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ORIGINAL ARTICLE

High genetic structuring of Tula hantavirus

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Abstract Tula virus (TULV) is a vole-associated hantavirus with low or no pathogenicity to humans. In the present study, 686 common voles (*Microtus arvalis*), 249 field voles (*Microtus agrestis*) and 30 water voles (*Arvicola* spec.) were collected at 79 sites in Germany, Luxembourg and France and screened by RT-PCR and TULV-IgG ELISA. TULV-specific RNA and/or antibodies were detected at 43 of the sites, demonstrating a geographically widespread distribution of the virus in the studied area. The TULV prevalence in common voles (16.7 %) was higher than that in field voles (9.2 %) and water voles (10.0 %). Time series data at ten trapping sites showed evidence of a lasting presence of TULV RNA within common vole populations for up to 34 months, although usually at low prevalence. Phylogenetic analysis demonstrated a strong genetic structuring of TULV sequences according to geography and independent of the rodent species, confirming the common vole as the preferential host, with spillover infections to co-occurring field and water voles. TULV phylogenetic clades showed a general association with evolutionary lineages in the common vole as assessed by mitochondrial DNA sequences on a large geographical scale, but with local-scale discrepancies in the contact areas.

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Introduction

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) were initially thought to be exclusively rodent-borne pathogens hosted by representatives of the families Muridae and Cricetidae [1]. The recent finding of novel hantaviruses in insectivores such as shrews and moles as well as bats raises questions about the origin and evolution of this group of viruses [2, 3].

Rodent-borne hantaviruses can cause two types of disease in humans, hantavirus cardiopulmonary syndrome (HCPS) and haemorrhagic fever with renal syndrome (HFRS). HCPS due to infection by New World hantaviruses, e.g., Sin Nombre virus (SNV) and Andes virus (ANDV), is associated with an average case fatality rate of about 40 % [4]. In Europe, hantaviruses are emerging pathogens with an increasing significance for human health [5], causing HFRS with differing case fatality rates, ranging from less than 1 % to 16 % [4, 6].

Hantaviruses associated with members of the vole genus *Microtus* have been detected in several parts of Europe, Asia and the North American continent. The North American California vole (*Microtus californicus*), meadow vole (*M. pennsylvanicus*) and prairie vole (*M. ochrogaster*) are known to harbour Isla Vista virus (ISLAV), Prospect Hill virus (PHV) and Bloodland Lake virus (BLLV), respectively [7]. In the Asian part of Russia and in China, three different hantaviruses have been detected in *Microtus* voles, with one species sometimes hosting more than one hantavirus: Khabarovsk virus (KHAV) was found in *M. fortis* and *M. maximowiczii*, Vladivostok virus (VLAV) in *M. fortis* and *M. oeconomus*, and Yuangjiang virus (YUJV) in *M. fortis* [8–12]. In Europe, two *Microtus*-associated hantaviruses have been described. Tula virus (TULV) was initially detected in common voles (*M. arvalis*) and sibling voles (*M. levis,* formerly *rossiaemeridionalis*) [13, 14]. A highly divergent TULV strain, designated as Adler virus, was recently discovered in common voles in Russia [15]. A second hantavirus, Tatenale virus, was found in field voles (*M. agrestis*) in England [16].

Like in all hantaviruses, the genome of TULV is partitioned into three segments: For TULV prototype strain Moravia, the small (S) segment, 1,831 nucleotides (nt) in length, codes for the nucleocapsid (N) protein, the medium-sized (M) segment of 3,694 nt codes for two glycoproteins (Gn und Gc), and the large (L) segment of 6,541 nt codes for the viral RNA-dependent RNA polymerase [17–19]. In addition, the S segment of all vole-derived TULV strains contains an overlapping open reading frame (ORF) coding for a putative nonstructural protein (NSs) that has been shown to enhance survival of the virus during passaging in interferon-competent cells [20, 21].

There is little knowledge about the pathogenicity of TULV for humans. TULV-specific antibodies have been detected in healthy blood donors in the Czech Republic [19] and in German forestry workers, a potential risk group for hantavirus infections [22]. Furthermore, one HFRS patient from Germany had TULV-specific neutralizing antibodies [23]. In addition, TULV RNA was detected in EDTA blood of an acutely infected, immunocompromised patient in the Czech Republic [24].

Corresponding to the wide distribution range of its main host, the common vole, TULV-specific nucleic acid has been detected in several European countries, but these studies usually included only one or a few trapping sites in a specific region (see Ref. [14] and [25–30] and references therein). Whereas most hantaviruses are host-specific and natural spillover infections are only rarely reported, TULV has been molecularly detected in a wide variety of other Arvicolinae species: *M. levis, M. gregalis*, *M. subterraneus*, *M. agrestis*, *Lagurus lagurus,* and *Arvicola* spec. [13, 31–35]. Aside from common voles, the most TULV infections have been reported in field voles (*M. agrestis*) in some places even without the presence of the main host species or with a larger number of infected field voles compared to sympatric common voles. It was therefore speculated that rather than being spillover infected, field voles might represent another host species that can enable successful TULV replication [29, 34].

The evolution of hantaviruses in relation to their rodent hosts is controversial. Often, the divergence of hantaviruses and their rodent hosts is interpreted as a consequence of co-evolutionary processes. Alternatively, host switching and subsequent adaptation processes are thought to be a reason for the observed divergence patterns [2, 7, 34, 36–38]. Previous investigations were mainly focused only at the species level of the reservoir, but co-evolutionary processes may also occur at the level of evolutionary divergence within species [7, 29, 34, 35, 37]. In Central and Eastern Europe, four main evolutionary lineages of common voles have been identified with both autosomal and mitochondrial DNA markers: the Western, Central, Italian and Eastern lineages [39–41]. So far, nothing is known about potential co-divergence between TULV and evolutionary lineages of the common vole.

Here, we describe large-scale serological and RT-PCR TULV screening of three potential reservoir species from

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Central and Western Europe, as well as phylogenetic analysis of TULV sequences and mitochondrial DNA sequences of common voles.

Materials and methods

Rodent trapping and dissection

Rodents were collected between 2004 and 2013 at 71 sites in 13 federal states of Germany, two sites in Luxembourg, and six sites in two administrative districts in France (Alsace and Midi-Pyrénées) in woodland and open field habitats (Fig. 1). Trapping of some of the animals has been described previously [42–45]. Rodent dissection was done according to standard protocols and resulted in the collection of heart, lung, liver, spleen, kidney, and brain, as well as tissue samples from the ear pinna and tail. Chest cavity fluid (CCF) was obtained by adding 1 ml of PBS [46]. For TULV-positive animals, TULV-negative animals from selected sites, and individuals with inconclusive morphological species identification, mitochondrial *cytochrome b* (*cyt b*) sequences were determined [47]. For samples that did not allow a morphological identification, a sex-determination PCR was performed following established protocols [48, 49].

Nucleic acid isolation

RNA extraction was performed using a modified QIAzol protocol. Briefly, RNA extraction was performed using 1 ml of QIAzol® Lysis Reagent (QIAGEN, Hilden, Germany) and sterilized steel beads of 0.5 cm diameter (Isometall, Pleidelsheim, Germany). After tissue homogenization, 200 µl of chloroform was added, and the sample was mixed and thereafter centrifuged for 15 min at 4 °C and 11,900 *g*. The resulting supernatant was mixed with 500 µl of cold isopropanol (-20 °C), incubated at -20 °C for 20 min, and centrifuged again at 4 °C for 10 min at 11,900 *g*. The resulting pellet was washed once with 1 ml

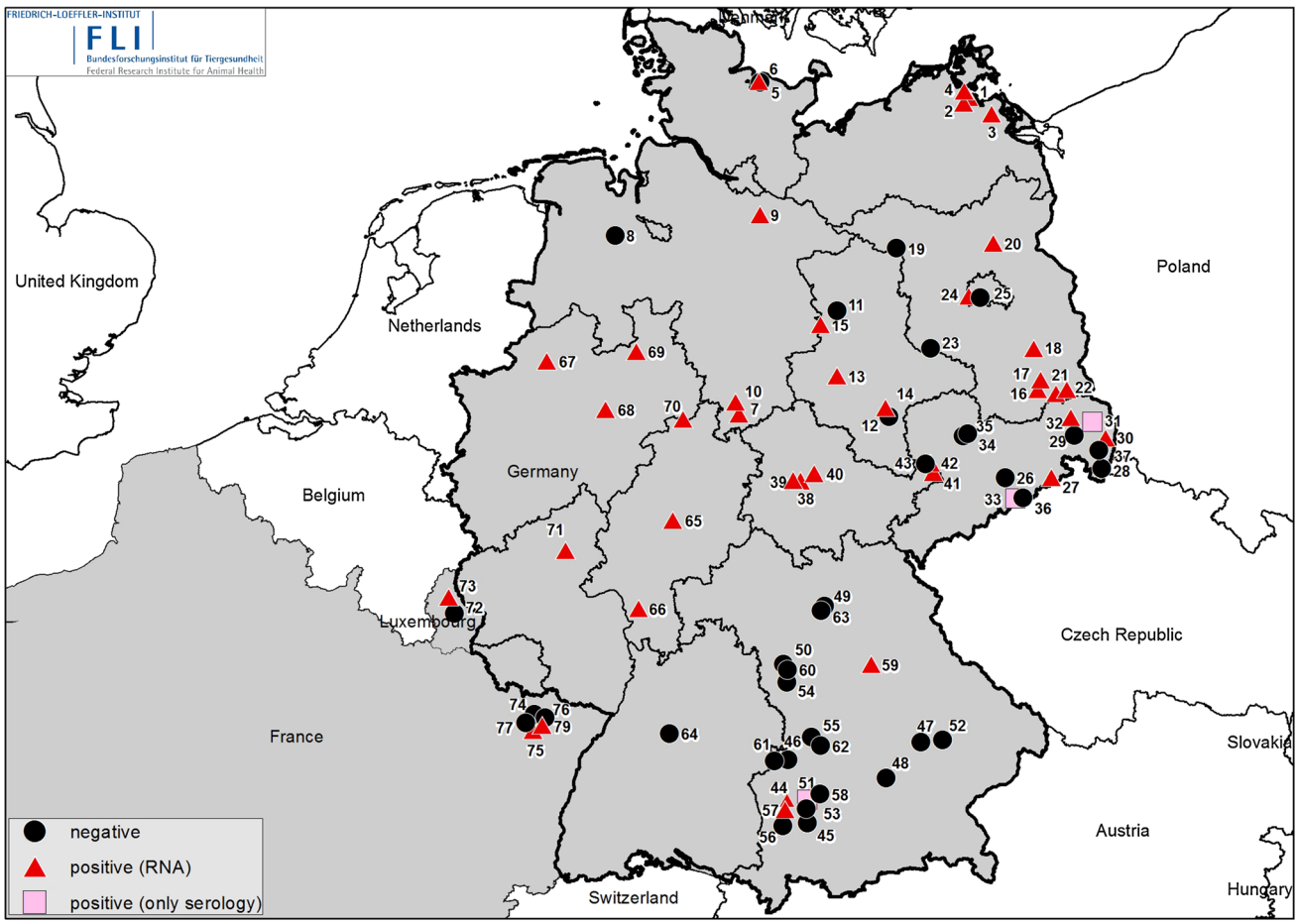


Fig. 1 Location of the main trapping sites in Germany, Luxembourg and France. An additional trapping site (#78) was located outside the range of this map in the Aveyron region in southern France

of 75 % ethanol and thereafter with 1 ml 99.8 % ethanol, dried at 56 °C for no more than 5 min, and resuspended in DEPC-treated water.

DNA for *cyt b* analysis was obtained from tissue samples using conventional chloroform DNA extraction or tail lysis overnight [47, 50]. Briefly, for extraction, all tissue samples were incubated overnight at 56 °C and 400 rpm in 300 µl of lysis buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.45 % NP40, 0.45 % Tween 20 and 1 mg of proteinase K per ml.

RT-PCR and serology

Hantavirus RT-PCR assays of lung tissue samples were done according to previously described protocols for the PUUV/TULV S segment [51]. TULV-N-specific antibodies were detected in the CCF by IgG-ELISA using yeast-expressed, purified recombinant N protein of TULV strain Moravia [22, 34].

Statistical analysis

The significance of prevalence differences between sexes was investigated using the χ^2 test. Because of the low number of RNA-positive water voles, we used Fisher's exact test to evaluate the significance of prevalence differences between species.

Sequence determination and phylogenetic analysis

DNA sequencing was performed by the dideoxy-chain termination method using a BigDye Terminator v1.1 Kit (Applied Biosystems, Darmstadt, Germany) and Genetic Analyser 3130 and 3130xl sequencers (Applied Biosystems). When the direct sequencing approach failed, sequences were obtained after insertion of the RT-PCR product into the pCR®-TOPO®-vector and transformation of TOP10 cells according to the manufacturer's instructions (TOPO-TA-Cloning Kit, Invitrogen, Darmstadt, Germany). At least two plasmids per RT-PCR product were sequenced.

All generated data were subjected to a BLAST-mediated comparison of the novel sequences with sequences available in GenBank (http://www.ncbi.nlm.nih.gov). All TULV sequences were included in subsequent phylogenetic analysis, and identical sequences were excluded. For common vole lineage analysis, one to three representatives from each trapping site were chosen, with the exception of site #27, where mtDNA of 29 individuals was investigated. All TULV and mtDNA sequences were deposited in the GenBank database with accession numbers KU139527-KU139605 and KU139696-KU139816, respectively (Supplementary Tables 4 and 5).

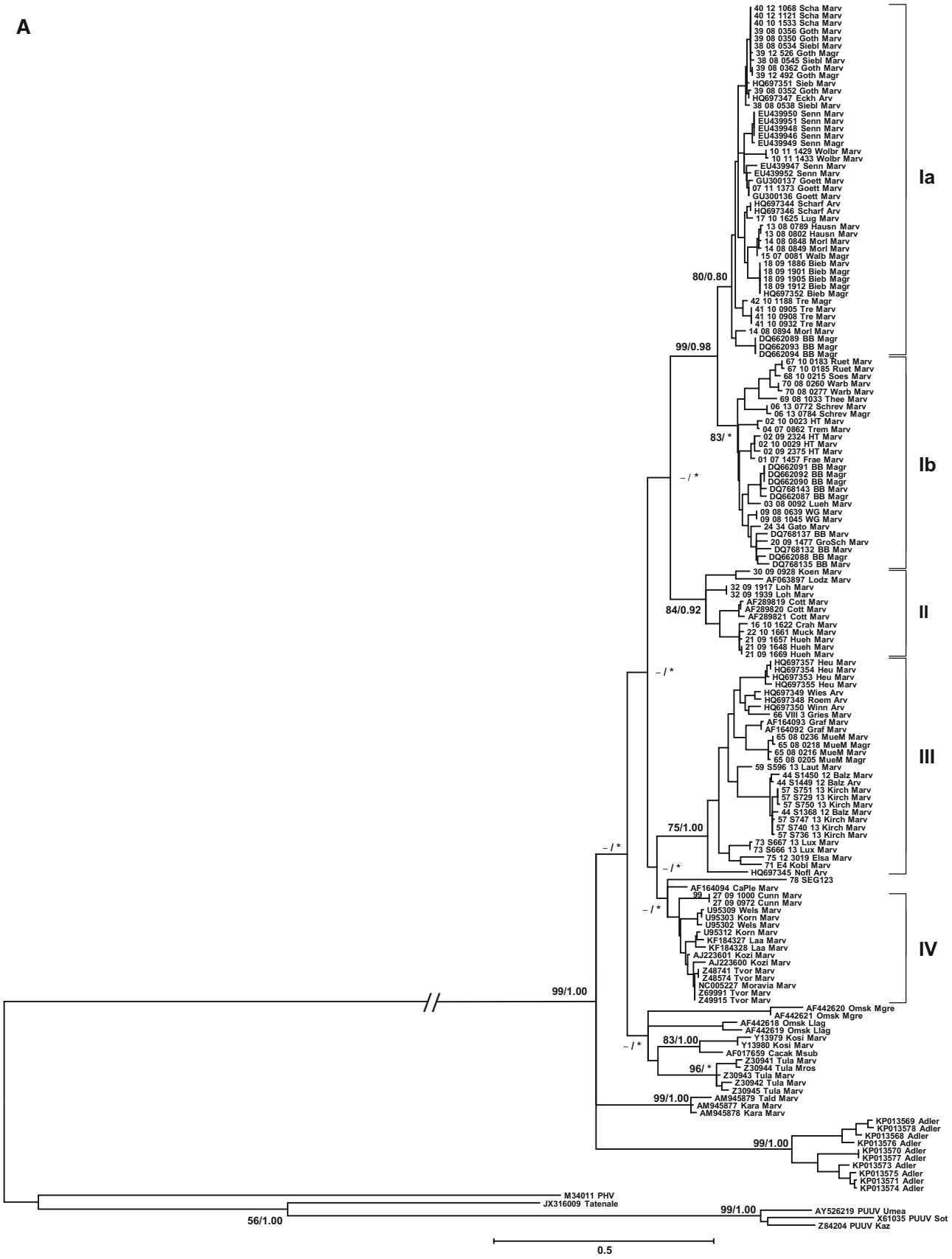
Nucleotide sequences were aligned using the ClustalW method implemented in BioEdit v7.2.5 [52] and revised manually. In addition to the novel sequences obtained in this study, representative sequences were obtained from GenBank, and these are labeled with accession numbers in Fig. 2 and Fig. 3. The final datasets used for analysis contained 158 S-segment sequences of 255-bp length for the TULV S-segment and 148 sequences of 763 bp from the *cyt b* gene of *M. arvalis*. Reference sequences for *cyt b* analysis were chosen as described in Ref. [34] and [53]. The outgroup sequences included Puumala virus (PUUV), Prospect Hill virus (PHV), and Tatenale virus for TULV and *M. gregalis* for the *cyt b* dataset. The best nucleotide substitution model to fit each data-set was determined with jModeltest v2.1.6 [54]. The Tamura and Nei model with a gamma-distribution model of among-site rate heterogeneity and a proportion of invariable sites (TrN+G+I) had highest scores for the TULV and *cyt b* data according to the Bayesian Information Criterion (BIC). MEGA 6 [55] was used for phylogenetic tree reconstruction based on maximum-likelihood (ML) algorithms with 1000 bootstrap replications. Phylogenetic relationships were also inferred using the Bayesian method implemented in MrBayes v3.2.2 [56] on the CIPRES platform [57], employing individual nucleotide substitution rate priors for each dataset. Four independent analyses were done for TULV and *cyt b* data, comprising each 10^7 generations of Markov chain Monte Carlo chains, sampled every 10^3 generations with a burn-in fraction of 25 %. For both datasets, the average standard deviation of split frequencies was lower than 0.01 in every run, and the potential scale reduction factor was in the range of 0.99-1.01 for all parameters, indicating that parameter convergence had occurred. Consensus trees were drawn with FigTree v1.4.2 [58].

Fig. 2 Maximum-likelihood (ML) phylogenetic tree based on TULV ▶ partial S-segment sequences (A) and association of TULV sequence clades with common vole evolutionary lineages (B). Novel sequences are labeled with sampling location, individual code, and host species. Published sequences obtained from GenBank are labeled with the accession number, location and host species. Bootstrap support for ML and posterior probabilities of Bayesian analyses are indicated for major branches only. — indicates bootstrap values <50; *indicates a different topology in Bayesian analysis. Clades Ia, Ib, II and III are major geographically coherent TULV clusters with adjacent distribution in Central Europe (see text). For GenBank accession numbers of the novel TULV sequences, see Supplementary Table 4

Results

Detection of TULV in common voles

To study the geographical distribution of TULV in its reservoir host, common voles were trapped in Germany, France and Luxembourg (Fig. 1, Table 1, Supplementary


A
40 12 1068 Scha Marv
40 12 1121 Scha Marv
40 10 1533 Scha Marv
39 08 0356 Goth Marv
39 08 0350 Goth Marv
38 08 0534 Siebl Marv
39 12 526 Goth Magr
38 08 0545 Siebl Marv
39 08 0362 Goth Marv
39 12 492 Goth Magr
HQ697351 Sieb Marv
39 08 0352 Goth Marv
HQ697347 Eckh Arv
38 08 0538 Siebl Marv
EU439950 Senn Marv
EU439951 Senn Marv
EU439948 Senn Marv
EU439946 Senn Marv
EU439949 Senn Magr
10 11 1429 Wolbr Marv
10 11 1433 Wolbr Marv
EU439947 Senn Marv
EU439952 Senn Marv
GU300137 Goett Marv
07 11 1373 Goett Marv
GU300136 Goett Marv
HQ697344 Scharf Arv
HQ697346 Scharf Arv
17 10 1625 Lug Marv
13 08 0789 Hausn Marv
13 08 0802 Hausn Marv
14 08 0848 Morl Marv
14 08 0849 Morl Marv
15 07 0061 Walb Magr
18 09 1886 Bieb Marv
18 09 1901 Bieb Magr
18 09 1905 Bieb Magr
18 09 1912 Bieb Magr
HQ697352 Bieb Magr
42 10 1188 Tre Magr
41 10 0905 Tre Marv
41 10 0908 Tre Marv
41 10 0932 Tre Marv
14 08 0894 Morl Marv
DQ662089 BB Magr
DQ662093 BB Magr
DQ662094 BB Magr
67 10 0183 Ruet Marv
67 10 0185 Ruet Marv
68 10 0215 Soes Marv
70 08 0260 Warb Marv
70 08 0277 Warb Marv
69 08 1033 Thee Marv
06 13 0772 Schrev Marv
06 13 0784 Schrev Magr
02 10 0023 HT Marv
04 07 0862 Trem Marv
02 09 2324 HT Marv
02 10 0029 HT Marv
02 09 2375 HT Marv
01 07 1457 Frae Marv
DQ662091 BB Magr
DQ662092 BB Magr
DQ662090 BB Magr
DQ768143 BB Marv
DQ662087 BB Magr
03 08 0092 Lueh Marv
09 08 0639 WG Marv
09 08 1045 WG Marv
24 34 Gato Marv
DQ768137 BB Marv
20 09 1477 GroSch Marv
DQ768132 BB Marv
DQ662088 BB Magr
DQ768135 BB Marv
30 09 0928 Koen Marv
AF063897 Lodz Marv
32 09 1917 Loh Marv
32 09 1939 Loh Marv
AF289819 Cott Marv
AF289820 Cott Marv
AF289821 Cott Marv
16 10 1622 Crah Marv
22 10 1661 Muck Marv
21 09 1657 Hueh Marv
21 09 1648 Hueh Marv
21 09 1669 Hueh Marv
HQ697357 Heu Marv
HQ697354 Heu Marv
HQ697353 Heu Marv
HQ697355 Heu Marv
HQ697349 Wies Arv
HQ697348 Roem Arv
HQ697350 Winn Arv
66 VIII 3 Gries Marv
AF164093 Graf Marv
AF164092 Graf Marv
65 08 0236 MueM Marv
65 08 0218 MueM Magr
65 08 0216 MueM Marv
65 08 0205 MueM Magr
59 S596 13 Laut Marv
44 S1450 12 Balz Marv
44 S1449 12 Balz Arv
57 S751 13 Kirch Marv
57 S729 13 Kirch Marv
57 S750 13 Kirch Marv
44 S1368 12 Balz Marv
57 S747 13 Kirch Marv
57 S740 13 Kirch Marv
57 S736 13 Kirch Marv
73 S667 13 Lux Marv
73 S666 13 Lux Marv
75 12 3019 Elsa Marv
71 E4 Kobl Marv
HQ697345 Nofl Arv
78 SEG123
AF164094 CaPle Marv
27 09 1000 Cunn Marv
27 09 0972 Cunn Marv
U95309 Wels Marv
U95303 Korn Marv
U95302 Wels Marv
U95312 Korn Marv
KF184327 Laa Marv
KF184328 Laa Marv
AJ223601 Kozi Marv
AJ223600 Kozi Marv
Z48741 Tvor Marv
Z48574 Tvor Marv
NC005227 Moravia Marv
Z69991 Tvor Marv
Z49915 Tvor Marv
AF442620 Omsk Mgre
AF442621 Omsk Mgre
AF442618 Omsk Llag
AF442619 Omsk Llag
Y13979 Kosi Marv
Y13980 Kosi Marv
AF017659 Cacak Msub
Z30941 Tula Marv
Z30944 Tula Mros
Z30943 Tula Marv
Z30942 Tula Marv
Z30945 Tula Marv
AM945879 Tald Marv
AM945877 Kara Marv
AM945878 Kara Marv
KP013569 Adler
KP013578 Adler
KP013568 Adler
KP013576 Adler
KP013570 Adler
KP013577 Adler
KP013573 Adler
KP013575 Adler
KP013571 Adler
KP013574 Adler
M34011 PHV
JX316009 Tatenale
AY526219 PUUV Umea
X61035 PUUV Sot
Z84204 PUUV Kaz
Ia
Ib
II
III
IV
80/0.80
99/0.98
83/ *
– / *
84/0.92
– / *
75/1.00
– / *
– / *
99/1.00
– / *
83/1.00
96/ *
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56/1.00
99/1.00
0.5

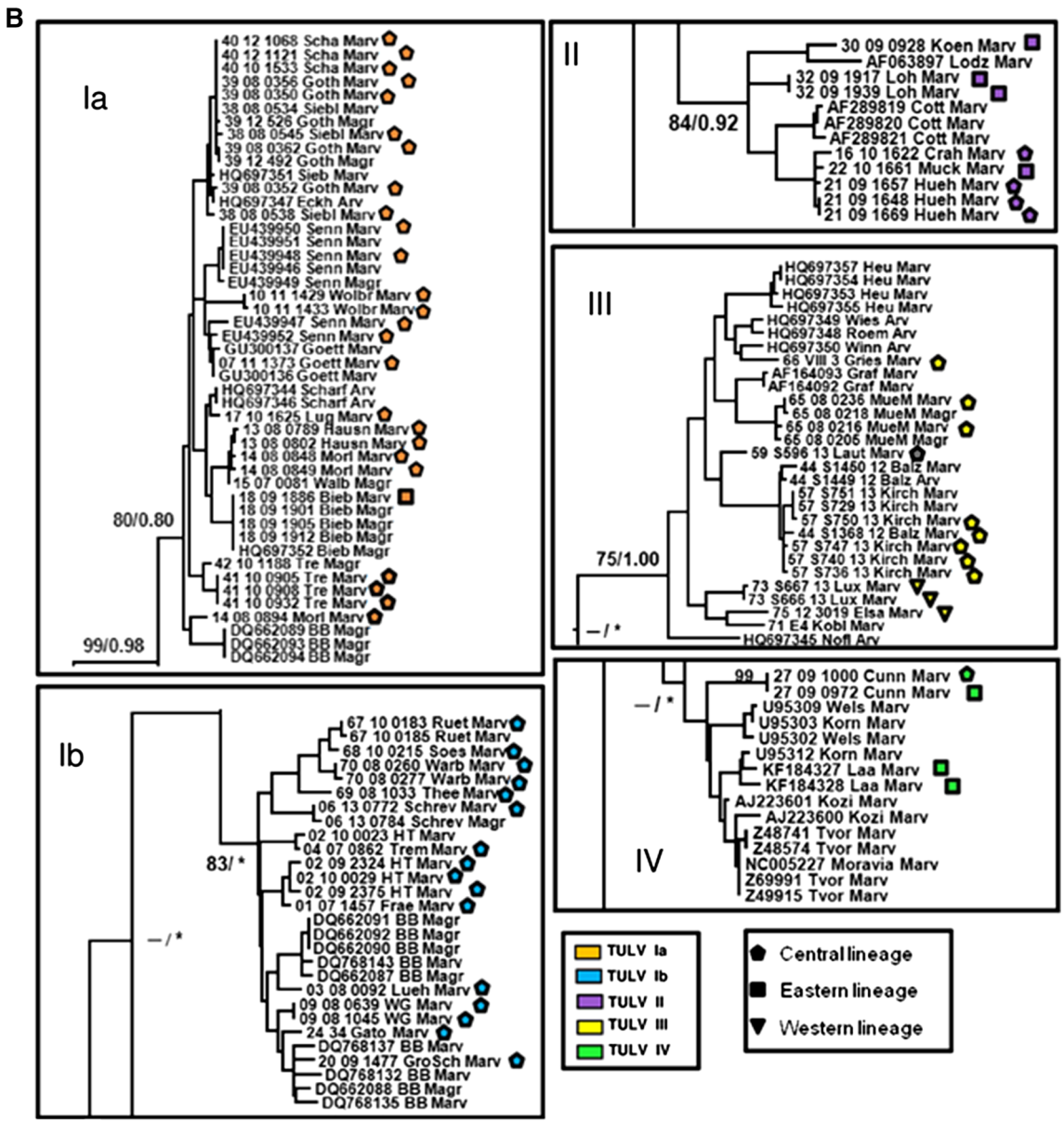

Fig. 2 continued

Table 1). The 686 common voles that were collected originated from 51 sites in 47 districts of 13 federal states in Germany (n = 654), six sites in two administrative regions of France (n = 26) and one site in Luxembourg (n = 6).

A total of 115 (16.7 %) common voles had signs of a previous or ongoing TULV infection (Table 1). Hantavirus-specific RNA and IgG antibodies were detected in 107 of 685 (15.6 %) and 49 of 670 (7.3 %) animals, respectively. Nine common voles (0.9 %) were positive in ELISA only without any detectable RNA. When focusing on the 15 locations where ten or more animals had been captured, the molecular prevalence ranged from 0 % to 37.5 % (mean: 14.2 %) and the seroprevalence ranged from 0 % to 19.0 % (mean: 5.3 %). TULV RNA could be detected in common voles from all three countries (Fig. 1, Supplementary Table 1). In contrast, TULV-specific antibodies were detected only in voles from Germany and Luxembourg. TULV-reactive antibodies were not found in any of the 26 animals from France, although TULV-RNA was detected in five animals originating from three trapping sites (Fig. 1, sites #75, #78, #79). For the RT-PCR-positive vole from site #79, no TULV sequence data were obtained after repeated attempts. Two out of six common voles from Luxembourg were RNA

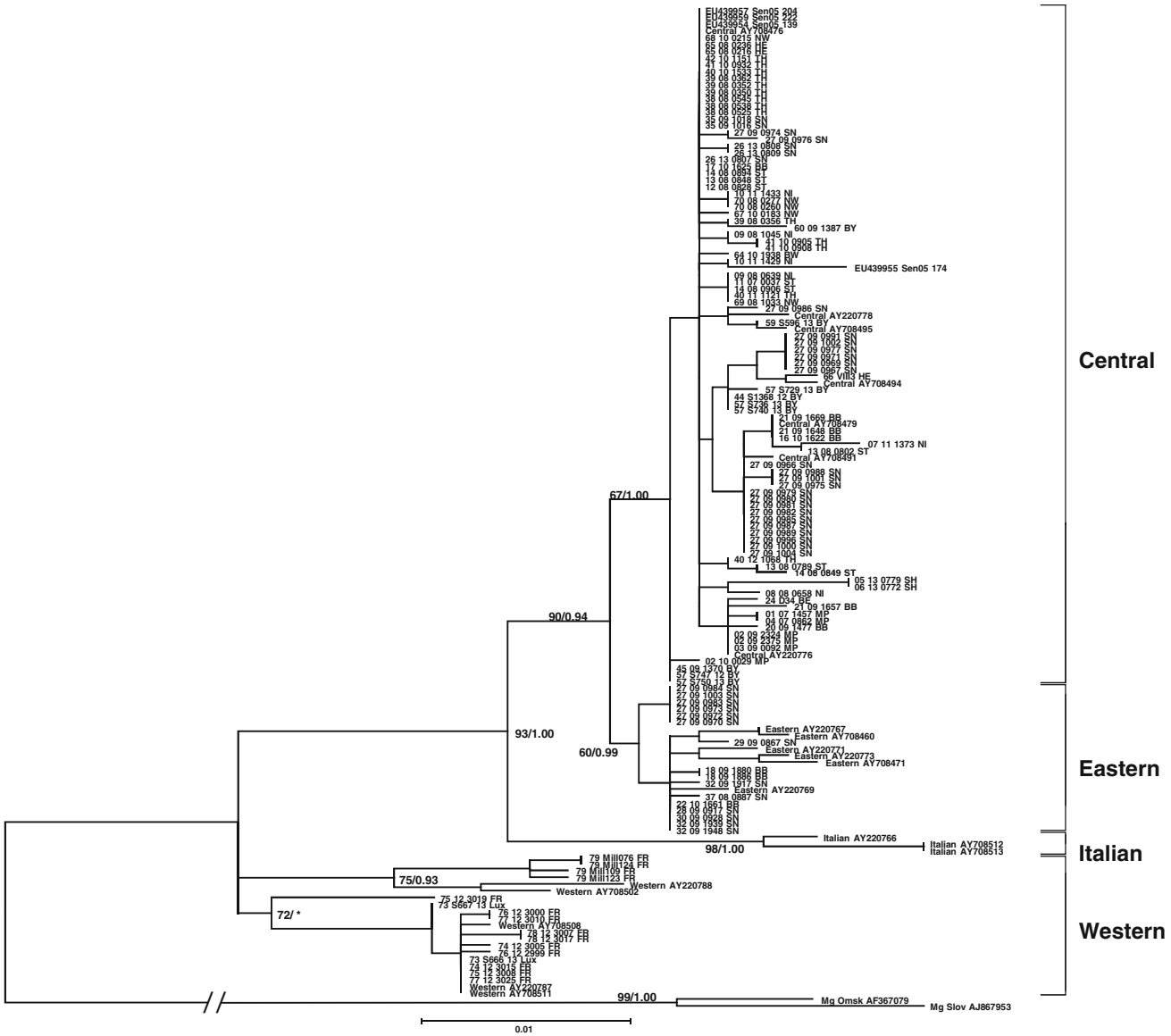

Fig. 3 Phylogenetic relationships of European *Microtus arvalis* based on *cytochrome b* sequences. The maximum-likelihood (ML) tree contains novel and published common vole sequences as references for the evolutionary lineages Central (C), Eastern (E), Italian (I) and Western (W), analogous to those reported in references 34 and 53. Labels for novel sequences start with the sampling location number, individual code and geographic region. Reference sequence labels specify the lineage and GenBank accession number. *M. gregalis* was used as an outgroup. Bootstrap support for ML and posterior probabilities of Bayesian analysis are indicated for major branches only. — indicates bootstrap values <50; *indicates a different topology in Bayesian analysis. For GenBank accession numbers of the *cytochrome b* sequences, see Supplementary Table 5

positive, one of them with parallel detection of TULV-reactive antibodies. In Germany, antibodies and/or TULV RNA could be detected in common voles at 34 of 51 trapping sites, with the majority of infected animals being antibody-negative (Supplementary Table 1). A total of 109 animals from Germany were positive for TULV-specific RNA and antibodies, and seven and 58 animals were exclusively antibody and RT-PCR positive, respectively. In addition, male common voles were more frequently found to be TULV positive than females (19 % vs. 14.7 %) (Table 2), but the difference was not significant ($\chi^2 = 2.319$, df = 1, $p = 0.128$).

Consecutive trappings of voles at one site in France (#75) and nine of 14 sites in Germany (#09, #10, #27, #39, #40, #41, #44, #57, #65) revealed a continuous presence or re-appearance of TULV infections (Supplementary Table 2). The presence of TULV RNA was monitored and detected in the common vole populations for one month (sites #10, #27, #44, #65), several months

Table 1 Results of serological and RT-PCR investigations for *Microtus arvalis* (Marv), *Microtus agrestis* (Magr) and *Arvicola* spec. (Arv) collected in Germany, Luxembourg and France

Species	No. of trapping sites	Total no. of voles analyzed	Sex		Weight range (g)		No. positive by serology only / total no. analyzed	No. positive by RT-PCR only /total no. analyzed	No. positive in both /total no. analyzed	Total no. positive by serology/ total no. analyzed	Total no. positive by RT-PCR /total no. analyzed	Total no. positive by serology and/ or RT-PCR
			Male	Female	Min	Max						
Marv	58	686	320	367	5	48	9/670	67/685	40/668	49/670	107/685	115/686
							1.3 %	9.8 %	6.0 %	7.3 %	15.6 %	16.8 %
Magr	37	249	134	115	16	47	10/246	4/249	9/246	19/249	13/249	23/249
							4.1 %	1.6 %	3.7 %	7.6 %	5.2 %	9.2 %
Arv	6	30	14	16	21	132	2/29	1/30	0/30	2/29	1/30	3/30
							6.9 %	3.3 %	0 %	6.9 %	3.3 %	10.0 %

[1] no sequence for one animal

(sites #9, #41, #57 and #75), and up to several years (sites #39, #40).

Detection of TULV in field voles

Field voles were successfully collected at 37 locations in Germany (n = 249). In total, 23 of 249 (9.2 %) field voles were found to have TULV-specific RNA and/or antibodies (Table 1). Thirteen field voles from four trapping sites in three German federal states were RT-PCR positive (5.2 %), and virus-specific antibodies were also present in nine of them. Ten additional animals from five federal states were positive by ELISA, but no viral RNA was detectable (Table 1, Supplementary Table 1). When focusing on the seven trapping sites with 10 or more field voles captured, the molecular prevalence ranged from 0 % to 14.0 % (mean 2.3 %), and the seroprevalence ranged from 0 % to 16.0 % (mean: 3 %). Similar to common voles, TULV infections were detected more often in males (11.2 %) than in females (7.0 %) (Table 3), but this difference was not significant ($\chi^2 = 1.326$, df = 1, $p = 0.25$).

At five of six trapping sites (#1, #18, #39, #44, #65) with TULV-positive field voles, TULV RNA was also detected in sympatrically occurring common voles. At the remaining site (#42) all four common voles were TULV negative. Inversely, at five of 11 sites with TULV-infected common voles (sites # 1, #18, #39, #44, #65), sympatric field voles showed signs of TULV infection. However, at five of the six sites without TULV-positive field voles, only 1-3 animals were found to be infected. At the remaining site (#41), all 21 field voles that were trapped were TULV negative.

TULV infection in water voles

Water voles were successfully trapped at four sites in Germany (n = 26) and both sites in Luxembourg (n = 4; Table 1). Two of the 29 water voles were positive in TULV-ELISA, but no RNA could be amplified from those animals (Table 1, Supplementary Table 1). A TULV sequence could be obtained from one additional animal, although there was no reaction in the ELISA. All three TULV-positive water voles originated from Bavaria, Germany, but the two seropositive animals and the RT-PCR-positive animal were captured at different trapping sites about 12 km apart (Fig. 1, sites #44 and #51).

At five of the six trapping sites, water voles occurred sympatrically with common voles (sites #26, #73), field voles (site #51) or both species (sites #44 and #65). At site #44, detection of TULV RNA in the single trapped water vole was accompanied by detection of TULV-specific RNA and antibodies in common voles and TULV-specific antibodies in field voles (Supplementary Table 1). At site

Table 2 Sex differences in the detection of TULV-specific RNA and antibodies in the vole species *Microtus arvalis* (Marv), *Microtus agrestis* (Magr) and *Arvicola* spec. (Arv)

	No. positive / total no. analyzed (percentage)					
	Male			Female		
	Serology	RT-PCR	Serology and/or RT-PCR	Serology	RT-PCR	Serology and/or RT-PCR
Marv	28/310 (9.0)	57/319 (17.9)	61/320 (19.1)	21/361 (5.8)	50/366 (13.7)	54/367 (14.7)
Magr	13/132 (9.8)	8/134 (6.0)	15/134 (11.2)	6/114 (5.3)	5/115 (4.3)	8/115 (7.0)
Arv	1/13 (7.7)	0/14	1/14 (7.1)	1/16 (6.3)	1/16 (6.3)	2/16 (12.5)

Table 3 Mean nucleotide (A) and amino acid (B) sequence identity of the S segment and nucleocapsid protein sequences, respectively, between the TULV clades in Central and Western Europe (for definition of the clades, see Fig. 2; for their geographical origin see Fig. 4)

	Ia	Ib	II	III	IV
A					
Ia	94.2 %	-	-	-	-
Ib	87.9 %	91.5 %	-	-	-
II	82.8 %	81.9 %	91.6 %	-	-
III	81.8 %	79.6 %	79.7 %	88.1 %	-
IV	80.2 %	80.7 %	82.2 %	83.3 %	92.0 %
B					
Ia	99.8 %	98.4 %	94.5 %	89.9 %	89.9 %
Ib	-	97.7 %	94.3 %	89.2 %	89.1 %
II	-	-	99.0 %	91.1 %	91.9 %
III	-	-	-	97.5 %	94.7 %
IV	-	-	-	-	98.2 %

#51, TULV-specific antibodies were detected in two of the 17 water voles, but not in the three field voles trapped there. At two sites (#65, #73), TULV infections were detected in common voles or common and field voles, but not in the few water voles collected sympatrically. At the remaining two sites (#26, #72) none of the water voles and common voles showed signs of a TULV infection.

Sequence comparison and phylogenetic analysis of TULV sequences

To analyze the phylogenetic relationships between TULV strains, S segment sequences of all RT-PCR-positive voles were compared to existing TULV sequences from Germany and other European countries. The phylogenetic tree revealed a strong geographic structuring of TULV sequences that was independent of the rodent species of origin (Fig. 2). Main cluster I represents the majority of TULV sequences originating from Germany and comprises two parapatric sister clades: Ia (central and eastern Germany) and Ib (northern, central and western Germany).

Main cluster II consists of novel sequences from the eastern part of Germany, published sequences from one site in this part of Germany [23], and prototype strain Lodz from Poland [59]. Cluster III contains sequences from southern Germany, Luxembourg, the Alsace region of France and a water-vole-derived TULV sequence from Switzerland [35]. TULV sequences from a trapping site in eastern Germany close to the Czech border (site #27), from Austria, and from the Czech Republic form cluster IV. The phylogenetic position of the novel sequence from southern France (site #78) could not be determined with confidence.

The intra-cluster proportion of variable sites ranged from 5.8 % to 11.9 % at the nt level and 0.2 % to 2.5 % at the amino acid level (Table 3). In contrast, the inter-cluster variability reached more than 20 % at the nt level and more than 10 % at the amino acid level, which exceeds the threshold level of 7 % established by the International Committee on Taxonomy of Viruses for hantavirus species definition based on the entire N protein sequence [60]. Sequence similarity at the nt and amino acid level was highest between the two subclades from Germany (Ia and Ib) and clade II from Eastern Germany and Poland (Tables 3A and 3B). The greatest differences were found between clusters Ia/Ib and III/IV, which differed on average by 8.9 % at the amino acid level and 20.3 % at the nt level (Tables 3A and 3B).

Co-segregation of TULV with evolutionary divergence in *M. arvalis*

To examine potential associations of TULV divergence with evolutionary divergence in the common vole, the *cyt b* sequences of selected animals were used for phylogenetic analysis together with reference sequences representing the evolutionary lineages Eastern, Central, Western and Italian (Fig. 3). The vast majority of *cyt b* sequences of common voles from Germany were identified as belonging to the Central lineage, and all investigated voles from France and

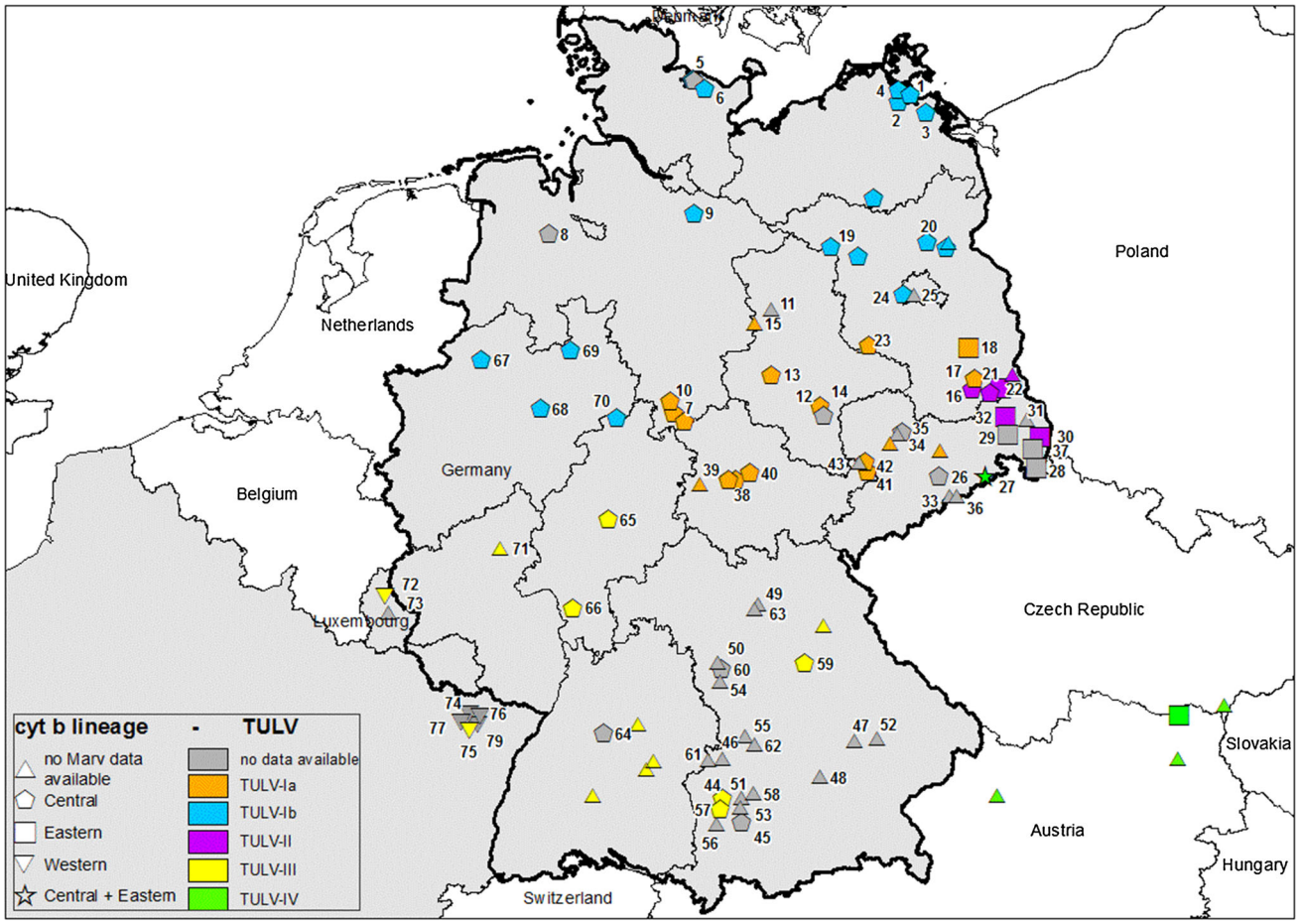

Fig. 4 Map showing the trapping sites of common voles infected with TULV of clusters Ia, Ib, II, III and IV and belonging to the evolutionary lineages Central, Eastern and Western

Luxembourg belonged to the Western lineage (Fig. 3 and Fig. 4). Interestingly, Central and Eastern evolutionary lineage *cyt b* sequences were identified in close proximity in the easternmost part of Germany, with sympatric occurrence of both lineages at site #27 (Fig. 4).

TULV clades showed a general association with certain evolutionary lineages of common voles. All animals harboring TULV of clusters Ia or Ib belonged to the Central lineage, except for one animal from Biebersdorf (site #18; Eastern lineage). TULV cluster III originated from common voles mainly of the Central lineage, with two from Luxembourg and one from France belonging to the Western lineage (sites #73 and #75). Sequences of cluster II are associated with the Eastern lineage but were also found in Central individuals trapped close to sites with Eastern lineage voles in western Brandenburg and southwestern Saxony (sites #28 and #79). TULV Cluster IV originated from Eastern-lineage hosts with one sequence from an animal of the Central lineage trapped at Cunnersdorf (site #27), Saxony. The TULV sequence from southern France originated from an animal of the Western lineage.

Discussion

Host specificity and spillover of TULV

In this study, we investigated three largely co-distributed vole species for the presence of TULV infections. The overall TULV prevalence for common voles (16.7 %) was higher than that for field voles (9.2 %) and water voles (10.0 %). In addition, the molecular prevalence for TULV differed significantly between species ($p < 0.001$; two-sided Fisher's exact test) and was again highest in common voles (15.6 %) compared to field voles (5.2 %) and water voles (3.3 %). Similarly, a previous study has shown a very low prevalence of TULV in water voles [35]. This may indicate a host preference of TULV for the common vole and the detection of TULV RNA in other vole species such as field and water voles being the result of spillover infections during the acute phase with active virus replication. In line with this assumption, almost all TULV-RNA-positive field voles were co-trapped with common voles or trapped at locations where TULV-RNA-positive

common voles were detected earlier and TULV sequences were relatively similar to those from common voles.

This observation of different prevalence in common voles versus field and water voles might also have been due to different interspecies interactions. Similar ecological characteristics and use of similar habitats can promote interspecific contact between common voles and other species directly (fighting) or indirectly (feces and urine) [61, 62]. Common voles and field voles might coexist in the same habitat, while encounters between water voles and other microtine species usually lead to a consistent dominance of the water vole and exclusion of permanent *Microtus* populations during the breeding season [63]. The water voles trapped in our study seemed to be sympatric with *Microtus* species at five out of six trapping sites, but these two species (water voles and *Microtus* spp.) had not shared the same habitat for an extended period of time (Supplementary Table 2). This might explain why we could detect TULV RNA in water voles at only one location.

A host function of field voles was discussed in previous studies, as TULV RNA was detected at higher prevalence in field voles than in common voles or in multiple field vole individuals in the absence of common voles [29, 34]. In this study, the molecular prevalence in field voles was in general much lower than in common voles. Furthermore, 53 % of field voles with TULV-reactive IgG antibodies were TULV-RNA negative, which is indicative of an earlier infection with virus clearance, but TULV-RNA could be detected in 81.6 % of TULV-IgG-positive common voles, which is typical of persistent infection in the reservoir. Furthermore, almost all field voles from sites without sympatrically occurring common voles or with only TULV-negative common voles were TULV negative.

The overall results of this study support the notion that common voles act as the preferential host for TULV. Collectively, these data do not support the idea that field voles serve as reservoir hosts with an equal ability to support TULV replication and persistence. Additional studies are needed to determine whether field voles may temporally function as a reservoir host with low viral load.

TULV in common vole populations

TULV was detected at 39 of 62 (62.9 %) sites with common voles, but with rather low prevalence compared to other vole-associated hantaviruses. While our results settle well within the reported range of TULV RNA prevalence of 8 %-37 % [18, 64], other vole-associated hantaviruses such as PUUV have been reported to reach a molecular prevalence of 60 %-100 % in reservoir host populations [65, 66]. The TULV seroprevalence of no more than 19 % observed here is much lower than antibody prevalence of up to 100 % reported for other Cricetidae- and Murinae-associated hantaviruses [66–71].

Our data suggest that – once in the common vole population – TULV seems to persist for months if not years. Persistence of hantaviruses in vole populations over several years has been shown for PUUV in bank voles. Distinct virus types were detectable over several years, independent of population density and virus prevalence [66]. Social behaviour, such as forming of colonies and kin clustering in winter, might contribute substantially to transmission and virus persistence, especially between closely related animals [72]. Our investigations have shown initial evidence of long-lasting presence of TULV in vole populations. Future systematic rodent-monitoring studies are needed to study potential intrinsic and extrinsic effects on TULV prevalence. Further studies should consider the potential oscillation of TULV prevalence in its reservoir according to season and long-term population dynamics [see ref. 66].

Discrepancy between serological and RT-PCR detection of TULV in the reservoir

This study shows a significant difference between seropositivity (49/670, 7.3 %) and molecular detection of TULV in common voles (105/685, 15.3 %) ($\chi^2 = 20.812$; df = 1; $p < 0.001$). One reason for this discrepancy might be a large number of acute TULV infections among common voles where no antibody response has been induced so far. This might be true in times of a high frequency of TULV transmission, perhaps during the peaks of the vole population. Alternatively, TULV might induce only a weak antibody response in the host, possibly due to an early innate immune response, as has been reported for *in vitro* investigations on the TULV-related PHV [73]. Furthermore, using a recombinant N protein originating from TULV strain Moravia for detection of other TULV strains in the ELISA might have caused a lower sensitivity of the IgG ELISA. Our sequence analysis (aa 225-307) including the hypervariable region of the N proteins [18] of members of TULV clades I to IV revealed sequence differences of up to 10.6 % at the amino acid level to the N protein of prototype strain Moravia (Supplementary Table 3). Additionally, the sensitivity of the ELISA might be influenced by the selection of the secondary antibody and setting of the cutoff value.

For nine adult common voles (weight, 18-36 g) TULV-reactive antibodies were found, but no viral nucleic acid, although TULV RNA was detected in other individuals from the same trapping sites and season. The detection of IgG antibodies in the absence of viral RNA may indicate virus clearance instead of a persistent infection. Similar

results were obtained in a study of PUUV in bank voles trapped in northern Sweden and for SNV in wild deer mice (*Peromyscus maniculatus*) [74, 75]. Furthermore, some studies of SEOV have suggested that, depending on the age of the reservoir upon infection, a persistent infection may not always be established [76]. Alternatively, this could be a sign of viral RNA load fluctuation during the course of infection.

In summary, our study showed a strong discrepancy between RT-PCR and serological detection of TULV infection in its reservoir. Future serological studies using antigens from different TULV strains should allow their role in the observed lower sensitivity of the ELISA compared to the RT-PCR to be tested. In addition, in-depth studies are needed to clarify the role of the rodent immune system in TULV infection and possible age effects.

Phylogeography of TULV and its reservoir

The detection of 79 novel TULV sequences from a large geographic area provided detailed insights into the high sequence variability and genetic structuring of TULV in Central Europe. Our study also allows an initial comparison of the phylogeography of the virus and its reservoir rodent host. In cluster II, the sequence of prototype strain Lodz from Poland is flanked by TULV sequences originating from common voles of the Eastern lineage, mostly found in countries located east of Germany, and four individuals of the Central lineage trapped at the eastern German border where Central and Eastern vole lineages meet [39, 40, 77–79]. In addition, our study shows the existence of distinct genetic clusters of TULV in close proximity to each other. This is consistent with the genetic isolation of common vole populations, even on a small geographic scale [40, 80, 81], and may indicate processes of virus-host adaptation.

In conclusion, this study shows that TULV is widespread in Central European common vole populations. TULV RNA was more frequently found in common voles than in field and water voles, confirming the common vole as the reservoir host and suggesting that infection of other vole species is most likely due to spillover. We readily detected TULV RNA in voles at different sites, but we were less successful in detecting specific antibodies. Although this could be a methodological problem, mechanisms leading to a reduced adaptive immune response cannot be excluded, and this provides an interesting target for further studies. The overall prevalence of TULV was not nearly as high as reported for the related PUUV in its bank vole reservoir. For further assessment of the potential involvement of population dynamics on TULV prevalence additional studies are needed. These monitoring studies would also have to consider potential consequences of TULV infection on the fitness of the vole reservoir. The initial finding of large-scale associations of some TULV clades with different evolutionary lineages of common voles indicates the need for future studies in the contact areas to study potential (co-) evolutionary processes in more detail.

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