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Extensive Host Sharing of Central European Tula Virus[▽]

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To examine the host association of Tula virus (TULV), a hantavirus present in large parts of Europe, we investigated a total of 791 rodents representing 469 *Microtus arvalis* and 322 *Microtus agrestis* animals from northeast, northwest, and southeast Germany, including geographical regions with sympatric occurrence of both vole species, for the presence of TULV infections. Based on serological investigation, reverse transcriptase PCR, and subsequent sequence analysis of partial small (S) and medium (M) segments, we herein show that TULV is carried not only by its commonly known host *M. arvalis* but also frequently by *M. agrestis* in different regions of Germany for a prolonged time period. At one trapping site, TULV was exclusively detected in *M. agrestis*, suggesting an isolated transmission cycle in this rodent reservoir separate from spillover infections of TULV-carrying *M. arvalis*. Phylogenetic analysis of the S and M segment sequences demonstrated geographical clustering of the TULV sequences irrespective of the host, *M. arvalis* or *M. agrestis*. The novel TULV lineages from northeast, northwest, and southeast Germany described here are clearly separated from each other and from other German, European, or Asian lineages, suggesting their stable geographical localization and fast sequence evolution. In conclusion, these results demonstrate that TULV represents a promiscuous hantavirus with a large panel of susceptible hosts. In addition, this may suggest an alternative evolution mode, other than a strict coevolution, for this virus in its *Microtus* hosts, which should be proven in further large-scale investigations on sympatric *Microtus* hosts.

Hantaviruses (genus *Hantavirus*, family *Bunyaviridae*) are characterized by a tripartite RNA genome of negative polarity. The small (S) genome segment of about 1.7 kb encodes the nucleocapsid (N) protein that is associated as a multimer with the viral RNA genome. The medium (M) segment of about 3.6 kb encodes a glycoprotein precursor that is cotranslationally cleaved at a highly conserved WAASA motif into the G1 and G2 envelope glycoproteins. These proteins form oligomers which mediate the interaction of the virus with the cellular receptor. The large (L) segment of about 6.5 kb encodes the RNA-dependent RNA polymerase that functions as transcriptase and replicase (for a review, see reference 57).

In general, hantaviruses are harbored by persistently infected rodent reservoir hosts which shed the hantaviruses by urine, feces, and saliva. Therefore, the major route of transmission to humans is by inhalation of aerosols originating from virus-contaminated urine or feces (for a review, see reference 58). The high stability of hantaviruses in nature allows indirect transmission and underlines the importance of environmental factors on the frequency of transmission (31). An alternative route of virus transmission to humans is by rodent bites (10). Human-to-human transmission has exclusively been observed for the South American Andes virus (42).

The congruent phylogenetic affinities of hantaviruses and their corresponding reservoir hosts are currently explained by a virus-host coevolution hypothesis (46). According to this theory, each hantavirus species is associated with a single rodent species or a closely related species of the same genus. This close host/pathogen association is also believed to determine viral properties and therefore the pathogenicity in humans (73).

Tula virus (TULV) has primarily been identified in the European common voles *Microtus arvalis* and *Microtus rossiaemeridionalis* collected from the Tula area of Russia, located 200 kilometers south of Moscow, and in *M. arvalis* in western Slovakia, near the town of Malacky (43, 61). Subsequently, TULV has been detected in other related rodent species of the subfamily Arvicolinae, i.e., *M. agrestis*, *Microtus gregalis*, *Micro-*

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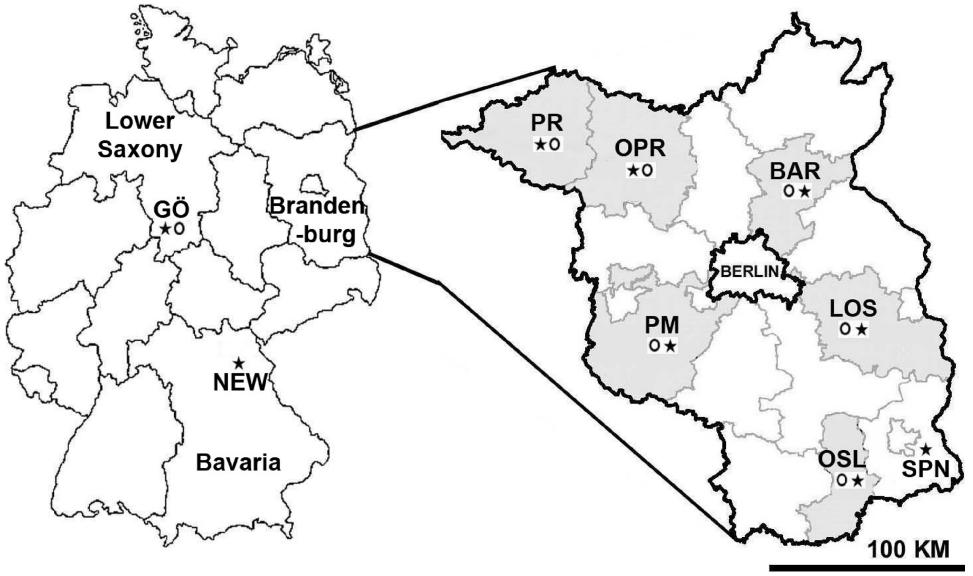

FIG. 1. Geographical localization of the trapping sites in six districts in Brandenburg, northeast Germany, one location in Lower Saxony, northwest Germany, and one location in Bavaria, southeast Germany. In addition, the localization of trapping sites of *M. arvalis*, where TULV sequences were detected in a previous study, is given (32). GÖ, Göttingen; NEW, Neustadt/Waldnaab; PR, Prignitz; OPR, Ostprignitz-Ruppin; BAR, Barnim; PM, Potsdam-Mittelmark; LOS, Oder-Spree; OSL, Oberspreewald-Lausitz; SPN, Spree-Neisse.

tus (*Pitymys*) *subterraneus*, and *Lagurus lagurus* (56, 65; A. E. Dekonenko and V. V. Yakimenko, unpublished data) (GenBank accession number AF442620-21). TULV-positive rodents have been detected in a number of European countries, such as Russia, Slovakia, Croatia, the Czech Republic, Austria, Poland, Belgium, Germany, France, Hungary, The Netherlands, and Slovenia (4, 6, 21, 30, 32, 35, 43, 44, 47, 51, 56, 61, 65, 66).

Hantaviruses associated with the genus *Microtus* (subfamily Arvicolinae) have currently only rarely been associated with human disease. Although the North American viruses Prospect Hill virus (PHV), isolated from the meadow vole *Microtus pennsylvanicus* (38), Bloodland Lake virus, detected in the prairie vole *Microtus ochrogaster* (22), and Isla Vista virus (ISLAV), detected in the Californian vole *Microtus californicus* (64), have not been shown to cause significant disease in humans, experimental infections of nonhuman primates with PHV caused an acute nephropathy (74). Similarly, the Eurasian hantavirus species Khabarovsk virus hosted by the reed vole *Microtus fortis* (23) and TULV are believed to have no or low pathogenicity for humans. However, a few human TULV infections have been reported, with two case reports about potential TULV-induced disease in humans (32, 59, 70, 71). This low frequency in the detection of human TULV infections might be explained by its low pathogenic potential (36); however, human TULV infections might have been overlooked because their differentiation from infections with the TULV-cross-reactive Puumala virus (PUUV) would require a neutralization assay (70).

Here we report on data from the first longitudinal monitoring study of TULV in Europe, with samples collected over a period of 12 years. These investigations showed the sympatric occurrence of TULV in *M. arvalis* and *M. agrestis* at several localities and in different years. Phylogenetic analysis of the TULV sequences demonstrates a geographical clustering which is independent from the rodent reservoir species. This provides strong evidence for isolated replication and transmission cycles of TULV in both species, along with frequent multiple spillover infections of TULV between *M. arvalis* and *M. agrestis* populations.

MATERIALS AND METHODS

Rodents. Between 1994 and 2005, *Microtus* rodents were trapped in six districts of the Federal State of Brandenburg, northeast Germany, and in 2005, they were trapped at one site (Sennickerode [SEN]) in the district Göttingen (GÖ) in the Federal State of Lower Saxony, northwest Germany. Additional rodents were trapped between 1994 and 1997 at four different military training areas, including Grafenwöhr, district Neustadt/Waldnaab (NEW), located in the Federal State of Bavaria, southeast Germany (Fig. 1 and Tables 1 and 2). Captured rodents were weighed and measured, and their gender and species were identified. For serological analysis, transudate samples were collected from the thoracic cavity of rodents from Brandenburg. From rodents trapped from 1994 to 1997 in Brandenburg or Bavaria, only brain or kidney samples were available. From rodents trapped from 2002 to 2005 in Brandenburg and Lower Saxony, lung, heart, liver, spleen, kidney, and brain samples were collected. Tissue samples were stored at −20°C until processing with reverse transcriptase PCR (RT-PCR). Morphological species determination of TULV RT-PCR-positive animals was confirmed by using a PCR specific for the mitochondrial cytochrome *b* gene (11).

Serological screening. To detect hantavirus-specific immunoglobulin G (IgG) antibodies, rodent transudates were screened by using enzyme-linked immunosorbent assays (ELISA) and Western blot (WB) tests based on *Saccharomyces cerevisiae*-expressed His-tagged N proteins of PUUV strain Vranica/Hällnäs (9, 50) and TULV strain Moravia (44; M. Mertens, R. Petraityte, K. Sasnauskas, R. Friedrich, and R. G. Ulrich, unpublished data). Assays were performed as described previously (11). Selected seroreactive samples were confirmed by focus reduction neutralization test analysis, according to protocols described earlier (3).

Statistical analysis. Potential associations between gender and age, using body mass as a proxy, and seropositivity to hantavirus in voles were analyzed using the chi-square test, Mann-Whitney test, or Fisher exact test included in the software package SPSS (SPSS version 12.0.1, SPSS Inc., Chicago, IL) and by Win Episcope 2.0 (69).

RT-PCR and sequencing. RNA from all animals from Lower Saxony and Bavaria and from TULV-seroreactive animals from Brandenburg was extracted

TABLE 1. Serological reactivity of transudates from *Microtus arvalis* and *M. agrestis* trapped during 1994 to 1997 at different trapping sites in Brandenburg

Species	Trapping year	No. of trapped rodents/no. of serologically reactive transudates of these rodents (%)										
		OPR trapping sites					PR trapping sites					Total
		Kyritz	Segeletz	Nackel	Breddin	Subtotal	Bendelin	Granzow	Glöwen	Schönhagen	Subtotal	
M. arvalis	1994	12/1	0	0	0	12/1 (8)	34/2	22/4	0	0	58/6[b] (10)	70/7 (10)[b]
	1995	1/0	42/8	7/1	0	53/9[a] (17)	15/2	61/12	35/17	31/8	142/39 (27)	195/48 (25)
	1996	34/3	0	0	10/2	44/5 (11)	16/3	0	2	6/1	24/4 (17)	68/9 (13)
	1997	1/0	0	0	0	1/0	10/3	0	4/0	29/3	43/6 (14)	44/6 (14)
	Total	48/4	42/8	7/1	10/2	110/15[a] (14)	75/10	83/16	41/17	66/12	267/55[b] (21)	377/70[a,b] (19)
M. agrestis	1994	0	0	0	0	0	11/3	24/1	0	0	35/4 (11)	35/4 (11)
	1995	0	0	1/1	0	1/1 (100)	11/0	0	0	0	11/0	12/1 (8)
	1996	1/1	0	2/0	0	3/1 (33)	0	0	0	0	0	3/1 (33)
	1997	0	0	0	0	0	0	0	0	0	0	0
	Total	1/1	0	3/1	0	4/2 (50)	22/3	24/1	0	0	46/4 (9)	50/6 (12)

[a] Total numbers contain three additional seronegative *M. arvalis* rodents trapped in 1995 in Netzeband, Germany.
[b] Total numbers contain two additional seronegative *M. arvalis* rodents trapped in 1994 in Perleberg, Germany.

from brain, heart, or lung tissue samples using commercial kits (11). Single-step and nested RT-PCRs using primers targeting the S segment were performed with primer pairs DOBV-M6/DOBV-M8, PUUV 342/cPUUV 1102, SNMa1/MaS4C, and S1/S10PC and nested primers PUUV390/cPUUV721 as described previously (11, 55, 61, 62). The partial M segment was amplified using primers C1, 5′-CC CCCTGATTGTCCTGGTGTAG-3′, and C2, 5′-CCAACTCCTGAACCCCAT GC-3′ (corresponding to nucleotides [nt] 2369 to 2390 and nt 3011 to 3031 of PUUV strain Sotkamo, GenBank accession number X61034). Direct sequencing of the purified PCR products was done using the S and M segment-specific primers described above. GenBank accession numbers of the novel TULV sequences are shown (see Table 4).

Sequence comparison and phylogenetic analysis. Nucleotide sequences were aligned in BioEdit 5.0.9 (17) and revised manually. Phylogenetic relationships among nucleotide sequences were reconstructed with neighbor-joining (NJ) (54) and maximum likelihood (ML) algorithms implemented in PAUP* 4.0b (67) using two PUUV strains as the outgroup and 5,000 bootstrap replicates. Hierarchical likelihood ratio tests and the Akaike information criterion (1) implemented in Modeltest 3.06 (48) were used to estimate the most suitable model of nucleotide substitution. The best substitution model for the S segment was the general time-reversible (GTR) model with gamma distribution (68) with the following parameters: substitution rate matrix, A ↔ C, 4.1689; A ↔ G, 14.7655; A ↔ T, 3.1028; C ↔ G, 0.6093; C ↔ T, 22.7757; and G ↔ T, 1.0000; gamma distribution shape parameter, 0.1931. The base frequencies were estimated as follows: A, 0.3318; C, 0.2114; G, 0.1966; and T, 0.2602. For the M segment, the best substitution model was the GTR model with invariable sites and gamma distribution (52). The following parameters for the model were estimated: substitution rate matrix, A ↔ C, 4.8675; A ↔ G, 16.5929; A ↔ T, 1.5562; C ↔ G, 4.9664; C ↔ T, 36.3308; and G ↔ T, 1.0000; gamma distribution shape parameter, 0.5635; proportion of invariable sites, 0.4103; base frequencies, A, 0.3568; C, 0.1519; G, 0.1657; and T, 0.3256.

Estimates of evolutionary raw divergence and standard error estimates (500 bootstrap replicates) over sequence pairs between groups were obtained by pairwise analysis, as supplied by MEGA4 (37). Codon positions included were first plus second plus third plus noncoding. All positions containing gaps and missing data were eliminated from the data set (complete deletion option).

Morphological species designations of TULV-positive rodents were verified by performing BLAST searches of the novel cytochrome *b* sequences with sequences available in GenBank (http://www.ncbi.nlm.nih.gov). The cytochrome *b* sequences of TULV-positive *M. agrestis* and *M. arvalis* were further compared to those from larger surveys of genetic diversity in these rodents across Germany and neighboring countries (14, 19, 25). Representative sequences from all evolutionary lineages present in Europe in *M. arvalis* and *M. agrestis* were obtained from GenBank. Technical details on phylogenetic reconstructions from cytochrome *b* are given elsewhere (15, 16, 19).

RESULTS

Rodent trapping in the longitudinal study and cytochrome *b* analysis of *Microtus* spp. Between 1994 and 1997, a total of 427 *Microtus* rodents, with 377 *M. arvalis* rodents, 115 male and 262 female, and 50 *M. agrestis* rodents, 24 male and 26 female, were trapped at 10 sites in two different districts (Ostprignitz-Rup-

TABLE 2. Serological reactivity of transudates from *Microtus arvalis* and *M. agrestis* trapped during 2002 to 2005 at different trapping sites in Brandenburg[a]

Species	Trapping year	No. of trapped rodents/no. of serologically reactive transudates of these rodents (%)						
		BAR trapping sites			LOS trapping site Wendisch	PM trapping site MRZ	OSL trapping site Altdöbern	Total
		Bernau	EBE	Subtotal				
M. arvalis	2002	47/0	1/0	48	0	0	1/0	49
	2003	0	0	0	0	0	0	0
	2004	0	1/1	1/1 (100)	0	0	6/0	7/1 (14)
	2005	0	0	0	9/0	1/0	0	10
	Total	47/0	2/1	49/1 (2)	9/0	1/0	7/0	66/1 (2)
M. agrestis	2002	3/0	14/4	17/4 (24)	7/0	45/3 (7)	4/0	73/7 (10)
	2003	15/0	16/0	31/0	8/0	21/1 (5)	0	60/1 (2)
	2004	1/0	13/0	14/0	21/0	15/1 (7)	2/0	52/1 (2)
	2005	0	26/1	26/1 (4)	13/0	36/1 (3)	10/0	85/2 (2)
	Total	19/0	69/5	88/5 (6)	49/0	117/6 (5)	16/0	270/11 (4)

[a] EBE, Eberswalde; MRZ, Marzehns.

pin [OPR] and Prignitz [PR]) in the Federal State of Brandenburg, northeast Germany (Fig. 1 and Tables 1 and 2). In addition, a total of 336 *Microtus* rodents, including 58 male and 8 female *M. arvalis* rodents and 122 male and 148 female *M. agrestis* rodents, were trapped at five sites in four other districts (Barnim [BAR], Oder-Spree [LOS], Potsdam-Mittelmark [PM], and Oberspreewald-Lausitz [OSL]) in the Federal State of Brandenburg between 2002 and 2005 (Fig. 1 and Tables 1 and 2). Altogether, *M. arvalis* and *M. agrestis* were trapped at nine sites in Brandenburg, with a sympatric occurrence of *M. arvalis* and *M. agrestis* in the same year of trapping (Tables 1 and 2). In 2005, 16 *M. arvalis* rodents, 5 female and 11 male, and 2 male *M. agrestis* rodents were trapped at a single site in Lower Saxony (SEN, GÖ) (Fig. 1). From 1994 to 1997, 10 *M. arvalis* rodents were trapped at one site in the military training area Grafenwöhr (NEW) in Bavaria (Fig. 1).

Sequencing of cytochrome *b* genes of selected *M. arvalis* and *M. agrestis* rodents from Brandenburg and Lower Saxony confirmed the morphological species determination (see Table 4). Phylogenetic reconstructions of rodent host relationships based on the mitochondrial cytochrome *b* gene showed a clear differentiation between *M. arvalis* and *M. agrestis* and an additional substructure among voles from different European regions (Fig. 2). All *M. arvalis* rodents from the German study sites belonged to the Central evolutionary lineage of this species, and all *M. agrestis* rodents from these sites belonged to the Western lineage. This is in agreement with the general distribution of these lineages, as determined in larger surveys of the two species (14, 19, 25).

In total, 791 *Microtus* rodents were trapped at 17 sites located in eight different districts in northeast, southeast, and northwest Germany.

Serological analysis of rodent transudates. An initial IgG ELISA screening using recombinant PUUV N protein as the antigen revealed a total of 35 seroreactive animals out of 763 animals (4.6%) from Brandenburg (data not shown). For 28 out of 35 samples (80%), the immunoreactivity was confirmed by a corresponding PUUV WB test (data not shown). In parallel, all transudate samples were tested by an IgG ELISA using recombinant TULV N antigen (Tables 1 and 2). Using this test format, TULV-reactive antibodies were detected in 71 of 443 *M. arvalis* transudates (16%) and 17 of 320 *M. agrestis* transudates (5.3%), demonstrating a much higher level of sensitivity for the TULV ELISA than that for the PUUV test. For 25 out of 30 TULV- and PUUV-IgG ELISA-reactive samples (83%), the endpoint titer was higher in the TULV assay (Table 3). For the majority (75 of 88; 88%) of transudates, the TULV ELISA reactivity was confirmed in a TULV WB test (data not shown). The *M. arvalis*-seroreactive samples originated from both male (31 of 173; 17.3%) and female (40 of 270; 14.8%) animals. In contrast, seroreactivity in *M. agrestis* transudates demonstrated a strong gender bias, with 15 out of 146 male (10.3%) but only 2 out of 174 female (1.1%) animals being seroreactive, which proved to be statistically significant (chi-square test, P of <0.001; odds ratio, 8.9; Fisher's exact test, P of <0.001). In contrast, the differences in the TULV seroprevalence between the genders in *M. arvalis* were not statistically significant (chi-square test, P of 0.416; odds ratio, 1.210; Fisher's exact test, P of 0.513). The body mass of *M. agrestis*, which was used as a proxy for the age of the animals, was found to be positively associated with the seropositivity (Mann-Whitney test, P of 0.0058), whereas this was not statistically significant for *M. arvalis* (Mann-Whitney test, P of 0.0703).

To prove the validity of the ELISA and WB tests, five *M. arvalis* and four *M. agrestis* transudates were tested by focus reduction neutralization assays using TULV, PUUV, Dobrava virus, and Saaremaa virus. For all nine transudates, the highest endpoint titer was observed for TULV, confirming TULV infections in both rodent species (Table 3).

The serological investigations demonstrated the presence of TULV-reactive antibodies in both *M. arvalis* and *M. agrestis*, with differences depending on the species, the trapping site, the gender, and the age of the rodents.

RT-PCR analysis of tissue samples. For 19 out of 20 seroreactive animals from Brandenburg whose brain, heart, or lung tissue samples were available, the investigations with S segment-specific RT-PCR or nested RT-PCR revealed specific amplification products (Table 4). The positive samples originated from four male and three female *M. arvalis* rodents and 11 male and one female *M. agrestis* rodents. One sample (Mu/04/151) from a seropositive female *M. agrestis* rodent tested negative in all RT-PCRs used. Sequencing of the amplification products resulted in the identification of TULV sequences in samples from both *M. arvalis* and *M. agrestis* (Table 4). As expected for RT-PCR investigations of lung samples, sequence information for all tested animals was obtained, with the majority of sequences being about 700 nt in size. Interestingly, the nested RT-PCR approach was successful for all tested brain samples from seven animals when no other tissue samples were available. In addition, for four out of five investigated heart samples, partial S segment-specific sequences were obtained (Table 4).

Screening of lung samples from all 16 *M. arvalis* and 2 *M. agrestis* rodents from Lower Saxony (district GÖ, site SEN) by S segment-specific RT-PCR revealed six positive *M. arvalis* results from one female and five male animals. In addition, one male *M. agrestis* animal was PCR positive (Table 4). Larger PCR fragments (from 1,347 nt up to 1,708 nt in length) (Table 4) could be derived from all positive samples.

Out of the 10 *M. arvalis* animals from Bavaria (Grafenwöhr, NEW), two kidney samples allowed the amplification of TULV S segment sequences of 334 and 1,832 nt in length (Table 4).

In addition to the S segment sequences, M segment sequences were obtained by RT-PCR amplification of the region spanning nt 2369 to 3031 (numbering according to PUUV strain Sotkamo, GenBank accession number X61034) for four *M. arvalis* animals, three from Lower Saxony and one from Brandenburg, and five *M. agrestis* animals from Brandenburg.

In summary, TULV S and M segment sequences were detected in both *M. arvalis* and *M. agrestis*.

Phylogenetic analyses of TULV sequences. In contrast to the rodent data, phylogenetic analyses of both the S and M segments revealed very strong geographical but no host-specific affinities of TULV sequences. Phylogenetic trees reconstructed from 80 sequences of a 333-nt-long S segment (nt positions 355 to 686 in TULV strain Lodz-2, GenBank accession number AF063897) demonstrated that the novel TULV sequences from northwest and southwest Brandenburg and Lower Saxony (lineages Germany I and II) were

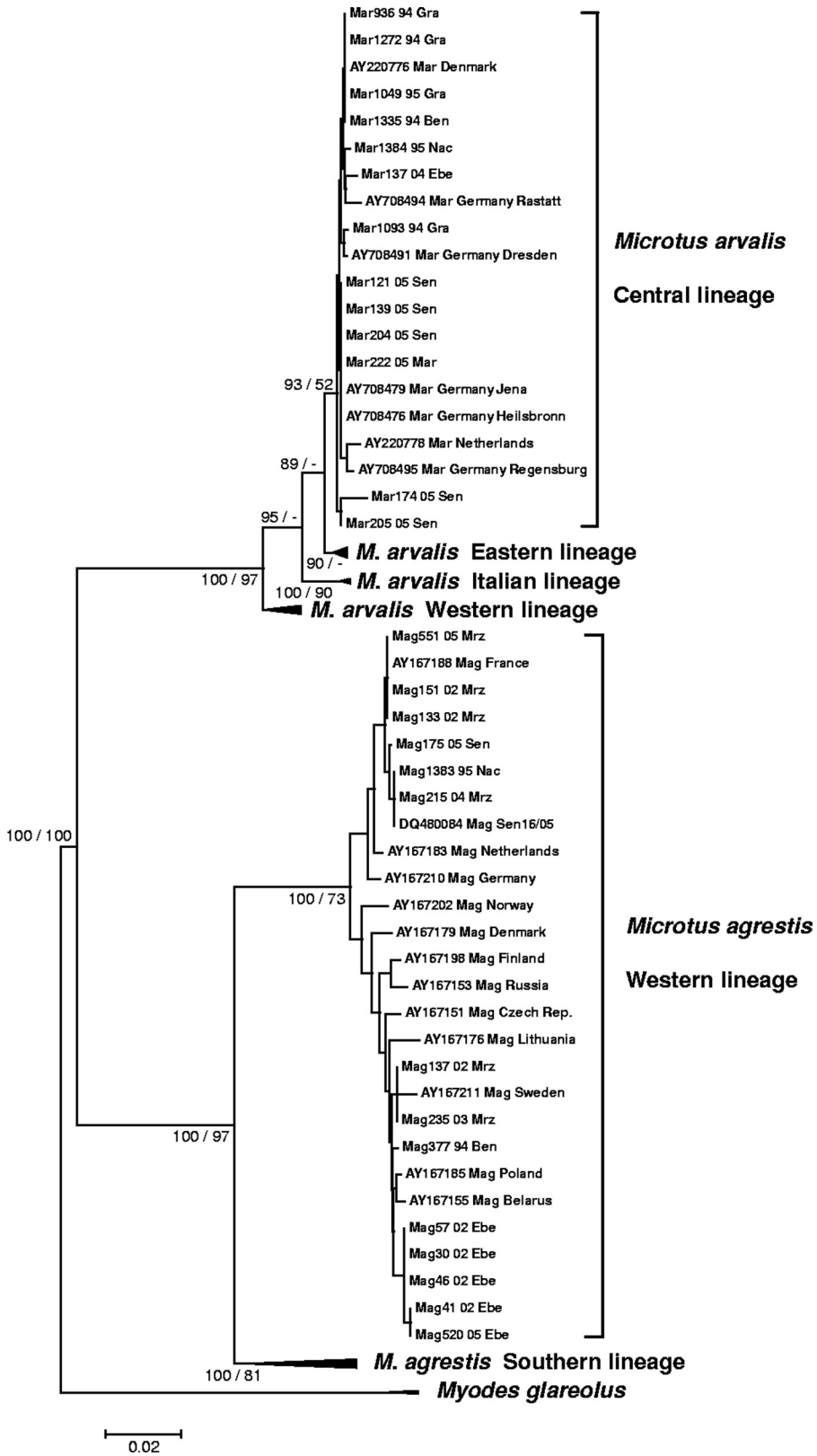

FIG. 2. NJ tree reconstructed from cytochrome *b* DNA sequence data of TULV-positive *M. arvalis* and *M. agrestis*, with two sequences from the closely related *Myodes glareolus* (host of PUUV) as the outgroup. All new sequences from this study (labels beginning with Mar or Mag) belong to the Central lineage in *M. arvalis* (8, 14, 19) or to the Western lineage in *M. agrestis* (25, 26). The robustness of nodes in phylogenetic trees based on NJ and ML algorithms was tested with 5,000 bootstrap replicates each. Only bootstrap values of >50% for main branches connecting major evolutionary lineages are displayed for NJ (before slash) and ML (after slash). Consistent with larger surveys of *M. arvalis* and *M. agrestis*, relationships among sequences within evolutionary lineages are unresolved and phylogenetically unstable due to insufficient variation in cytochrome *b* (for an example, see reference 14). The accession numbers of cytochrome *b* sequences used for comparison are given in Table 8.

TABLE 3. Immunoreactivity of transudates from *Microtus arvalis* and *M. agrestis* in TULV and PUUV IgG ELISA and focus reduction neutralization tests

Rodent no.[a]	Species	Endpoint titer results[b]					
		IgG ELISA		FRNT			
		TULV	PUUV	TULV	PUUV	SAAV	DOBV
Mar1738_95_Gra	*M. arvalis*	**200**	<200	**160**	40	<40	<40
Mar1782_95_Glo	*M. arvalis*	**400**	200	**320**	<40	<40	<40
Mar1831_95_Glo	*M. arvalis*	200	200	**160**	80	40	80
Mar2053_95_Scho	*M. arvalis*	400	400	**320**	40	40	<40
Mar2057_95_Scho	*M. arvalis*	**800**	200	**320**	80	40	<40
Mag377_94_Ben	*M. agrestis*	**3,200**	800	**80**	<40	<40	<40
Mag544_94_Gra	*M. agrestis*	**800**	200	**40**	<40	<40	<40
Mag1383_95_Nac	*M. agrestis*	ND	400	**>1,280**	40	<40	<40
Mag30_02_Ebe	*M. agrestis*	**1,600**	800	**640**	160	<40	<40
Positive control (anti-PUU B.N)	Human	ND	ND	<40	160	<40	<40
Negative control (NHS-007)	Human	ND	ND	<40	<40	<40	<40

[a] The rodent numbers reflect the species (Mar, *M. arvalis*; Mag, *M. agrestis*), the number of the rodent, trapping year, and trapping site in the different districts (Glo, Glöwen, Prignitz; Scho, Schönhagen, Prignitz; Gra, Granzow, Prignitz; Nac, Nackel, Ostprignitz-Ruppin; Ebe, Eberswalde, Barnim; Ben, Bendelin, Prignitz); for geographical localization of trapping sites, see Fig. 1.

[b] The highest endpoint titer for each sample is highlighted in boldface. FRNT, focus reduction neutralization test; SAAV, Saaremaa virus; DOBV, Dobrava virus; ND, not determined.

clearly separated from other German lineages originating from Bavaria, southeast Germany (Germany IV), and southeast Brandenburg (Germany III) (Fig. 3A). A more detailed evaluation of clusters Germany I and II showed a geographical clustering of these sequences irrespective of the rodent host species (Fig. 3B). The sublineage IA from the trapping sites Granzow and Bendelin (district PR) and Nackel (district OPR) was predominantly detected in *M. arvalis* but also in one *M. agrestis* animal. A similar pattern was observed for TULV infections in *Microtus* from SEN (district GÖ; lineage Germany II). In Eberswalde (EBE) (district BAR; sublineage IB), TULV infections were found mostly in *M. agrestis* but also in one *M. arvalis* animal. At the trapping site Marzehns (MRZ) (district PM) in southwest Brandenburg, exclusively TULV-infected *M. agrestis* animals were found between 2002 and 2005, but no TULV-infected *M. arvalis* animal was found (Tables 1, 2, and 4).

The analysis of M segment nucleotide sequences (nt positions 2390 to 3010) (Fig. 4) and corresponding amino acid sequences (amino acid [aa] positions 780 to 985 of GPC in the G2 part) supported the patterns detected in the S segment (data not shown). The novel sequences from Germany were clearly distinct from all M segment sequences published so far. Again, the *M. arvalis*-associated sequence from Mar137_04_EBE (district BAR) grouped together with four *M. agrestis*-associated TULV sequences in the cluster Germany I. Similarly, the Germany II clade consisted of TULV M sequences associated with *M. arvalis* and *M. agrestis*.

Therefore, phylogenetic analysis revealed a geographical, but not host-specific, clustering of TULV S and M segment sequences from two different *Microtus* species. In addition, these investigations showed for the first time that TULV is (probably) able to establish an isolated transmission cycle in *M. agrestis*.

Molecular analysis of S and M segment nucleotide sequences. A pairwise comparison of TULV S and M segment nucleotide sequences from animals within the clusters of TULV revealed a high level of divergence. The levels of the average nucleotide (amino acid) partial S segment sequence divergence in groups Germany I and Germany II were up to 7.2% (0.2%) and 5.4% (0.2%), respectively (Table 5). Similarly, sequences of clusters Russia I, Russia II, and Russia III showed divergence levels of 5.3 to 6.8% (0 to 2.3%). In contrast, the other clusters, including Germany III, Slovakia III/Czech Republic, Austria I and II, Poland, and Slovakia I and II, displayed lower average intercluster divergence levels of 0 to 1.9% (0 to 0.3%).

The divergence level of the S segment nucleotide sequences between the clusters from Germany was surprisingly high (about 12 to 20%) (Table 6). The divergence level of about 18% between sequences from clusters I and II and those from cluster III is particularly important, as all sequences in clusters I (districts OPR, PR, and BAR) and III (district Spree-Neisse [SPN]) and about one-half of the sequences in cluster II (districts PM and OPR) originated from closely related geographical sites in Brandenburg (Fig. 1). The nucleotide sequence divergence level of TULV sequences from other European regions also ranged from 17 to 20%. Interestingly, the amino acid sequence divergence level between sequences from clusters Germany I and II and those from cluster Germany IV was higher than the divergence levels for all other sequences from Europe.

The full-length open reading frame (ORF) of the N protein made up of 430 codons was able to be amplified from four RT-PCR-positive *Microtus* sequences from SEN (district GÖ; SEN 174, 175, 204, and 205) and one from Grafenwöhr (district NEW; AF164093). A putative second ORF encoding a hypothetical nonstructural protein, NSs, made up of 90 aa (corresponding to nt 84 to 356) was determined for these four sequences of *Microtus* from SEN and the one from Grafenwöhr. The entire N-encoding sequence in the samples from Bavaria was 84.2 to 84.6% identical at the nucleotide level and 95.3 to 95.8% identical at the amino acid level to the corresponding sequences from SEN (data not shown). For the N ORF, the nucleotide and amino acid sequences of Mar204_05_SEN, Mar205_05_SEN, and Mar174_05_SEN dif-

TABLE 4. GenBank accession numbers for TULV S and M segment sequences and cytochrome *b* sequences from *Microtus arvalis* and *M. agrestis* from Brandenburg, Lower Saxony, and Bavaria[a]

Rodent no.[b]	District	Species	Sex	Tissue type	Positive S segment result(s) for RT-PCRs[c]	Length of final fragment (nt)	GenBank accession no. for indicated sequences		
							Cytochrome *b*	S segment	M segment
Mar1335_94_Ben	PR	*M. arvalis*	M	Brain	N	287	DQ768133	EF409820	ND
Mag377_94_Ben	PR	*M. agrestis*	M	Lung	P, D	696	DQ662096	DQ662088	ND
Mar1272_94_Gra	PR	*M. arvalis*	M	Brain	N	287	DQ768138	EF409819	ND
Mar936_94_Gra	PR	*M. arvalis*	F	Brain	D, N	624	DQ768136	DQ768137	ND
Mar1093_94_Gra	PR	*M. arvalis*	M	Brain	D, N	624	DQ768134	DQ768135	ND
Mar1049_95_Gra	PR	*M. arvalis*	F	Brain	N	287	DQ768139	EF409818	ND
Mar1384_95_Nac	OPR	*M. arvalis*	F	Brain	D, N	624	DQ768131	DQ768132	ND
Mag1383_95_Nac	OPR	*M. agrestis*	M	Brain	N	287	DQ768140	EF409821	ND
Mag20_97_Grf	NEW	*M. arvalis*	ND	Kidney	S, S1	1832	ND	AF164093	ND
Mag28_97_Grf	NEW	*M. arvalis*	ND	Kidney	D	334	ND	AF164092	ND
Mag30_02_Ebe	BAR	*M. agrestis*	M	Lung	P, D	703	DQ662098	DQ662090	DQ665814
Mag41_02_Ebe	BAR	*M. agrestis*	M	Lung	P, D	703	DQ662099	DQ662091	DQ665815
Mag46_02_Ebe	BAR	*M. agrestis*	M	Lung	P, D	702	DQ662095	DQ662087	DQ665812
Mag57_02_Ebe	BAR	*M. agrestis*	M	Lung	P, D	713	DQ662100	DQ662092	DQ665813
Mag133_02_Mrz	PM	*M. agrestis*	F	Lung	P, D	696	DQ662101	DQ662093	ND
Mag137_02_Mrz	PM	*M. agrestis*	M	Heart	P, D	713	DQ662102	DQ662094	ND
Mag235_03_Mrz	PM	*M. agrestis*	M	Lung	D, N	624	DQ662097	DQ662089	ND
Mag215_04_Mrz	PM	*M. agrestis*	M	Lung/heart	N	287	DQ768145	EF409816	ND
Mar137_04_Ebe	BAR	*M. arvalis*	M	Lung/heart	P, D	725	DQ768142	DQ768143	DQ768144
Mag551_05_Mrz	PM	*M. agrestis*	M	Lung[d]	D, N	624	DQ768147	DQ768148	DQ768149
Mag520_05_Ebe	BAR	*M. agrestis*	M	Lung/heart	N	287	DQ768146	EF409817	ND
Mag175_05_Sen	GÖ	*M. agrestis*	M	Lung	P, D, P5′, P3′	1670	EU439956	EU439949	ND
Mar121_05_Sen	GÖ	*M. arvalis*	F	Lung	P, D, P3′	1347	EU439953	EU439946	ND
Mar139_05_Sen	GÖ	*M. arvalis*	M	Lung	P, D, P3′	1347	EU439954	EU439947	EU439960
Mar174_05_Sen	GÖ	*M. arvalis*	M	Lung	P, D P5′, P3′	1666	EU439955	EU439948	ND
Mar204_05_Sen	GÖ	*M. arvalis*	M	Lung	P, D, P5′, P3′	1700	EU439957	EU439950	EU439961
Mar205_05_Sen	GÖ	*M. arvalis*	M	Lung	P, D, P5′, P3′	1708	EU439958	EU439951	EU439962
Mar222_05_Sen	GÖ	*M. arvalis*	M	Lung	P, D, P3′	1347	EU439959	EU439952	ND

[a] M, male; F, female; ND, not determined.

[b] The rodent numbers reflect the species (Mar, *M. arvalis*; Mag, *M. agrestis*), the number of the rodent, trapping year, and trapping site in the different administrative districts (for geographical localization of trapping sites, see Fig. 1).

[c] Positive reactions in the three different RT-PCRs used are indicated. D, primer pair DOBV-M6/DOBV-M8; P, PUUV 342/cPUUV 1102; N, nested primer PUUV390/cPUUV721; P3′, PUUV1104/cPUUV1758; P5′, PUU_Fpuni/PUUV c740; S1, S1/S10PC; S, SNMa1/MaS4C.

[d] The heart sample from this animal was not reactive in RT-PCR.

fered from 0.1 to 0.5% and 0 to 0.5%, respectively. The sequence divergence of these sequences from *M. arvalis* at the nucleotide and amino acid levels was slightly higher than that of Mag175_05_SEN from *M. agrestis* trapped at the same site and time, with 0.8 to 1.2% and 0.5 to 1.0%, respectively (Tables 5 and 6).

For the partial M segment, within-group calculations resulted in cluster Germany I having an average nucleotide divergence level of up to 3.5% (amino acid divergence level, 0.4%) and in cluster Germany II having an average nucleotide divergence of 4.9% (amino acid divergence, 0%), whereas in clusters Czech Republic and Poland, the average nucleotide divergences were 1.3% (amino acid divergence, 0%) and 0%, respectively (Table 5). The divergence pattern observed for the M segment sequences seems to be comparable to that found for the S segment sequences (Table 7). However, this conclusion does not take into account that different numbers of sequences were available for the various clusters.

As expected, the nucleotide and amino acid sequence divergences of S and M segment sequences from other *Microtus*-associated viruses, i.e., Yakeshi virus, Fusong virus, Vladivostok virus, PHV, ISLAV, and Khabarovsk virus, were found to be much higher.

These investigations revealed a surprisingly high level of nucleotide sequence divergence between the cluster of the novel German TULV sequences and TULV sequences from other European countries.

Comparison of the amino acid sequences of the N and G2 proteins and identification of clade-specific amino acid residue patterns. A comparison of the partial N protein spanning aa 105 to 215 or aa 121 to 215 (according to numbering in the N protein of TULV, strain Moravia, GenBank accession number Z69991) of the novel sequences revealed only three amino acid exchanges, comprising residues with identical (M188L and R213K in Mar1384_95) or similar (A208T in Mag235_03; data not shown) properties.

The observed higher level of amino acid sequence divergence between sequences from clusters Germany I and II and those from cluster Germany IV is reflected also in the amino acid sequence alignment of the N protein between aa residues 231 and 332 (numbering according to TULV reference strain NC_005227), showing the sequences from Germany IV to be much more similar to sequences from Slovakia, Czech Republic, and Croatia (Fig. 5). However, the other TULV sequences from Germany had a more unique amino acid sequence pattern than those from clusters Germany I and II. Interestingly, TULV sequences from trapping sites from the districts BAR (site EBE) and PR/

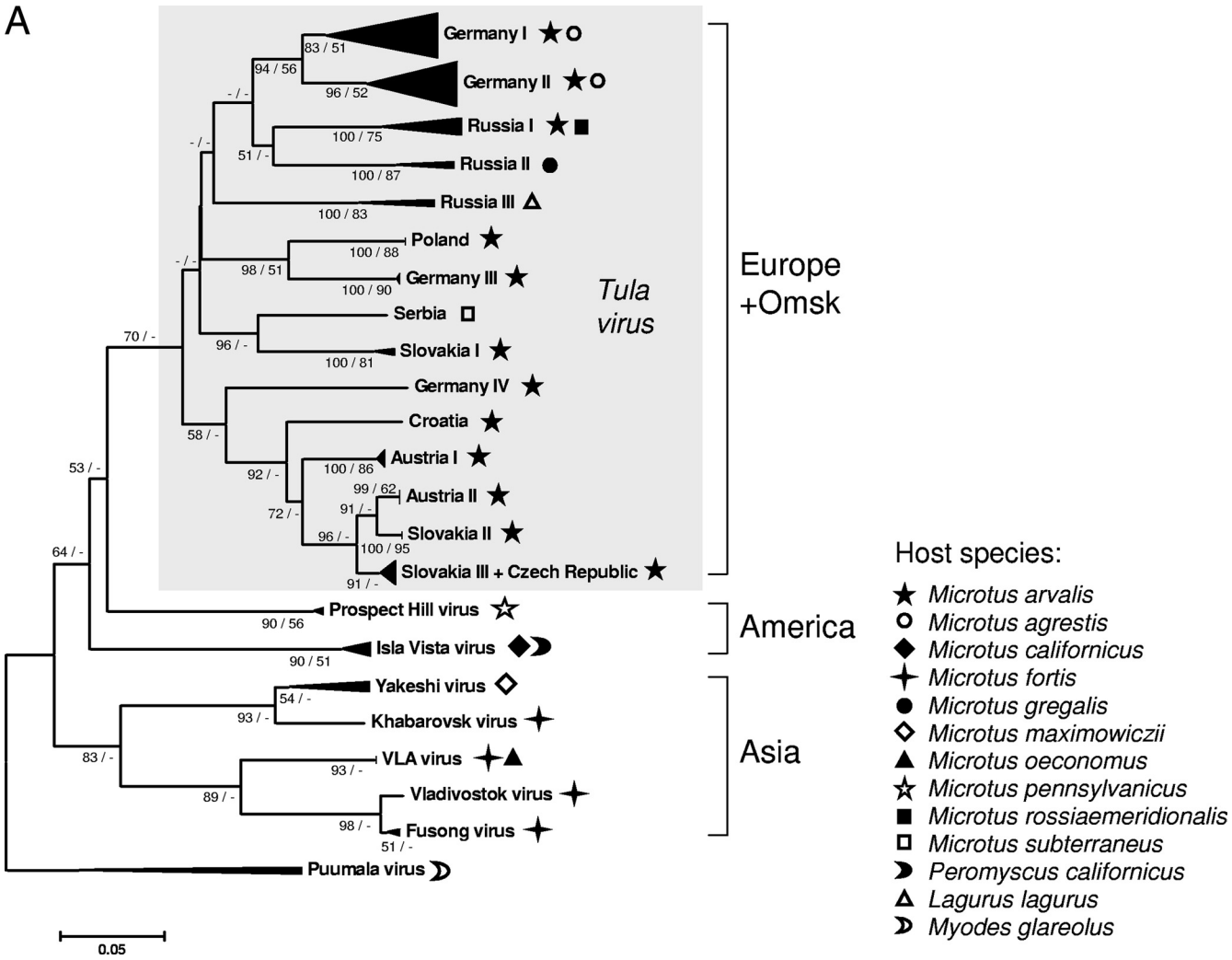

FIG. 3. (A) Phylogenetic tree (NJ tree) based on partial S segment nucleotide sequences of TULV and related viruses and information on their respective rodent hosts. Two partial S segments from PUUV (host, *Myodes glareolus*) were used as outgroups. For visibility only, clusters of phylogenetically closely related sequences were condensed to triangles (size proportional to the number of sequences) in this figure and labeled according to their geographical origin. NJ and ML algorithms were used for tree reconstruction, and the robustness of branching patterns in phylogenies was tested with 5,000 bootstrap replicates each (see Materials and Methods). Bootstrap values of >50% are given before slashes for NJ and after slashes for ML. Details of statistics for the TULV part of the phylogeny are shown in panel B. TULV records are restricted to Europe and the Omsk region in the Asian part of Russia, whereas related viruses in related rodents were detected in America and Asia. (B) The TULV part of the phylogenetic tree with the new Germany I and II clusters in detail are shown. Each cluster comprises closely related TULV lineages from both *M. arvalis* and *M. agrestis* trapped at the same locality. These phylogenetic clusters of sequences from the same locality are well supported compared to the deeper nodes connecting clusters from different regions, which is in agreement with fast evolutionary change in TULV and no recent exchange among regions. The cluster Russia I with sequences from the type locality of TULV shows the same phenomenon in the sibling species *M. arvalis* and *M. rossiaemeridionalis*, whereas the clusters Russia II and III comprise clearly distinct TULV sequences derived from different hosts caught in the same geographical region. The accession numbers of S segment sequences of TULV and other hantaviruses used for comparison are given in Table 9.

OPR (sites Granzow, Bendelin, and Nackel) had unique amino acid residues at positions 248 and 258.

A comparison of the G2 part covering aa positions 780 to 985 of the GPC (numbering in GPC of TULV, strain Moravia, according to GenBank accession number Z66538) confirmed the geographical but not host-specific clustering of TULV sequences from Germany (data not shown). Conservative amino acid exchanges were observed at position 834 with neutral V and I amino acid residues (in both *M. agrestis* and *M. arvalis* from trapping site EBE) and L residues (in both *M. agrestis* and *M. arvalis* from trapping sites MRZ and SEN) and at position 885 with neutral L amino acid residues (in both *M. arvalis* and *M. agrestis* from trapping site EBE as well as in the other five TULV sequences from Europe) and I residues (in both *M. agrestis* and *M. arvalis* from trapping sites MRZ and SEN). Interestingly, at position 834, the other five TULV sequences from Poland, Czech Republic, and Serbia had a conserved V residue. At position 976, all other European TULV strains showed a V residue, whereas the German strains had an I residue.

The amino acid sequence comparison of TULV sequences

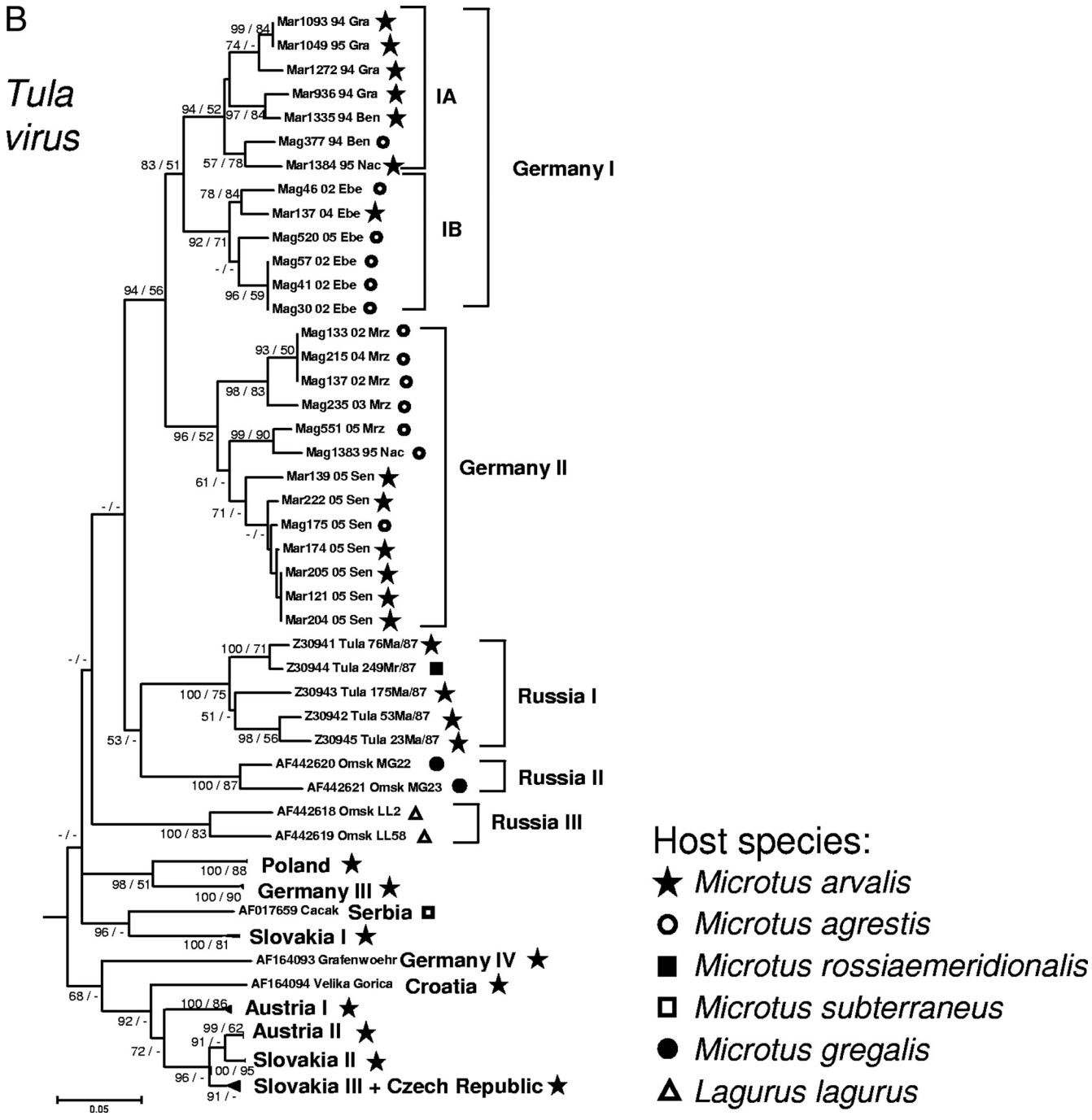

FIG. 3—*Continued*.

from certain sites resulted in the identification of trapping site-specific amino acid signatures.

DISCUSSION

In this study, we demonstrated for the first time that TULV occurs in Germany simultaneously in two different *Microtus* species, *M. arvalis* and *M. agrestis*, which is apparently uncommon for hantaviruses. However, TULV seems to be a very special hantavirus, as it has been found in a large number of different species, including *M. rossiaemeridionalis*, *M. agrestis*, *M. gregalis*, *M. subterraneus*, and *Lagurus lagurus* (43, 56, 65; Dekonenko and Yakimenko, unpublished), after its initial description in *M. arvalis* (43, 61). Similarly, other Old World hantaviruses, like Seoul virus, and various North and South American hantaviruses have been found in multiple rodent reservoir hosts (for reviews, see references 8a and 40a). These observations raise the question whether the occurrence of han-

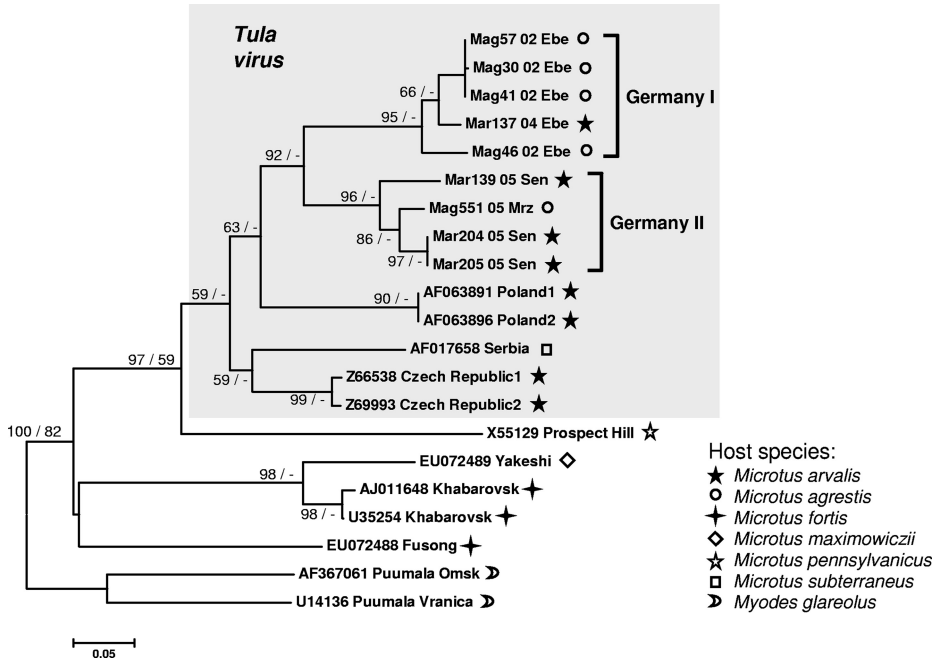

FIG. 4. NJ tree reconstructed from partial M segment nucleotide sequences of 21 TULV and related viruses. Consistent with S segment data, the new TULV sequences from Germany form two clusters according to their geographical location, independent of the respective rodent host species. The robustness of phylogenetic trees resulting from NJ and ML algorithms was tested with 5,000 bootstrap replicates each. Bootstrap values of >50% of nodes are given before slashes for NJ and after slashes for ML. The accession numbers of M segment sequences of TULV and other hantaviruses used for comparison are given in Table 10.

TABLE 5. Intercluster difference in the sequences shown in the phylogenetic trees

Cluster designation[a]	% Intercluster difference for indicated segments[b]			
	Partial S		Partial M	
	266 nt	88 aa	614 nt	206 aa
S				
Germany I (13)	7.2	0.2		
Germany II (13)	5.4	0.2		
Germany III (3)	0.3	0		
Germany IV (1)				
Russia I (5)	6.1	0.9		
Russia II (2)	5.3	2.3		
Russia III (2)	6.8	0		
Poland (2)	0	0		
Slovakia I (3)	1.9	0		
Slovakia II (4)	0	0		
Slovakia III/Czech Republic (9)	1.3	0.3		
Austria I (5)	0.3	0		
Austria II (4)	0	0		
Croatia (1)				
Serbia (1)				
Yakeshi (3)	5.0	0.6		
Fusong (2)	1.1	0.9		
VLA (3)	0	0		
Prospect Hill (2)	0.8	1.9		
Isla Vista (4)	2.2	0.1		
M				
Germany I (5)			3.5	0.4
Germany II (4)			4.9	0
Poland (2)			0	0
Czech Republic (2)			1.3	0
Serbia (1)				
Yakeshi (1)				
Fusong (1)				
Khabarovsk (2)			1.0	0.5
Prospect Hill (1)				

[a] The number of singular sequences for each cluster is shown in parentheses.
[b] Intercluster difference was measured using within-group average calculation with *P* distance models.

taviruses in different related rodent hosts might be a general phenomenon which has frequently been overlooked so far due to the lack of large-scale screenings of sympatrically occurring animals.

This paper also describes the first comprehensive study of the presence of TULV in three different regions of Germany. The initial serological detection of TULV-specific antibodies in *M. arvalis* and *M. agrestis* by ELISA using a novel homologous N antigen was confirmed by RT-PCR investigations targeting the S and M segments. RT-PCR using S segment-specific primers resulted in the detection of TULV RNA not only in lung tissue samples but also in heart and/or kidney tissue samples, which is in line with our previous observations in PUUV-infected bank voles (11). Interestingly, we were able to detect TULV-specific RNA in brain samples of seven animals from which no other tissues were available, even after storage at −20°C for 7 to 10 years. Previously, Black Creek Canal virus, a North American, *Sigmodon hispidus*-transmitted hantavirus causing hantavirus cardiopulmonary syndrome (53), was detected in rodent brain samples (24). The observed presence of TULV and Black Creek Canal virus in the brain of their natural hosts might be explained by crossing of the blood-brain barrier due to infection of newborn animals lacking an intact blood-brain barrier or of animals with pathological changes in the central nervous system microenvironment, resulting in a blood-brain barrier dysfunction. Alternatively, the presence of the virus in the brain might be mediated by infected migrating Trojan horse-like cells, such as monocytes. If crossing of the brain barrier is an outstanding property of TULV, as it is also in terms of host specificity and pathogenicity, the finding of

TABLE 6. Nucleotide and amino acid divergence among clusters of analyzed partial S segments of TULV and closely related hantaviruses[a]

	Germany I (13)	Germany II (13)	Germany III (3)	Germany IV (1)	Russia I (5)	Russia II (2)	Russia III (2)	Poland (2)	Slovakia I (3)	Slovakia II (4)	Slovakia III/ Cz.Rep. (9)	Austria I (5)	Austria II (4)	Croatia (1)	Serbia (1)	Yakeshi (3)	Fusong (2)	VLA (3)	Prospect Hill (2)	Isla Vista (4)
Germany I		12.3±1.6	18.0±2.0	19.8±2.0	17.3±1.9	17.6±1.9	18.2±1.9	19.2±2.1	18.8±2.0	18.7±2.0	18.9±2.0	18.0±2.0	18.5±2.0	19.0±2.0	17.0±1.9	26.9±2.4	27.5±2.5	27.0±2.4	22.7±2.2	26.2±2.3
Germany II	0.2±0.1		18.5±2.1	20.8±2.2	15.4±1.7	17.2±1.9	19.2±2.0	19.3±2.1	17.5±2.1	20.6±2.2	20.5±2.2	19.0±2.2	20.8±2.2	18.0±2.1	17.4±2.1	27.2±2.5	27.7±2.5	30.5±2.6	23.5±2.3	25.7±2.3
Germany III	1.2±1.2	1.2±1.2		20.1±2.2	18.4±2.1	17.7±2.1	16.9±2.1	10.0±1.8	17.9±2.2	18.7±2.3	17.8+2.2	17.2±2.2	18.7±2.3	18.4±2.3	19.9±2.3	26.8±2.5	26.3±2.6	25.4±2.5	22.1±2.4	23.0±2.5
Germany IV	4.6±2.2	4.6±2.2	3.4±2.0		20.2±2.2	19.5±2.1	19.2±2.1	17.7±2.2	17.7±2.2	15.8±2.1	14.6±2.1	14.1±2.1	14.7±2.1	17.7±2.3	18.0±2.2	24.8±2.5	26.9±2.5	25.6±2.4	19.9±2.3	25.2±2.5
Russia I	0.5±0.4	0.5±0.3	1.6±1.2	5.0±2.2		15.6±1.9	18.9±2.1	19.1+2.2	17.7±2.0	21.3±2.2	21.5±2.2	20.4±2.2	20.6±2.2	21.1±2.2	18.9±2.1	28.0±2.5	28.6+2.4	28.0±2.5	24.1±2.3	24.9±2.4
Russia II	2.4±1.3	2.4±1.3	2.8±1.7	4.0±2.1	2.7±1.3		18.2±2.0	18.8±2.3	17.5±2.0	19.4±2.2	19.7±2.2	20.7±2.4	19.0±2.1	19.0±2.2	19.0±2.2	28.8±2.6	27.8±2.6	27.6±2.4	23.1±2.3	22.3±2.3
Russia III	1.2±1.1	1.2±1.1	2.3±1.5	5.7±2.5	1.6±1.2	3.4±1.7		18.4±2.2	20.1±2.2	19.0±2.0	19.1±2.0	19.3±2.1	19.4±2.1	22.4±2.1	19.9±2.2	27.4±2.5	26.7±2.5	24.1±2.4	26.9±2.4	21.9±2.3
Poland	2.4±1.5	2.4±1.5	1.1±1.1	2.3±1.6	2.7±1.6	1.7±1.3	3.4±1.9		18.2±2.3	18.4±2.2	16.0±2.1	16.7±2.1	18.4±2.2	20.3±2.3	17.3±2.1	25.1±2.4	28.2±2.6	25.2±2.5	21.4±2.3	22.0±2.5
Slovakia I	0.1±0.1	0.1±0.1	1.1±1.2	4.5±2.2	0.5±0.3	2.3±1.3	1.1±1.1	2.3±1.5		17.3±2.3	17.0±2.3	17.3±2.2	17.7±2.3	19.4±2.3	11.5±1.9	26.3±2.5	24.4±2.5	26.7±2.5	22.6±2.5	23.0±2.4
Slovakia II	3.5±1.9	3.5±1.9	2.3±1.5	2.3±1.6	3.9±1.9	4.0±2.0	4.5±2.2	2.3±1.5	3.4±1.9		4.4±1.2	9.2±1.7	2.3±0.8	9.8±1.8	18.8±2.2	24.8±2.5	26.9±2.5	24.4±2.4	18.4±2.2	22.8±2.4
Slovakia III/ Cz.Rep.	3.7±1.9	3.7±1.9	2.4±1.5	2.4±1.6	4.0±1.9	4.1±2.0	4.7±2.2	2.4±1.5	3.6±1.9	0.2±0.2		7.2±1.5	3.6±1.0	10.0±1.8	18.2±2.2	25.2±2.5	28.1±2.6	24.8±2.4	18.0±2.2	22.5±2.3
Austria I	3.5±1.9	3.5±1.9	2.3±1.5	2.3±1.6	3.9±1.9	4.0±2.0	4.5±2.2	2.3±1.5	3.4±1.9	0	0.2±0.2		8.5±1.7	10.5±1.9	15.4±2.1	24.4±2.6	28.0±2.6	24.4±2.5	19.0±2.3	25.2±2.6
Austria II	3.5±1.9	3.5±1.9	2.3±1.5	2.3±1.6	3.9±1.9	4.0±2.0	4.5±2.2	2.3±1.5	3.4±1.9	0	0.2±0.2	0		9.4±1.7	18.0±2.2	25.1±2.6	28.8±2.6	24.8±2.4	16.9±2.1	23.3±2.4
Croatia	3.5±1.9	3.5±1.9	2.3±1.5	2.3±1.6	3.9±1.9	4.0±2.0	4.5±2.2	2.3±1.5	3.4±1.9	0	0.2±0.2	0	0		18.8±2.3	26.1±2.6	30.3±2.7	27.4±2.6	18.8+2.2	23.4±2.5
Serbia	0.1±0.1	0.1±0.1	1.1±1.2	4.5±2.2	0.5±0.3	2.3±1.3	1.1±1.1	2.3±1.5	0	3.4±1.9	3.6±1.9	3.6±1.9	3.6±1.9	3.4±1.9		24.9±2.4	23.7±2.5	26.3±2.4	20.3±2.3	24.3±2.6
Yakeshi	14.1±3.8	14.1±3.8	15.2±3.9	16.3±4.1	14.5±3.8	16.3±4.0	14.0±3.8	16.3±4.1	14.0±3.8	15.2±3.9	15.2±3.9	15.2±3.9	15.2±3.9	15.2±3.9	14.0+3.8		21.1±2.3	20.9±2.2	23.6±2.4	25.4±2.4
Fusong	14.3±3.8	14.3±3.8	15.3±3.9	16.5±4.1	14.7±3.8	16.5±4.0	14.2±3.8	16.5±4.1	14.2±3.8	15.3±4.0	15.3±4.0	15.3±4.0	15.3±4.0	15.3±4.0	14.2±3.8	2.1±1.3		12.6±2.0	23.5±2.6	26.8±2.5
VLA	13.7±3.8	13.7±3.8	14.8±3.9	15.9±4.1	14.1±3.8	15.9±4.0	13.6±3.8	15.9±4.1	13.6±3.8	14.8±3.9	14.8±3.9	14.8±3.9	14.8±3.9	14.8±3.9	13.6±3.8	1.5±1.1	0.6±0.6		22.2±2.4	24.7±2.5
Prospect Hill	11.5±3.2	11.5±3.2	10.2±3.1	11.4±3.4	11.8±3.2	13.1±3.5	11.4±3.3	11.4±3.4	11.4±3.3	10.2+3.2	10.2+3.2	10.2+3.2	10.2+3.2	10.2+3.2	11.4±3.3	7.8±2.8	8.0±2.9	7.4±2.8		21.7±2.3
Isla Vista	7.5±2.6	7.5±2.6	8.5±2.8	9.1±3.0	7.8±2.7	9.1±2.8	7.4±2.7	9.1±2.9	7.4±2.7	8.2±2.8	8.2±2.8	8.2±2.8	8.2±2.8	8.2±2.8	7.4±2.7	10.0±3.2	10.2±3.2	9.7±3.2	8.0±2.7	

[a] Nucleotide divergence, above diagonal; amino acid divergence, below the diagonal; analyzed partial S segments of TULV, boxed. The number of singular sequences for each cluster is shown in parentheses. The number of base differences and standard error estimates (500 bootstrap replicates) per site from averaging all sequence pairs among groups are hidden. All results are based on the pairwise analysis of 76 sequences, with 266-nt positions corresponding to 88 aa in the final data set.

TULV RNA in the brain should be proven in a comparative study of different tissue samples from a larger number of voles.

Phylogenetic analyses of the novel S and M segment sequences indicated that these *Microtus*-borne sequences belong to TULV and are clearly separated from sequences originating from other *Microtus*-borne viruses such as PHV, ISLAV, Yakeshi virus, Khabarovsk virus, VLA virus, Vladivostok virus, and Fusong virus.

The high nucleotide divergence level of 12 to 20% among the three novel TULV clades from northeast, northwest, and southeast Germany, the already known clade from central east Germany (32), and clades from other European countries, as well as the Omsk region in the Asian part of Russia, is remarkable. This high level of sequence divergence was even observed among sequences from trapping sites that are only about 200 km or less apart from each other, i.e., in the districts SPN (clade Germany III) (32) and PM (clade Germany II). In contrast to the high level of nucleotide sequence divergence, the level of amino acid sequence divergence was much lower, e.g., only about 1% between clades Germany II and III. This is consistent with strong purifying selection. Taken together, these results showed the presence of at least four clades of TULV sequences in Germany, suggesting a quickly evolving virus species with a strong genetic substructure.

The detection of a large number of novel sequences forming clusters in the phylogenetic tree makes an analysis of the intercluster differences possible. The observed levels of intercluster differences of 5.4 and 7.2% for the novel S segment sequences and 3.5 and 4.9% for the novel M segment sequences of clades Germany I and Germany II, respectively, are in a similar range as those observed for TULV clades Russia I, II, and III. A previous study revealed a range of diversity of 1.5 to 4.9% for the S segment and 0.2 to 1.2% for the M segment between TULV strains circulating within a location of 20 km (43). It is interesting to note that the intercluster differences for 22 partial S segment PUUV sequences derived from bank voles from the city of Cologne, Germany, and for 10 partial S segment PUUV sequences from Lower Bavaria, southeast

TABLE 7. Nucleotide and amino acid divergence among clusters of analyzed partial M segments of TULV and related hantaviruses[a]

	Germany I (5)	Germany II (4)	Poland (2)	Czech R. (2)	Serbia	Yakeshi	Fusong	Khabarovsk (2)	Prospect Hill
Germany I		15.1±1.2	18.1±1.4	17.2±1.4	20.1±1.5	27.9±1.9	26.1±1.6	27.0±1.8	25.6±1.6
Germany II	1.1±0.7		17.4±1.4	18.8±1.4	17.8±1.5	27.6±1.8	23.9±1.6	25.5±1.7	24.7±1.7
Poland	1.5±0.8	2.0±0.9		17.8±1.5	18.1±1.5	26.1±1.8	23.9±1.7	26.3±1.8	23.5±1.6
Czech Republic	1.0±0.6	1.5±0.8	0.5±0.5		13.7±1.3	26.3±1.8	25.5±1.7	23.8±1.7	21.9±1.7
Serbia	1.5±0.7	2.0±0.9	1.0±0.6	0.5±0.5		26.5±1.8	24.4±1.7	26.9±1.8	21.8±1.6
Yakeshi	18.1±2.7	18.1±2.7	17.6±2.7	17.6±2.7	18.1±2.7		24.6±1.7	9.9±1.2	27.7±1.7
Fusong	14.2±2.3	14.2±2.3	15.2±2.3	14.7±2.3	15.2±2.4	10.3±2.2		22.8±1.6	27.7±1.8
Khabarovsk	17.4±2.7	17.4±2.7	16.9±2.6	16.9±2.6	17.4±2.7	1.7±0.9	9.6±2.1		26.6±1.8
Prospect Hill	15.6±2.5	15.7±2.5	14.7±2.5	14.7±2.5	14.2±2.4	19.6±2.9	18.1±2.7	18.9±2.8	

[a] Nucleotide divergence, above diagonal; amino acid divergence, below the diagonal; analyzed partial M segments of TULV, boxed. The number of singular sequences for each cluster is shown in parentheses. The number of base differences and standard error estimates (500 bootstrap replicates) per site from averaging all sequence pairs among groups are indicated. All results are based on the pairwise analysis of 76 sequences, with 614-nt positions and 206 aa in the final data set.

Germany, were only 1.2% and 3.1%, respectively (11, 12). Compared to these studies, the intercluster sequence differences of TULV clusters Germany I and II were doubled.

In contrast to previous investigations (45), no indication of a quasispecies population in the investigated *Microtus* animals has been obtained. The novel nucleotide sequence from Bavaria (Germany IV) clustered with nucleotide sequences from Austria, Slovakia, Czech Republic, and Croatia, and the previously described sequences from cluster Germany III grouped with sequences from Poland. Although the novel sequences represented by clusters Germany I and Germany II did not cluster with sequences from other parts of Europe in the phylogenetic tree, a multiple amino acid sequence alignment reflected some similarities in the amino acid sequences between the novel strains (clades Germany I and II) and those from clades Germany III and Poland (Fig. 5).

Phylogenetic reconstructions of rodent host relationships based on the mitochondrial cytochrome *b* gene showed a clear differentiation between *M. arvalis* and *M. agrestis*. This is in accord with the current taxonomy of *Microtus* (16). In both species, separation of populations during glacial cycles has led to intraspecific genetic divergence into several evolutionary lineages, which nowadays occupy large regions in Central Europe which do not overlap (19). These evolutionary lineages of *M. arvalis* and *M. agrestis* can be identified by phylogenetic analysis of the cytochrome *b* gene. The new sequences of *M. arvalis* presented here cluster clearly within the Central lineage, an old evolutionary lineage which currently inhabits most of Central Europe (Germany, Denmark, The Netherlands, and Switzerland) (19). Variation in cytochrome *b* was not high enough for a further geographical resolution among rodents at the regional level. All new sequences are distinct from the

TABLE 8. Cytochrome *b* sequences of *Microtus arvalis* and *M. agrestis* of different genetic lineages[a]

Lineage/outgroup	Strain	GenBank accession no.
M. arvalis Central lineage	Denmark	AY220776
	Germany Rastatt	AY708494
	Germany Dresden	AY708491
	Germany Jena	AY708479
	Germany Heilsbronn	AY708476
	Netherlands	AY220778
	Germany Regensburg	AY708495
M. arvalis Eastern lineage	Hungary	AY220769
	Slovakia	AY220767
	Austria	AY708460
	Czech Republic	AY708471
	Poland	AY220773
	Russia	AY220771
M. arvalis Italian lineage	Switzerland1	AY708512
	Switzerland2	AY708513
	Italy	AY220766
M. arvalis Western lineage	Spain1	AY220788
	Spain2	AY708502
	Belgium	AY708508
	France1	AY220787
	France2	AY708511
M. agrestis Western lineage	France	AY167188
	Mag Sen16/05 Sennickerode Germany	DQ480084
	Netherlands	AY167183
	Germany	AY167210
	Norway	AY167202
	Denmark	AY167179
	Finland	AY167198
	Russia	AY167153
	Czech Republic	AY167151
	Lithuania	AY167176
	Sweden	AY167211
	Poland	AY167185
	Belarus	AY167155
M. agrestis Southern lineage	Switzerland2	AY167161
	Switzerland1	AY167160
	Spain2	AY167163
	Spain1	AY167162
	Portugal	AY167186
M. glareolus outgroup	*M. glareolus* Omsk	AF367079
	M. glareolus Slovenia	AJ867953

[a] The different genetic lineages are included in the phylogenetic analysis shown in Fig. 2.

Eastern lineage of *M. arvalis*. The western border of this lineage is in Poland and the Czech Republic (19). All new cytochrome *b* sequences of *M. agrestis* clustered with the Western lineage of this species. The Western lineage in *M. agrestis* has a very large distribution range, encompassing most of Western, Central, and Eastern Europe and Scandinavia (25, 26). The absence of a substructure within the Western lineage is consistent with detailed analyses of cytochrome *b* in *M. agrestis* (25). Dedicated analyses of more variable genetic markers are needed to resolve the finer genetic structures that exist among local populations within evolutionary lineages of voles (8, 13, 19, 60). However, movements of individual *Microtus* animals are unlikely to exceed a few kilometers (60). The clustering of TULV strains according to locality within the clades Germany I and II is in accord with relatively low levels of migration among regional *Microtus* populations.

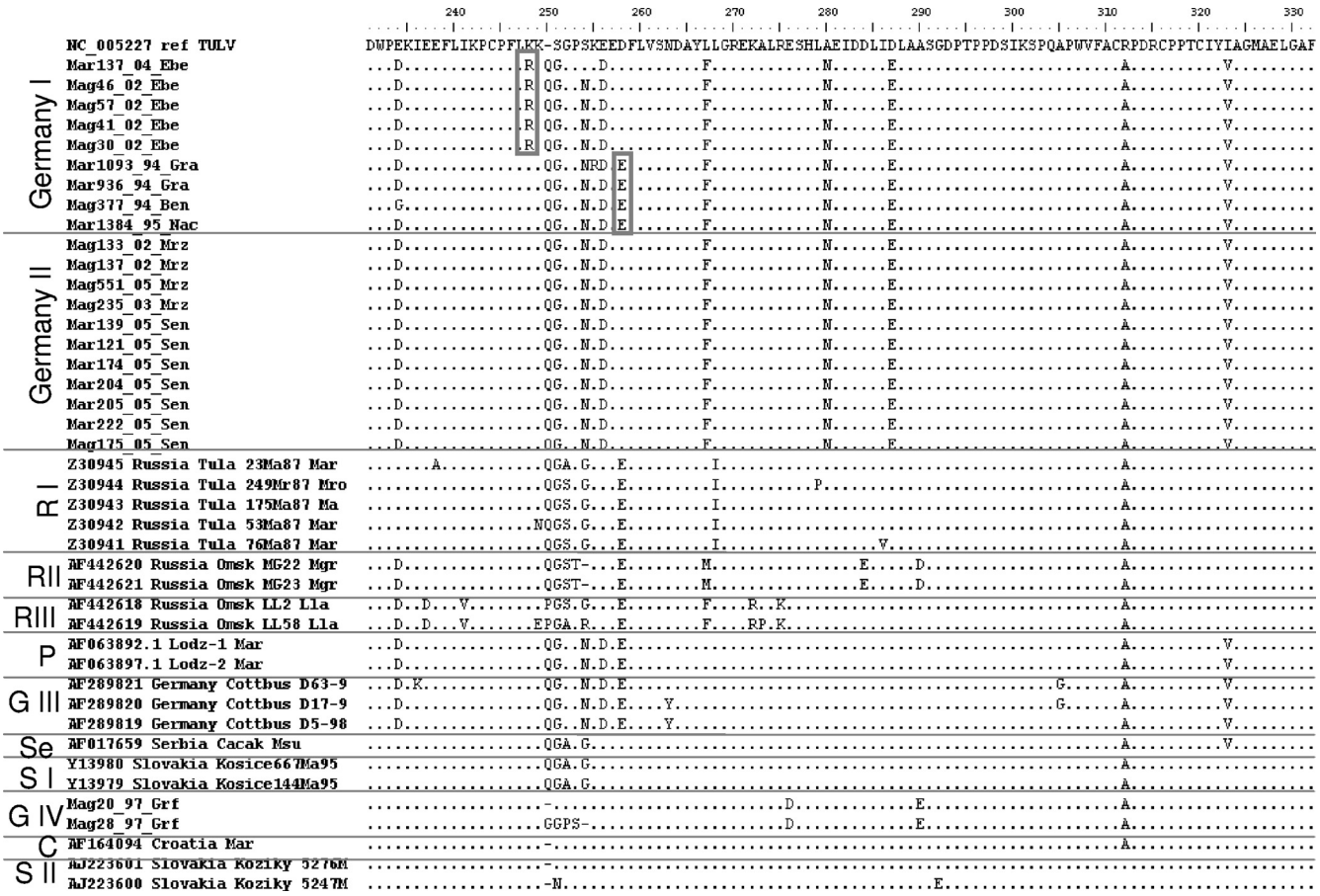

FIG. 5. Hypervariable region of the predicted nucleocapsid proteins of TULV (aa 231 to 332). TULV strain NC_005227 (71) was used as the leader strain for an amino acid alignment. Sequences were aligned in clusters according to the S segment tree in Fig. 3B, and identical amino acid residues in German subclusters are boxed. Amino acid sequences of TULV strains from clusters Austria I and II, Slovakia II, and Slovakia III/Czech Republic are identical to NC_005227 and not included in the alignment. Abbreviations: R I, Russia I; RII, Russia II; RIII, Russia III; P, Poland; G III, Germany III; Se, Serbia; S I, Slovakia I; G IV, Germany IV; C, Croatia; S II, Slovakia II.

The detection of naturally TULV-infected *M. agrestis* contrasts with a previous study, where attempts to infect this rodent species with TULV failed (33). The failure in this experimental setting might have been due to the use of a cell culture-adapted TULV (33), as previously observed for PUUV (39). In addition, the TULV strain Moravia used for the experimental infections contains a stop codon in the putative NSs ORF (71), which may reduce its potential activity as an interferon antagonist (28, 29). Interestingly, in our field study, TULV infections in *M. arvalis* were found at a similar frequency in male and female animals, whereas *M. agrestis* TULV-positive males were significantly more frequent than *M. agrestis* TULV-positive females. This might indicate gender-dependent limitations for the transmission of TULV by spillover infections from *M. arvalis* or by horizontal transmission between *M. agrestis* individuals mediated by gender-specific differences in the TULV-specific immune response, as previously demonstrated for PUUV patients (34). Further studies are required to prove whether differences in the immune response are responsible for the clearance or the establishment of a persistent infection in spillover-infected *M. agrestis*. Additionally, species-, age-, and gender-specific differences in behavior, such as aggression, territoriality, or social status, may contribute to the detected differences in the prevalences. Males tend to be more mobile and aggressive than females in many *Microtus* species (see reference 60 and references therein), which may increase the risk of infection for males overall. However, comparative analyses of space use and social behavior of *M. arvalis* and *M. agrestis* living in sympatry are needed to clarify whether these factors could cause gender-specific differences in infection rates or prevalences between species. In line with previous studies for other hantaviruses (for a review, see reference 39a), our investigations demonstrated a positive association of the age and seropositivity for *M. agrestis*.

Paleozoological and molecular investigations suggest a last common ancestor of *M. arvalis* and *M. agrestis* more than 0.5 million years ago (16, 27). Following the coevolution hypothesis, this ancient separation of the *Microtus* species should have resulted in very distinct genetic lineages of hantaviruses associated with *M. arvalis* and *M. agrestis*. Indeed, the coexistence of two different virus lineages has been described for Dobrava-Belgrade virus in natural foci with sympatric populations of *Apodemus agrarius* and *Apodemus flavicollis* in Slovenia and Slovakia (5, 63). Similar observations were made for different

TABLE 9. TULV S segment sequences included in the phylogenetic analysis[a]

Hantavirus lineage	Strain	GenBank accession no.
TULV Russia I	Tula 76Ma/87	Z30941
	Tula 53Ma/87	Z30942
	Tula 175Ma/87	Z30943
	Tula 249Mr/87	Z30944
	Tula 23Ma/87	Z30945
TULV Russia II	Omsk MG22	AF442620
	Omsk MG23	AF442621
TULV Russia III	Omsk LL2	AF442618
	Omsk LL58	AF442619
TULV Poland	Poland Lodz-1	AF063892
	Poland Lodz-2	AF063897
TULV Germany III	Germany Cottbus D5-98	AF289819
	Germany Cottbus D17-98	AF289820
	Germany Cottbus D63-98	AF289821
TULV Serbia	Cacak	AF017659
TULV Slovakia I	Slovakia Kosice 144/Ma/95	Y13979
	Slovakia Kosice 667/Ma/95	Y13980
TULV Croatia	Velika Gorica	AF164094
TULV Austria I	Austria O24	U95302
	Austria O52	U95303
	Austria O20	U95304
	Austria O64	U95309
	Austria O8	U95313
TULV Austria II	Austria K11	U95305
	Austria K58	U95311
	Austria K26	U95310
	Austria K64	U95312
TULV Slovakia II	Slovakia Malacky Ma32/94	Z48235
	Slovakia Malacky Ma370/94	Z68191
TULV Slovakia III and Czech Republic	Moravia5302v95	Z69991
	Moravia 5294Ma94	Z48741
	Moravia 5302Ma94	Z49915
	Moravia 5286Ma94	Z48573
	Moravia 5293Ma94	Z48574
	Slovakia Koziky 5247Ma/94	AJ223600
	Slovakia Koziky 5276Ma/94	AJ223601
PHV	Prospect Hill	M34011
	Prospect Hill PH-1	Z49098
ISLAV	Isla Vista Mca MC-SB-1	U31534
	Isla Vista MC-SB-47	U19302
	Isla Vista PC-SB-77	U31535
	Isla Vista PC-SB-46	U31530
Yakeshi virus	Yakeshi-Mm-182	EU072484
	Yakeshi-Mm-59	EU072483
	Yakeshi-Mm-31	EU072482
Khabarovsk virus	Khabarovsk	KHU35255
VLA virus	VLA/Nesterikha/Mf500/2005	AM930974
	VLA/Barguzin/Mo483/2005	AM930973
Vladivostok virus	Vladivostok	AB011630
Fusong virus	Fusong-Mf-731	EU072481
	Fusong-Mf-682	EU072480
PUUV	PUUV CRF366 Omsk	AF367071
	PUUV Balkan-1	AJ314600

[a] The phylogenetic analysis is shown in Fig. 3.

TABLE 10. TULV M segment sequences included in the phylogenetic analysis[a]

Virus lineage	Strain	GenBank accession no.
TULV-Poland1	Poland1	AF063891
TULV-Poland2	Poland2	AF063896
TULV-Serbia	Serbia	AF017658
TULV-Czech Republic1	Czech Republic1	Z66538
TULV-Czech Republic2	Czech Republic2	Z69993
PHV	Prospect Hill	X55129
Yakeshi virus	Yakeshi	EU072489
Khabarovsk virus	Khabarovsk	AJ011648
	Khabarovsk	U35254
Fusong virus	Fusong	EU072488
PUUV	PUUV Omsk	AF367061
	PUUV Vranica-Hällnäs	U14136

[a] The phylogenetic analysis is shown in Fig. 4.

New World hantaviruses (for a review, see reference 46). However, there is increasing evidence that besides a general coevolution of the reservoir host and the associated hantavirus species, host switch events might have been played an important role in hantavirus evolution. Such a host switch event has been postulated for the Arvicolinae-associated Khabarovsk virus (72). We found a clustering of TULV sequences depending on the geographical origin but not on the sympatrically occurring *M. arvalis* and *M. agrestis* hosts. In addition, our data may indicate an ongoing process of establishing *M. agrestis* as a novel reservoir host for TULV. These data thus indicate that spillover infections are less rare than believed so far. Moreover, our finding of TULV infections in *M. agrestis* at different time points without any presence of *M. arvalis* might even suggest an already established isolated replication and transmission cycle of TULV in *M. agrestis*. Taken together, these findings and the recent detection of novel hantaviruses in shrews and moles in different parts of the world suggest a richer evolutionary history and more complex transmission dynamics of hantaviruses (2, 20).

The potential for spillover infections between host species depends for instance on the frequency of cooccurrence determined by the specific ecological preferences. *M. arvalis* and *M. agrestis* both have very large distribution ranges in Europe, with regions of overlap extending from Spain into Russia (40). In many regions, *M. arvalis* prefers somewhat drier and more open habitats than *M. agrestis*, but cooccurrence is relatively common (18, 40, 41). Our trapping results confirmed the sympatric occurrence of *M. arvalis* and *M. agrestis* in exactly the same habitats at different places in northeast and northwest Germany. Information on the frequency and nature of interspecific interactions in the field is lacking for these species and other small rodents, but territoriality and the establishment of kin associations suggest aggressive interactions, with biting and scratching as the most likely route of transmission between species (7, 60).

In conclusion, this paper demonstrates that TULV is a promiscuous virus able to infect different *Microtus* species, including *M. arvalis*, *M. agrestis*, *M. rossiaemeridionalis*, *M. gregalis*, *M. subterraneus*, and other related species such as *Lagurus lagurus*. Moreover, initial evidence at one trapping site in Brandenburg suggests that TULV not only causes multiple spillover infections of *M. agrestis* but also seems to establish an isolated replication and transmission cycle in this putative novel reservoir host. Although we cannot rule out coevolutionary mechanisms, the observations described here may be interpreted against the background of the following two alternative evolution mechanisms for hantaviruses. (i) After initial multiple spillover infections of a hantavirus, e.g., TULV, from the established host to a sympatrically occurring potential novel host

in different geographical regions, host adaptation of the virus in the novel host at different geographical localizations may result in a convergent evolution. This would then lead to a change from a geographical clustering to a host-specific clustering of the hantavirus sequences. Therefore, the finally observed host-specific clustering of hantavirus sequences might be, under certain circumstances, misinterpreted as a coevolution mechanism. In line with this assumption, recent studies have postulated that similarities between the phylogenies of hantaviruses and their hosts also seem to result from preferential host switching and local host-specific adaptation (49). (ii) The geographical clustering of hantavirus, e.g., TULV, sequences might have been caused by an isolation-by-distance mechanism. If this hypothesis is true, one would postulate that the viruses are less adapted to their rodent host, e.g., representatives of the genus *Microtus*, allowing frequent spillover or host switch events in overlapping rodent populations. This might be supported by a strong similarity of host receptor molecules that may have evolved slowly since the separation of the different species in a rodent genus, e.g., *Microtus*.

Future investigations on different sympatrically occurring *Microtus* species should address the frequency of spillover and host switch events for TULV and may thus allow for the definition of the host range of TULV and its viral and host determinants. Similar investigations of other hantaviruses and their putative rodent or insectivore hosts should greatly improve our current knowledge on the molecular evolution and host adaptation of hantaviruses.

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