment. These primers were used in conjunction with specific LSC virus primers (denoted by arrows above hatched boxes) for RT–PCR followed by either nested or heminested PCR. In addition, two sets of specific LSC virus primers were designed to amplify the region joining the G1 and G2 diagnostic PCR fragments. Inosines within primer sequences are represented by the letter I. NCR, noncoding region; ORF, open reading frame.

mony analysis (using a 4:1 weighting of transversions to transitions) of the nt sequences placed LSC virus in a highly supported (99% bootstrap support) monophyletic clade with the two *Reithrodontomys*-associated hantaviruses, ELMC and RIOS (Fig. 2A). The other *Peromyscus*-associated hantaviruses (SN, MGL, and NY) occupied a separate clade with 100% bootstrap support. Maximum parsimony analysis of the deduced S segment aa sequences also showed two distinct and well-supported clades, with one consisting of LSC virus and the *Reithrodondomys* viruses and the other clade containing the other *Peromyscus*-borne viruses (Fig. 2B). Further support of this topology was demonstrated by maximum likelihood analysis of the nt sequences (Fig. 2C).

TABLE 1

LSC Virus and Other Hantaviruses: Percentages of Difference in Nucleotide and Amino Acid Identities[a]

A. S segment[b]

Virus	LSC	EMC	RS	SN	MGL	NY	BAY	BCC	MUL	CD	RM	LN	AND	PUU	HTN
LSC	—	19.7	22.0	23.2	23.4	23.2	25.6	24.6	24.6	26.8	24.7	25.3	24.8	32.1	39.6
EMC	9.1	—	20.5	23.7	23.7	23.0	23.7	24.8	24.7	24.1	23.8	25.7	23.7	32.8	39.0
RS	10.8	8.0	—	24.4	25.4	26.2	24.7	26.6	27.5	26.2	25.8	25.3	26.0	30.2	40.9
SN	14.8	15.3	16.6	—	16.4	15.9	23.2	24.5	24.9	23.9	23.8	23.8	23.7	32.4	38.8
MGL	14.6	15.8	17.3	6.3	—	14.7	22.9	24.3	24.5	23.9	23.5	25.2	21.5	32.2	38.4
NY	14.3	16.1	17.1	6.0	3.5	—	22.7	24.3	23.5	24.0	23.0	23.8	22.8	30.9	39.3
BAY	15.6	15.6	16.8	12.8	11.6	12.3	—	18.5	19.1	24.4	22.2	22.4	23.4	30.1	36.8
BCC	16.8	17.3	18.3	16.1	14.1	14.8	7.8	—	19.1	26.4	23.2	22.8	23.7	31.8	37.3
MUL	18.3	17.8	19.4	17.6	16.3	16.6	7.3	10.3	—	25.0	23.0	24.2	23.1	31.4	37.9
CD	19.7	18.4	20.2	16.8	16.5	16.5	18.4	19.2	18.6	—	24.0	25.5	22.6	32.8	39.3
RM	16.3	17.6	18.6	15.3	13.6	13.6	12.1	13.3	14.1	16.5	—	17.5	20.6	31.6	37.8
LN	17.3	18.1	19.4	14.8	13.6	13.1	12.8	14.6	15.1	16.8	7.0	—	20.8	30.9	36.4
AND	15.6	17.6	18.3	13.8	12.1	12.3	11.8	13.8	14.6	15.7	9.3	9.8	—	32.4	37.4
PUU	28.9	30.4	29.9	29.7	29.9	29.7	28.1	27.9	27.6	29.5	29.2	28.9	28.1	—	39.2
HTN	38.4	39.2	39.5	38.7	38.4	38.2	37.7	36.9	38.4	40.1	37.2	37.4	36.9	40.9	—

B. M segment[c]

Virus	LSC	EMC	SN	NY	BR	BAY	BCC	LN	AND	LEC	ORN	PUU	HTN
LSC	—	27.3	26.1	26.6	25.9	27.7	27.3	29.8	28.7	29.5	30.1	34.0	39.6
EMC	19.7	—	27.8	28.6	27.6	28.8	28.2	30.0	30.8	31.2	30.6	34.9	40.5
SN	16.9	19.7	—	19.2	19.6	27.1	26.6	28.8	28.1	27.9	28.7	33.5	41.6
NY	17.3	20.9	5.1	—	19.6	28.0	27.2	28.5	28.6	29.7	29.3	34.2	40.8
BR	17.6	20.1	6.7	6.8	—	27.8	26.6	27.7	28.7	28.4	28.4	33.4	40.5
BAY	20.8	22.1	18.3	20.3	19.8	—	22.3	29.5	28.6	29.3	29.4	34.8	40.8
BCC	20.8	22.5	19.4	20.8	20.4	11.3	—	28.3	28.3	29.7	29.4	35.2	40.9
LN	24.4	25.1	22.3	23.3	22.5	23.2	23.2	—	24.4	24.1	24.0	33.8	41.2
AND	24.0	25.4	20.8	22.4	21.6	22.8	23.5	12.7	—	21.3	21.2	34.4	41.1
LEC	23.6	25.3	20.9	22.0	21.5	23.5	23.5	12.9	6.4	—	18.9	35.7	41.6
ORN	24.4	26.1	21.6	22.4	22.0	23.5	23.6	13.2	7.6	4.6	—	34.7	42.1
PUU	32.2	32.4	32.4	32.5	32.2	34.4	33.9	33.4	32.5	32.5	32.7	—	41.4
HTN	41.7	42.8	44.6	44.9	43.8	44.3	44.9	44.5	44.2	43.4	44.2	45.5	—

[a] Values above the dashed lines represent the percentages of difference in nucleotide identities and those below represent the percentages of difference in amino acid identities. Values were calculated using the DISTANCES program of the Genetics Computer Group software. Virus abbreviations and GenBank Accession Nos.: LSC, Limestone Canyon, AF307322, AF307323; EMC, El Moro Canyon RM-97, U11427, U26828; RS, Rio Segundo RMx-Costa-1, U18100; SN, Sin Nombre NM H10, L25784, L25783; MGL, Monongahela-1, U32591; NY, New York RI-1, U09488, U36801; BAY, Bayou, L36929, L36930; BCC, Black Creek Canal, L39949, L39950; MUL, Muleshoe, U54575; CD, Caño Delgadito 574 and 757, AF000140, Fulhorst *et al.,* 1997; RM, Rio Mamore OM-556, U52136; LN, Laguna Negra, AF005727, AF005728; AND Andes, AF004660, Padula *et al.,* unpublished; LEC, Lechiguanas, AF028022; ORN, Oran, AF028024; PUU, Puumala Sotkamo, X61035, X61034; HTN, Hantaan 76-118, M14626, M14627.

[b] Percentages of difference for the S segment sequences were based on 1225 nucleotide and 403 amino acid multiple sequence alignments except for differences from CD, which were based on 1146 nucleotide and 381 amino acid alignments.

[c] Nucleotide comparisons were done on the open reading frame portion of the M segment sequence. Percentages of difference for the M segment sequences were based on 3355 nucleotide and 1119 amino acid multiple sequence alignments.

M segment sequence

The primers SM5, SM123C, and SM3 (Fig. 1) were used together with specific primers designed within the G1 and G2 diagnostic fragments to obtain an almost complete M segment sequence of 3489 nt, including 161 nt of the NCR after the glycoprotein precursor (GPC) stop codon (rodent 68273). Unlike the results obtained with the S segment data, pairwise comparison of the nt sequences (ORF portion) of virus M segments showed LSC virus to be slightly more related to the other *Peromyscus*-associated hantaviruses BR, SN, and NY (25.9 to 26.6% difference) than to the *Reithrodondomys*-borne ELMC virus (27.3%) (Table 1B). A similar pattern was seen on comparison of the deduced aa sequences, with the sequences of the *Peromyscus*-borne hantaviruses again being the closest to that of LSC virus, albeit with substantial differences of 16.9 to 17.6% in identity. The next closest viruses were ELMC (19.7% difference), followed by the South American sigmodontine-associated hantaviruses [BAY, BCC, LN, AND, Lechiguanas (LEC), and Oran (ORN)]. Although LSC virus demonstrated the closest relationship with the *Peromyscus*-borne hantavi-

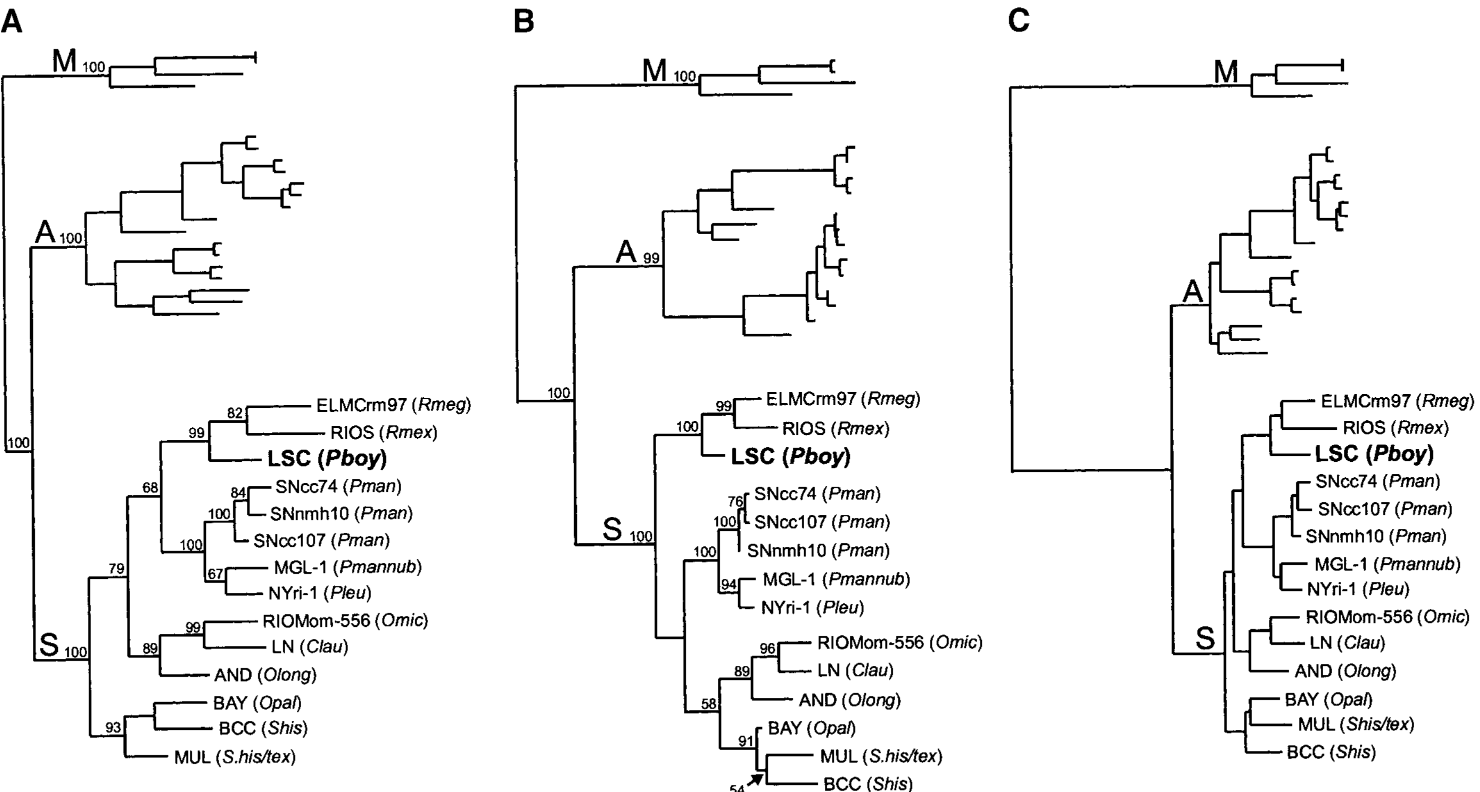


FIG. 2. Phylogenetic analysis of a 1209-nt region of S segment and the deduced 398-aa N protein sequence of LSC virus and the corresponding region from previously characterized hantaviruses. (A) Maximum parsimony analysis of nucleotide sequence. Analysis, using the heuristic search option and a 4:1 weighting of transversions over transitions, generated a single most parsimonious tree. Bootstrap analysis was conducted using 1000 replicates and values above 50% are shown at branch points. Horizontal distances represent nucleotide step differences (see bar scale), while vertical branches are for visual clarity only. (B) Maximum parsimony analysis of the deduced N protein aa sequence. The heuristic search option was used and bootstrap analysis of 1000 replicates was performed. A representative of four most parsimonious trees is shown; the topology of the four trees was identical except among the *Arvicolinae*-associated hantaviruses. (C) Maximum likelihood analysis of the nucleotide sequence. Horizontal lines represent substitutions/site (see bar scale). The three major groups of hantaviruses are designated M (*Murinae*), A (*Arvicolinae*), and S (*Sigmodontinae*), based on the rodent subfamily with which they are associated. Abbreviations for the rodent hosts associated with each hantavirus (in parentheses) include Rmeg, *Reithrodontomys megalotis;* Rmex, *Reithrodontomys mexicanus;* Pboy, *Peromyscus boylii;* Pman, *Peromyscus maniculatus;* Pmannub, *Peromyscus maniculatus nubiterrae;* Pleu, *Peromyscus leucopus;* Omic, *Oligoryzomys microtis;* Clau, *Calomys laucha;* Olong, *Oligoryzomys longicaudatus;* Opal, *Oryzomys palustris;* Shis, *Sigmodon hispidus;* and Shis/tex, *Sigmodon hispidus/texensis.* Viral sequences used in these analyses included Hantaan 76-118 (GenBank Accession No. M14626) and CUMC-B11 (U37768); Seoul SR-11 (M34881); Dobrava (L41916); Puumala Vindeln/L20Cg/83 (Z48586), Vranica (U14137), CG1820 (M32750), Udmurtia (Z21497), Evo/12Cg/93 (Z30702), Virrat//25Cg/95 (Z69985), Sotkamo (X61035), and CG 13891 (U22423); Khabarovsk (U35255); Tula Moravia/5302v/95 (Z69991), Malacky/Ma370/94 (Z68191), 249Mr/87 (Z30944), and 23Ma/87 (Z30945); Prospect Hill (M34011, X55128); prairie vole strain MO46 (U19303); Isla Vista PCB-77 (U31535); El Moro Canyon (ELMC) RM-97 (U11427); Rio Segundo (RIOS) RMx-Costa-1 (U18100); Limestone Canyon (LSC, AF307322); Sin Nombre (SN) Convict Creek 74 (L33816), NM H10 (L25784), and Convict Creek 107 (L33683); Monongahela-1 (MGL, U32591); New York (NY) RI-1 (U09488); Rio Mamore (RIOM) OM-556 (U52136); Laguna Negra (LN, AF005727); Andes (AND, AF004660); Bayou (BAY, L36929); Black Creek Canal (BCC, L39949); and Muleshoe (MUL, U54575).

ruses, it was still quite distant in comparison to the relationship of the other *Peromyscus*-associated hantaviruses to one another (only 5.1 to 6.8% difference). If the comparison is limited to the G1 aa coding region, LSC virus is even more divergent from the other *Peromyscus*-borne viruses (19.5% different from SN, BR, and NY).

The only M segment nucleotide sequence information that is available for Caño Delgadito virus is that for the 139-nt diagnostic fragment. Comparison of this sequence region with that of LSC virus shows that they differ by 28.8% and demonstrates that these M segments are not closely related.

Phylogenetic analysis of hantavirus M segment nt and deduced aa sequences provided less clear resolution of the virus relationships than was obtained with the S segment data. Maximum parsimony analysis (with 4:1 weighting of transversions to transitions) of the 3489-nt sequence of LSC virus and that of other known hantaviruses generated a well-supported (100%) monophyletic clade that included all of the previously described *Peromyscus*-associated hantaviruses (BR, SN, and NY) (Fig. 3A). The analysis placed LSC virus in a separate clade with ELMC, but with relatively weak bootstrap support (60%). The maximum parsimony aa analysis also strongly supported the monophyletic grouping of BR, SN, and NY viruses (Fig. 3B). However, there was little support for the monophyly of LSC virus and ELMC virus in this analysis, but instead LSC virus was placed on a separate branch, with some bootstrap support. A maximum likelihood analysis of the nt sequences was then conducted to try to more precisely resolve the phylogenetic relationship of LSC virus relative to the other known *Sigmodontinae*-associated hantaviruses (Fig. 3C). This analysis produced a tree topology similar to that seen with the nt and aa maximum parsimony analyses, but placed LSC virus on a separate individual branch as in the aa tree. Bootstrap analysis using maximum likelihood was not possible due to the computationally intensive nature of the analysis.

DISCUSSION

Previous studies have revealed a high prevalence of SNV-reactive antibody in *P. boylii* populations in the Southwest (Mills *et al.,* 1999). A study of the population dynamics and seropositivity patterns of these rodents strongly suggested that these hantaviral infections did not simply represent spillover infection from a different host species. In a 35-month mark–recapture study of rodent populations in Limestone Canyon in central Arizona, eight different species of rodents were captured and serologically tested for antibodies to SN virus (Abbott *et al.,* 1999). *P. boylii* was by far the predominant species captured (70%), followed by *P. truei* (18%). *P. boylii* also had the highest hantavirus antibody prevalence (20%), followed by *P. truei* (3%), with all other captured rodent species being negative for the presence of hantavirus antibody. These results, in addition to the fact that the few seropositive *P. truei* were captured when *P. boylii* densities were at their highest, are evidence that *P. boylii* is a hantavirus primary reservoir host and that these infections do not represent spillover infection in the brush mice from another sympatric rodent. The present study was aimed at determining the identity of the virus for which *P. boylii* is apparently the distinct rodent host.

Six representative *P. boylii* from the previous trapping study were selected, and diagnostic PCR fragments were amplified from the blood. All of the rodents were infected with the same virus, as the nt sequences for the virus partial N, G1, and G2 coding regions differed by less than 2% among the rodents, and the deduced aa sequences were all identical.

Extended sequences of the S and M viral segments allowed us to gain insight into the identity of this particular virus. The S segment sequence provided the clearest picture. Unexpectedly, the closest relationship to the *P. boylii* virus was shown to be the hantaviruses of *Reithrodontomys* origin. The difference in aa sequence was 9.1% to ELMC virus (*R. megalotis*) and 10.8% to RIOS virus (*R. mexicanus*), suggesting that the *P. boylii* virus was a quite distinct virus. Phylogenetic analysis of the S segment using three different methods supports this relationship by placing the *P. boylii* virus in a well-supported clade with the *Reithrodontomys* viruses, while the other *Peromyscus*-borne viruses occupy their own highly supported clade.

The unique nature of the *P. boylii* virus was further demonstrated by the M segment sequence pairwise comparisons. The closest known hantavirus aa sequence was 16.9% different, not to a *Reithrodontomys*-associated virus but to SN virus (*P. maniculatus*). In fact, the other *Peromyscus*-borne hantavirus sequences (NY and BR) were also closer to the *P. boylii* virus sequence than ELMC. Phylogenetic analysis of the M sequences showed LSC virus to be distinct from the other *Peromyscus*-associated hantaviruses, but did not clearly resolve the relationship of the virus relative to the *Reithrodontomys*- and other *Peromyscus*-borne viruses. The maximum parsimony nt tree placed the *P. boylii* virus in a monophyletic clade with ELMC. However, the maximum parsimony aa tree and the maximum likelihood nt tree both placed the *P. boylii* virus on a branch alone, separated from both ELMC and the *Peromyscus*-borne hantaviruses. However, poor bootstrap support was obtained for the placement of the *P. boylii* virus on either of the maximum parsimony-generated topologies.

Given the distinct nature of the *P. boylii* virus, we have proposed the name Limestone Canyon (LSC) virus after the geographic location of the initial detection of the virus. The S segment analyses indicate a clear relationship of LSC virus to the *Reithrodontomys*-borne viruses, which presum-

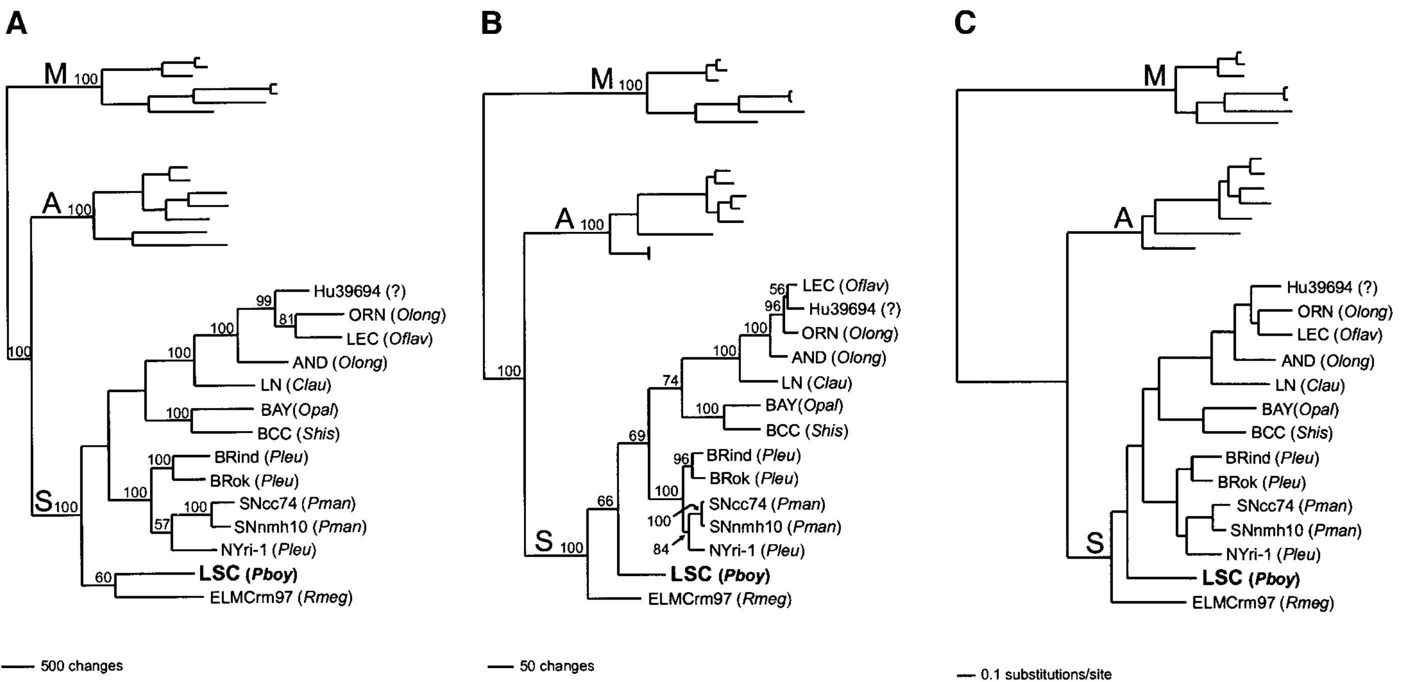


FIG. 3. Phylogenetic analysis of a 3489-nt region of the M segment and the deduced 1109-aa GPC sequence of LSC virus and the corresponding region from previously characterized hantaviruses. (A) Maximum parsimony analysis of nucleotide sequence. Analysis, using the heuristic search option and a 4:1 weighting of transversions over transitions, generated a single most parsimonious tree. Bootstrap analysis was conducted using 1000 replicates and values above 50% are shown at branch points. Horizontal distances represent nucleotide step differences (see bar scale), while vertical branches are for visual clarity only. (B) Maximum parsimony analysis of the deduced GPC aa sequence. The heuristic search option was used and bootstrap analysis of 1000 replicates was performed. A representative of two most parsimonious trees is shown; the topology of the two trees was identical except among the *Arvicolinae*-associated hantaviruses. (C) Maximum likelihood analysis of the nucleotide sequence. Horizontal lines represent substitutions/site (see bar scale). The three major groups of hantaviruses are designated M (*Murinae*), A (*Arvicolinae*), and S (*Sigmodontinae*), based on the rodent subfamily with which they are associated. Abbreviations for the rodent hosts associated with each hantavirus (in parentheses) include ?, unknown; Olong, *Oligoryzomys longicaudatus;* Oflav, *Oligoryzomys flavescens;* Clau, *Calomys laucha;* Opal, *Oryzomys palustris;* Shis, *Sigmodon hispidus;* Pleu, *Peromyscus leucopus;* Pman, *Peromyscus maniculatus;* Pboy, *Peromyscus boylii;* and Rmeg, *Reithrodontomys megalotis.* Viral sequences used in these analyses included Hantaan 76-118 (GenBank Accession No. M14627), HoJo (D00376), HV114 (L08753); Seoul 80-39 (S47716), SR-11 (M34882); Thailand (L08756); Dobrava (L33685); Puumala Vindeln/L20Cg/83 (Z49214), Vranica (U14136), Sotkamo (X61034), CG1820 (M29979), CG 13891 (U22418); Tula Moravia/5302v/95 (Z69993), Moravia/5286Ma/94 (Z66538); Prospect Hill (X55129); Human 39694 (Hu39694, AF028023); Oran (ORN, AF028024); Lechiguanas (LEC, AF028022); Andes (AND, Padula *et al.,* unpublished); Laguna Negra (LN, AF005728); Bayou (BAY, L36930); Black Creek Canal (BCC, L39950); Blue River (BR) Indiana (AF030551) and Oklahoma (AF030552); Sin Nombre (SN) Convict Creek 74 (L33684) and NM H10 (L25783); New York (NY) RI-1 (U36801); Limestone Canyon (LSC, AF307323); and El Moro Canyon (ELMC) RM-97 (U26828).

ably means that a host species-jumping event must have taken place during the long evolutionary history of these viruses. Extensive studies of hantavirus genetic sequences support a general scenario in which a single rodent species or subspecies is found to host each genetically distinct hantavirus, with occasional spillover infections occurring from the primary rodent host into other species, which result in only transient infections (Monroe *et al.,* 1999; Nichol, 1999). The finding of a small number of examples of actual host species-jumping events suggests that occasionally these spillover infections may result in establishment of a hantavirus in a new rodent reservoir and its evolutionary adaptation to that host (Monroe *et al.,* 1999; Vapalahti *et al.,* 1999). A previous example of this in North America is the *P. leucopus*-borne NY virus, which appears to be more phylogenetically related to the *P. maniculatus* viruses than to other *P. leucopus* viruses (Morzunov *et al.,* 1998). While this host-switching event appears to have involved rodents of the same genus, the LSC virus example described here suggests that virus host-switching can also occur between rodents of different genera. In the case of LSC virus, the switch likely involved an ancestor virus/rodent host of *Peromyscus* and *Reithrodontomys,* both members of the neotomine–peromyscine rodent group of North America (Engel *et al.,* 1998). A similar transgenus jump has also been recently suggested to explain the finding of the *Microtus fortis*-borne Khabarovsk virus being phylogenetically more closely related to viruses of lemmings and *Clethrionomys* voles than to other *Microtus*-borne viruses (Horling *et al.,* 1996; Vapalahti *et al.,* 1999; Nichol, 1999).

Phylogenetic analysis of mtDNA sequences of *Peromyscus* species suggests that *P. boylii* is not particularly unusual relative to the other *Peromyscus* species and is not more ancestral relative to *P. maniculatus* and *P. leucopus* (Engel *et al.,* 1998). Thus, even if the weakly supported virus tree topologies (obtained by maximum parsimony analysis of M segment deduced aa sequence or maximum likelihood analysis of M segment nt sequence data) that depict LSC virus as not being monophyletic with *Reithrodontomys*-borne hantaviruses are correct, the placement of LSC virus quite separate from and ancestral to the other *Peromyscus*-borne viruses would still be at odds with a strict virus–rodent coevolutionary scenario. If the evolutionary history of the LSC virus S and M segments are truly different, then the possibility of a segment reassortment event may also be considered. This appears to be supported by the pairwise comparisons in which the opposite scenario is seen from the S segment analysis, where the LSC virus S segment sequence is seen to be closer to the ELMC virus sequence than to those of the other *Peromyscus*-borne viruses. However, analysis of additional hantavirus sequences may be necessary before the precise phylogenetic relationships of ELMC and LSC viruses relative to other hantaviruses can be determined.

Finally, the RT–PCR detection of LSC virus RNA in blood specimens taken over an 8 month period from a naturally infected *P. boylii* is evidence of long-term infection and further supports the conclusion that *P. boylii* is the rodent host for this newly identified hantavirus. This rodent is found throughout much of the southwestern United States and down into central Mexico. Currently, there are no data linking this virus to human infections, but further monitoring and surveillance of infected individuals throughout the range of *P. boylii* will be necessary to rule out this possibility. Whether other unique *Peromyscus*-borne viruses exist will also require further testing of other species. As part of an HPS case investigation in Arizona, several hantavirus antibody-positive *P. eremicus* (cactus mice) were trapped on a river landing less than 1 acre in size, which was bordered by a high cliff wall on one side and the Colorado river on the other sides. No *P. maniculatus* were found during the trapping; however, the virus associated with the *P. eremicus* was shown by RT–PCR analysis to be a minor variant of Sin Nombre virus (Nichol, Ksiazek, Sanchez, Stevens, and Leslie, unpublished). While several hantaviruses have now been found to be associated with different *Peromyscus* species or subspecies, it is possible that not all of the 53 recognized *Peromyscus* species will harbor unique hantaviruses.

MATERIALS AND METHODS

Genetic testing and analysis

RNA extraction, RT–PCR, nested PCR, and product purification were done as previously described, except when amplifying products between 1.5 and 2.0 kilobases (kb) where extension times were increased from 2 to 3 min for both first- and second-round PCR (Johnson *et al.,* 1997, 1999).

Primers were used that amplified partial N and G1 protein coding regions of *Sigmodontinae*-associated hantaviruses (Johnson *et al.,* 1997). Primers designed to detect a region of the G2-coding sequence of BAY virus were also used (Morzunov *et al.,* 1995; Fulhorst *et al.,* 1997). Once sequence was obtained for each of these regions, specific RT-PCR primers were designed and used together or in various combinations with terminal and internal primers designed to target *Sigmodontinae*-associated hantaviruses. These were used in either nested or hemi-nested PCR formats to obtain larger fragments (Fig. 1).

After purification of the PCR products, direct sequencing was done on an ABI 377 sequencer using the dye termination cycle sequencing technique (Applied Biosystems, Inc., Foster City, CA). Sequencher version 3.1.1 (Gene Codes Corp., Ann Arbor, MI) was used for initial editing of the obtained sequences. These sequences were then compiled and analyzed with the Wisconsin Sequence Analysis Package version 10.0 (Genetics Computer Group, Inc., Madison, WI). Phylogenetic anal-

ysis was done using the PAUP* version 4.0b4a Macintosh computer software program (Swofford, 1998). Nucleotide analyses included maximum parsimony employing a 4:1 weighting of transversions to transitions and maximum likelihood. Amino acid sequence alignments were analyzed by maximum parsimony using the step matrix PROTPARS. For maximum likelihood analyses, an unweighted parsimony tree was first generated, and certain parameters estimated from this analysis (transition: transversion ratio, proportion of invariable sites, and gamma distribution value for variable sites) were entered into the maximum likelihood analysis to facilitate the run.

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