Host range and spillover infections of rodent- and insectivore-borne hantaviruses

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“When you understand the nature of a thing, you know what it's capable of.”

Eric Brooks

In *Blade* (David S. Goyer, 1998)

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Summary

Hantaviruses (family *Bunyaviridae*) are enveloped viruses with a segmented RNA genome of negative polarity. They can cause two different diseases in humans, the hemorrhagic fever with renal syndrome in Europe and Asia and the hantavirus cardiopulmonary syndrome in America. The transmission to humans is mainly indirect by inhalation of aerosolized virus-contaminated rodent excreta. In the last three decades the knowledge of hantaviruses has broadened significantly. In contrast to the initial assumption that hantaviruses are mainly carried by rodents, during the last years many novel hantaviruses were detected in shrews, moles and recently in bats. These findings raise important questions about the evolutionary history of hantaviruses, their host association and adaptation, the role and frequency of spillover infections and host switch events.

This study aims to prove the presence, geographical distribution and host association of the rodent-borne *Tula virus* (TULV) and the shrew-associated Seewis virus (SWSV) in Central Europe. For this purpose, novel laboratory techniques for molecular and serological hantavirus detection were developed.

Initially, a broad-spectrum molecular assay to identify small mammal species from Central Europe was developed. This novel assay is based on PCR amplification using degenerated primers targeting the *cytochrome b* (*cyt b*) gene, nucleotide sequence analysis of the amplified *cyt b* gene portion and followed by pairwise sequence comparison to published sequences using the BLAST function of GenBank. Different small mammal species prevalent in Central Europe could be determined by this new approach, including not only representatives of various Rodentia and Soricomorpha, but also representatives of the orders Erinaceamorpha, Lagomorpha, Carnivora and Chiroptera.

For characterization of insectivore-borne hantavirus *Thottapalayam virus* (TPMV), specific monoclonal antibodies were generated that detect native virus in infected mammalian cells. For the detection of TPMV-specific antibodies, Asian house shrew *Suncus murinus* immunoglobulin G (IgG)-specific antibodies were produced in laboratory mice and rabbit. Using this anti-shrew IgG and recombinant TPMV nucleocapsid (N) protein, an indirect enzyme-linked immunosorbent assay (ELISA) was developed allowing the detection of TPMV N protein-specific antibodies in immunized and experimentally TPMV infected shrews.

A Pan-Hantavirus SYBR-Green based reverse transcription real-time polymerase chain reaction assay (RT-qPCR) was developed for the search to novel hantaviruses. This novel assay was shown to detect RNA from different rodent-borne hantaviruses in tissues of naturally-infected animals, but failed in the detection of SWSV in shrews. By this novel RT-qPCR and other conventional RT-PCR approaches, TULV infections were identified for the first time in the Eurasian water vole *Arvicola amphibius* from different regions in Germany and Switzerland. The phylogenetic analyses of the different partial TULV small (S)-, medium (M)- and large (L)-genome segment sequences from *A. amphibius*, with those of *Microtus arvalis*- and *M. agrestis*-derived TULV lineages, revealed a geographical, but host-independent clustering and may suggest multiple TULV spillover or a potential host switch from *M. arvalis* or *M. agrestis* to *A. amphibius*.

In a further comprehensive study, different shrew species (*Sorex araneus*, *S. minutus*, *S. coronatus*, and *S. alpinus*) were collected in Germany, Czech Republic, and Slovakia and screened by another L-segment-targeting Pan-Hantavirus RT-PCR approach. This screening revealed hantavirus L-segment sequences in a large number of *S. araneus* and a few *S. minutus* indicating a broad geographical distribution of this hantavirus. For detailed analyses, S-segment sequences were obtained, from *S. araneus* and *S. minutus*. The sequences demonstrated their similarity to SWSV sequences from Hungary, Finland, Austria and Germany. A detailed phylogenetic analysis showed low intra-cluster sequence variability, but high inter-cluster divergence suggesting a long-term SWSV evolution in local shrew populations. Moreover, in 28 of the 49 SWSV S-segment sequences an additional putative open reading frame (ORF) on the opposite strand to the N protein-encoding ORF was identified that has never been observed in any other hantavirus.

In conclusion, the investigations demonstrated a broad geographical distribution and multiple spillover infections of rodent-borne TULV and shrew-borne SWSV in Europe. The finding of putative spillover transmissions described here and in other studies underline the current problem of the hantavirus reservoir host definition. In contrast to the hypothesis of a long-standing hantavirus–rodent (small mammal) host coevolution, the investigations support a more dynamic evolutionary history of hantavirus diversification including spillover infections and host-switch events. In future *in vitro* and *in vivo* infection studies as well as field studies has to define factors determining the host specificity of these hantaviruses.

Introduction

A brief history of the discovery of hantaviruses

Thottapalayam virus (TPMV) isolated from the Asian house shrew *Suncus murinus* captured in Tamil Nadu, India, was the first discovered hantavirus, but initially characterized as an arbovirus (CAREY et al. 1971). After this initial finding, it took more than 30 years until this virus was confirmed to be a hantavirus (SONG et al. 2007a). Historically, the discovery of hantaviruses is associated with the Korean war in 1951–1953, where a hemorrhagic fever (KHF) of unknown origin, with more than 3000 cases among soldiers, was reported. After a long-term research on the causative agent of this outbreak, the *Hantaan virus* (HTNV), was discovered in 1978 (LEE et al. 1978). It was identified as a rodent-borne virus, associated with the striped field mouse *Apodemus agrarius,* and classified as the prototype virus of a new genus called *Hantavirus* within the family *Bunyaviridae* (LEE et al. 1981; MCCORMICK et al. 1982; WHITE et al. 1982; SCHMALJOHN and DALRYMPLE 1983). After the discovery of HTNV additional hantaviruses were identified in Europe, Asia and America, e.g., *Puumala virus* (PUUV), *Seoul virus* (SEOV)*, Prospect Hill virus* (PHV) and *Sin Nombre virus* (SNV) (BRUMMER-KORVENKONTIO et al. 1980; LEE et al. 1982; LEE et al. 1985; NICHOL et al. 1993). Since this, 426 hantavirus strains or lineages were described and are currently listed in GenBank (http://www.ncbi.nlm.nih.gov). Out of about 50 hantaviruses 23 species are currently accepted by the International Committee on Taxonomy of Viruses within the genus *Hantavirus* (PLYUSNIN et al. 2011).

Hantavirus structure and genome organization

Hantaviruses are enveloped viruses with a single-stranded RNA genome (Figure 1A-B). The RNA genome of negative polarity is represented by three segments of different size. The small (S)-segment of 1.6-2.0 kilobases (kb) encodes for a nucleocapsid (N) protein 48-54 kilodalton (kDa). The medium (M)-segment of 3.5-3.6 kb encodes a glycoprotein precursor (GPC), which is co-translationally cleaved into two surface glycoproteins Gn (G1; ca. 70kDa) and Gc (G2; ca. 55kDa). The RNA-dependent RNA polymerase (RdRp) of ca. 250kDa is encoded by the large (L)-segment of approximately 6.5 kb (Figure 1C).

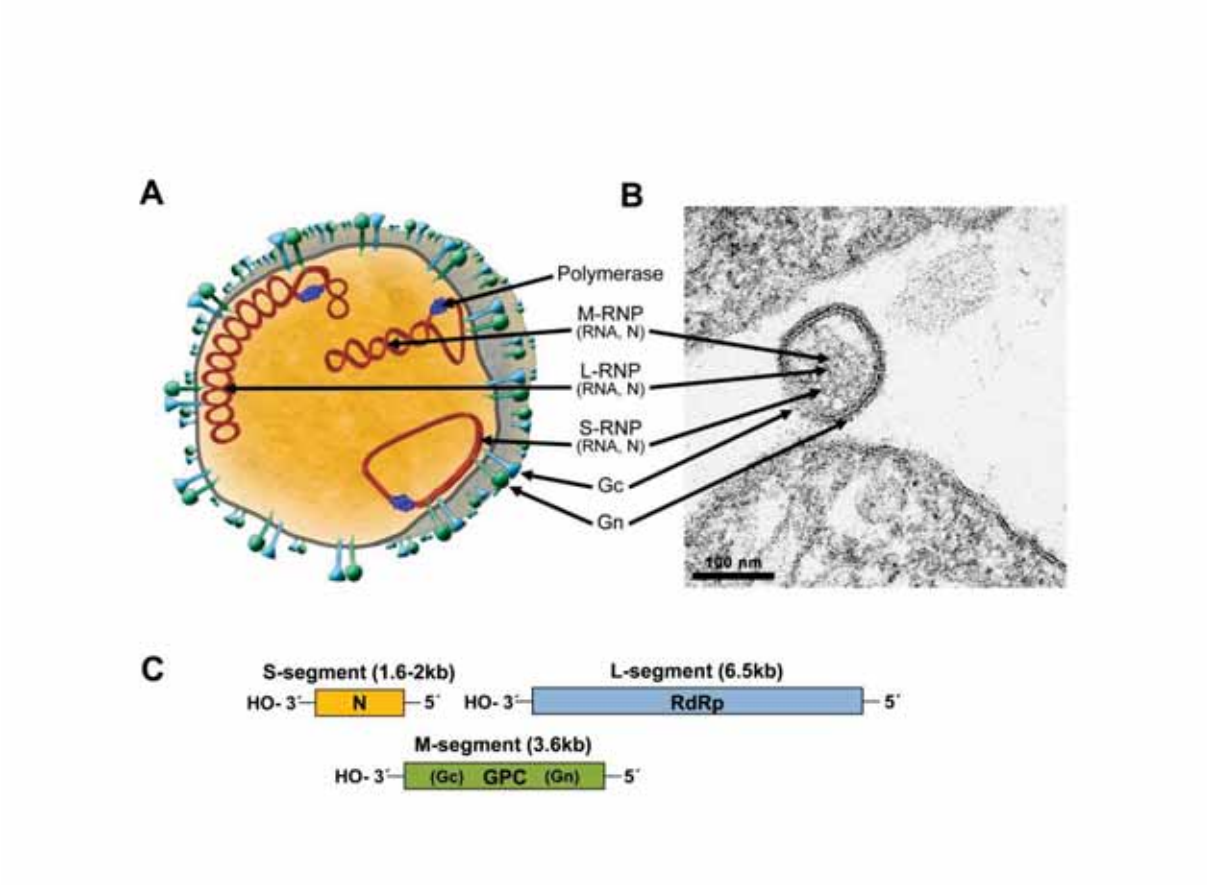

Figure 1 Structure (A), electron microscopic image (B) and genome organisation of a hantavirus (C). Abbreviations: GPC, glycoprotein precursor; Gn and Gc, amino- and carboxy-terminal glycoproteins; kb, kilobases; nm, nano meter, N, nucleocapsid protein; RdRp, RNA-dependent RNA polymerase; RNA, ribonucleic acid; RNP, ribonucleoprotein; L, large; M, medium; S, small. Electron microscopic image of TULV-infected Vero E6 cells was provided by Dr. H. Granzow (B) and the graphic design (A) was made by M. Jörn (Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany).

The consensus-nucleotide sequences of the L-, M-, and S-segments, AUCAUCAUCUG at the noncoding 3' end and UAGUAGUA at the noncoding 5' end, are terminal complementary and thereby forming panhandle-like, bunyavirus-typic structures (SCHMALJOHN and DALRYMPLE 1983; ELLIOTT et al. 1991; ANTIC et al. 1992; PLYUSNIN et al. 1996c). Comparisons of the three genome segments, from different hantaviruses, showed that the entire RdRp-coding part of the L-segment is higher conserved at nucleotide (nt) and amino acid (aa) level then the M- and S-segment coding sequences and the corresponding GPC and N protein (ANTIC et al. 1992; XIAO et al. 1994 and Table 1).

The N protein is the most abundant viral protein synthesized early during the infection. It protects the viral genomic RNA (v)RNA of negative polarity from nuclease degradation and is involved in the formation of the ribonucleoprotein complex. The RNA encapsidation is mediated

through N protein trimerization with a coiled-coil structure. This motif is localized within the first 75 amino (N)-terminal aa region and highly conserved in hantaviruses (ALMINAITE et al. 2006). The N protein possesses a RNA-binding domain at amino acid (aa) residues 175 to 217. Glutamic acid, tyrosine and serine residues at positions 192, 206 and 217 were found to be essential for RNA interaction (XU et al. 2002; SEVERSON et al. 2005). Carboxy (C)-terminal 100 aa-long fragments of HTNV and PUUV N proteins showed *in vitro* full RNA binding capacity, but competition experiments, using transfer (t)RNA, indicated that binding of RNA by the N protein is nonspecific (GOTT et al. 1993).

Table 1 Pairwise nucleotide sequence identities between the entire N-, GPC- and RdRp-encoding S-, M- and L-segment sequences (above the diagonal) and amino acid sequence identities between the corresponding proteins (below the diagonal) of insectivore-borne hantaviruses *Thottapalayam virus* (TPMV), Imjin virus (MJNV) and Rockport virus (RKPV) and different rodent-borne hantaviruses.

	% identity with virus species									
Segment and virus species	HTNV	SEOV	DOBV	PUUV	TULV	SNV	ANDV	TPMV	MJNV	RKPV
S-segment ORF										
HTNV	-	74.5	73.9	60.9	63.1	62.4	63.0	52.6	53.5	62.0
SEOV	83.2	-	73.8	59.6	62.5	62.8	63.0	52.3	53.7	62.0
DOBV	83.0	81.6	-	61.6	61.7	62.9	61.2	52.5	52.7	61.8
PUUV	60.5	61.9	61.0	-	73.0	67.2	68.3	53.1	52.3	66.8
TULV	63.1	62.6	62.8	79.2	-	70.3	68.3	53.1	53.2	68.4
SNV	62.7	62.0	62.5	70.0	73.5	-	76.6	52.8	53.7	69.5
ANDV	65.1	64.8	64.4	72.1	74.7	86.0	-	55.1	53.6	71.0
TPMV	46.4	45.1	46.2	44.0	44.2	47.7	46.5	-	66.7	52.8
MJNV	45.8	44.6	45.3	44.2	43.5	46.3	46.8	69.7	-	52.5
RKPV	63.2	62.7	64.1	71.4	76.5	76.9	79.0	46.8	44.7	-
M-segment ORF										
HTNV	-	71.8	71.3	57.3	58.6	57.4	57.8	50.0	50.7	58.1
SEOV	77.0	-	70.8	57.7	58.6	56.6	57.5	50.6	51.0	57.2
DOBV	77.3	77.1	-	57.7	58.0	58.1	57.5	50.4	51.0	57.0
PUUV	52.6	52.6	52.1	-	71.5	65.4	64.9	50.5	50.3	63.1
TULV	54.5	53.8	54.3	78.3	-	67.1	65.5	51.3	51.5	65.0
SNV	54.2	52.3	53.2	66.0	69.0	-	71.3	50.7	50.4	62.9
ANDV	54.2	53.5	53.6	65.7	67.2	77.7	-	52.3	50.7	62.8
TPMV	41.7	41.2	42.5	40.9	41.7	41.8	42.6	-	68.7	50.8
MJNV	42.4	42.0	41.9	41.5	42.6	41.5	43.4	71.7	-	50.6
RKPV	52.9	52.8	52.4	61.7	62.7	62.1	62.2	41.8	41.3	-
L-segment ORF										
HTNV	-	74.3	74.7	65.9	65.2	65.8	65.9	62.3	62.3	65.8
SEOV	85.0	-	74.9	66.5	65.8	66.4	65.8	61.6	61.8	65.6
DOBV	85.2	85.4	-	66.5	66.0	66.7	66.0	61.9	62.4	66.0
PUUV	68.9	68.6	69.5	-	75.0	70.8	71.2	62.8	62.8	70.9
TULV	68.4	68.8	68.8	85.0	-	71.3	71.4	62.5	62.1	70.4
SNV	69.2	69.0	69.5	77.8	78.5	-	75.4	62.0	62.3	71.4
ANDV	68.6	68.1	68.2	77.2	78.1	86.7	-	61.8	62.6	71.0
TPMV	62.3	61.9	61.8	61.6	61.4	61.9	62.1	-	74.2	61.4
MJNV	62.2	61.3	61.5	61.6	61.4	61.8	61.4	81.6	-	63.1
RKPV	67.8	67.8	68.5	75.7	76.2	77.9	76.5	61.2	61.5	-

Abbreviations and accession numbers: HTNV, *Hantaan virus* (NC_005218, NC_005219, NC_005222); SEOV, *Seoul virus* (NC_005236, NC_005237, NC_005238); DOBV, *Dobrava-Belgrade virus* (NC_005233, NC_005234, NC_005235); PUUV, *Puumala virus* (NC_005224, NC_005223, NC_005225); TULV, *Tula virus* (NC_005227, NC_005228, NC_005226); SNV, *Sin Nombre virus* (NC_005216, NC_005215, NC_005217); ANDV, *Andes virus* (NC_003466, NC_003467, NC_003468); TPMV (NC_010704, NC_010708, NC_010707); MJNV (EF641805, EF641799, EF641807); RKPV (HM015223, HM015219, HM015221).

A further functional domain is localized at the 57 C-terminal aa segment, which interacts with an apoptosis enhancer (LI et al. 2002). The hantavirus N protein exhibit immunodominant and conformational cross-reactive epitopes within the first 100 aa of the N-terminus (JENISON et al. 1994; ELGH et al. 1995; YAMADA et al. 1995; YOSHIMATSU et al. 1996; ARAKI et al. 2001). A large panel of monoclonal antibodies (mAbs) has been developed, that bind to epitopes within aa positions 1-61, 166-175 and 226-293, of the N protein of different hantaviruses (DZAGUROVA et al. 1995; LUNDKVIST et al. 1996b; YOSHIMATSU et al. 1996; MAZZAROTTO et al. 2009). Several mAbs were reactive with epitopes within the 80 and 120 N-terminal aa of the N protein, confirming the immunodominant nature of the N-terminal region of N (YOSHIMATSU et al. 1996; LUNDKVIST et al. 2002; ZVIRBLIENE et al. 2006; KUCINSKAITE-KODZE et al. 2011). Furthermore, a hypervariable region of the N protein, between aa residues 244 and 273 (PLYUSNIN et al. 1994; HUGHES and FRIEDMAN 2000) has been described, which includes epitopes for monoclonal antibodies in human patient sera (LUNDKVIST et al. 1995). Interestingly, the N protein has been found to induce protective immunity in animal models (YOSHIMATSU et al. 1993b; LUNDKVIST et al. 1996a; ULRICH et al. 1998; DARGEVICIUTE et al. 2002; DE CARVALHO NICACIO et al. 2002). In line, N protein specific cytotoxic T cells can protect nude mice against HTNV infection (YOSHIMATSU et al. 1993b). Alternatively, in human patients the clinical manifestation of the hantavirus infection is thought to be caused by an immunpathogenesis (SCHONRICH et al. 2008). Using peripheral blood lymphocytes of HTNV-infected patients, CTL epitopes have been identified in the N protein at aa positions 12-20 and 421-429 of HTNV (VAN EPPS et al. 1999).

The Gn and Gc glycoproteins are type-I integral transmembrane proteins with C-terminal hydrophobic anchor domains (ELLIOTT et al. 1991; SPIROPOULOU 2001). The GPC encoded by the M-segment has a conserved C-terminal WAASA-motif, which is involved in the co-translational cleavage of the precursor polypeptide, into the envelope glycoproteins Gn and Gc (LOBER et al. 2001). Gn and Gc exhibit cytoplasmic tails of about 150 aa and 10 aa in length. The long cytoplasmic tail of the Gn protein contains a highly conserved YRTL-motif, which is proposed to be a glycoprotein trafficking signal and the tail of the Gc contains an endoplasmic reticulum retention signal (KKXX-motif) (SPIROPOULOU 2001). The Gn and Gc proteins possess several cysteine residues, e.g. for *Tula virus* (TULV) Gc 15 are described (PLYUSNIN et al. 1995). Four N-glycosylation sites (NXS/T) in the GPC, with three in Gn (aa positions 142, 358 and 410) and one in Gc (aa position 939), are conserved for all hantaviruses (SPIROPOULOU 2001). Moreover, an immunoreceptor tyrosine-based activation signal (ITAM-motif), which is present in several

receptors of the immune system, was predicted in the C-terminal cytoplasmic region of Gn (SCHONRICH et al. 2008). Several mAbs are generated against Gn and Gc (DANTAS et al. 1986; ARIKAWA et al. 1989; DZAGUROVA et al. 1995; LIANG et al. 1997; YU et al. 2006), but only for Gc, two epitopes were identified at aa positions 916- 924 and 954-963 (KOCH et al. 2003). Recombinant glycoproteins and DNA vaccines, could induce a protective humoral immune response in animal models (SHOPE et al. 1981; ZHANG et al. 1989; SCHMALJOHN et al. 1990; ARIKAWA et al. 1992; LUNDKVIST et al. 1993). Protective immunity is mediated by glycoprotein specific neutralizing antibodies, and a responsible epitope (LTKTLVIGQ-motif) was identified near the C-terminus of HTNV Gn (SCHMALJOHN et al. 1990; LIANG et al. 1997; LIANG et al. 2003). In contrast, immunpathogenic relevant CTL epitopes in the SNV Gc have been reported (KILPATRICK et al. 2004). The cytoplasmic tails of *New York virus* (NYV), *Andes virus* (ANDV) and HTNV Gn have been shown to be degraded by the cellular proteasome, which results in more efficient processing and presentation of Gn-derived epitopes to CTLs (SEN et al. 2007).

The RdRp, encoded by the L-segment, functions as a replicase, transcriptase and endonuclease (PLYUSNIN et al. 1996c). The primary function of this enzyme is the synthesis of mRNA and the synthesis of negative-stranded vRNA. The RdRp comprises conserved motifs at aa positions 884-902, 964-980, 1050-1077, 1091-1101, 1152-1164 and 1171-1181, which are found in all hantaviruses, including an XDD motif that is essential for catalytic activity (KUKKONEN et al. 2005; KANG et al. 2009c). More detailed information about molecular or biochemical characteristics of the RdRp, are not available, because no efficient heterologous expression of the RdRp has been obtained and no suitable reverse genetic system has been established so far.

In addition, to the N, Gn, Gc and RdRp, an additional putative nonstructural protein encoded by an open reading frame (ORF) overlapping the N protein encoding sequence, could be found in PUUV, PHV, TULV, SNV, ANDV, NYV, but not in HTNV and *Dobrava-Belgrade virus* (DOBV) (PLYUSNIN and MORZUNOV 2001). This putative nonstructural protein might have an important function for these viruses in the reservoir host (ULRICH et al. 2002; RANG et al. 2006) and seems to be involved in blocking the innate immune response (JAASKELAINEN et al. 2007; JAASKELAINEN et al. 2008).

Hantavirus infections in humans

Hantaviruses can be transmitted from reservoir animals to humans (zoonosis), where the human represents a dead-end host. Only for the South American ANDV a person-to-person transmission was described (PINNA et al. 2004). Almost all reported human infections in the past are related to infections with rodent-borne hantaviruses. For insectivore-borne hantaviruses the pathogenicity for humans is still unclear.

Hantaviruses can cause two different diseases in humans, the hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia (LINDERHOLM and ELGH 2001) and the hantavirus cardiopulmonary syndrome (HCPS) in North and South America (ENRIA et al. 2001). For Africa, few reports of hantavirus-specific antibodies and cases of putative HFRS in humans are available (GONZALEZ et al. 1984; COULAUD et al. 1987; GONZALEZ et al. 1989; RODIER et al. 1993; KLEMPA et al. 2010). For Australia to date no human hantavirus infections, but hantavirus-specific antibodies in rodents have been reported (LEDUC et al. 1986; BI et al. 2005).

The HCPS is characterized by an incubation time ranging from 9 to 24 days followed by a febrile phase with fever, muscular pain, malaise, headache, dizziness, anorexia, nausea, vomiting and diarrhea. After this phase, the disease proceeds fast within 4-24 hours with presentation of hypotension, oliguria, pulmonary edema and shock. The patients die within the next 24-48 hours or recover apparently fully after two months (ENRIA et al. 2001). The case-fatality rate of HCPS ranges from 35% in North and up to 50% in South America (ENRIA et al. 2001; MACNEIL et al. 2011).

The characteristic clinical picture of HFRS, after an incubation period of 1 to 8 weeks, is fever, chill, headache, myalgia, nausea, abdominal pain, thrombocytopenia and hematuria. The renal impairment is a typical sign of HFRS and is indicated by oliguria and rising serum concentrations of creatinine and urea. A life-threatening electrolyte imbalance during the renal involvement and acute kidney failure is described (METTANG et al. 1991; KULZER et al. 1992). Hemorrhagic manifestations and respiratory symptoms were sometimes reported (LINDERHOLM and ELGH 2001). The case-fatality rate of HFRS cases in Asia ranges from 10-15%. Compared to the described symptoms of HFRS in Asia, another mild to moderate form of HFRS, also called *Nephropathia epidemica*, is prevalent in Europe, with case-fatality rates lower than 1%. This disease is caused by PUUV, which is responsible for the majority of the human cases in Europe (HEYMAN et al. 2011). In addition, in South-East Europe, the Balkan region and Greece, an

Apodemus flavicollis-associated DOBV lineage (DOBV-Af) causes more severe disease, like HFRS in Asia, with case-fatality rates from 10-12% (SCHONRICH et al. 2008; KRUGER et al. 2011). Human infections with the Caucasus field mouse (*A. ponticus*)-associated DOBV-Ap have resulted in more moderate than severe HFRS in the southern part of European Russia (KLEMPA et al. 2008). Mild-to-moderate human disease in central and eastern Europe has been associated with infections by the DOBV-Aa lineage carried by *A. agrarius* (SIBOLD et al. 2001; KLEMPA et al. 2005; KLEMPA et al. 2008).

The clinical characteristics of human hantaviral disease are significantly affected by the causative hantavirus species. In addition to the typical renal dysfunction, human infections with pulmonary involvement (HCPS-like symptoms) are also reported for PUUV infected patients in Europe (CLEMENT et al. 1994; CARAMELLO et al. 2002; HOIER et al. 2006). Regarding this, the clear separation in two distinct syndromes should be at least reconsidered (RASMUSON et al. 2011). Human hantavirus infections are sometimes unrecognized or diagnosed as leptospirosis. Several studies identified risk groups, which have a higher probability to get in contact with rodents or rodent excreta and therefore, to contract a hantavirus infection. Such risk groups are forestry workers (ZOLLER et al. 1995; MERTENS et al. 2011b), farmers (VAPALAHTI et al. 1999a), mammalogists (LUNDKVIST et al. 2000; KELT and HAFNER 2010) and soldiers (CLEMENT et al. 1996; MARKOTIC et al. 2002; SONG et al. 2009b). However, the number of comprehensive studies of risk groups is limited and affected by the lack of information of the seroprevalence rates in matched general population control groups.

Hantavirus transmission and pathogenesis in rodents

Most of the members within the family *Bunyaviridae* are vector-borne zoonotic viruses. They are transmitted by arthropods to humans. In contrast to that, hantaviruses are carried by rodents, shrews and moles. Recently single hantavirus sequences have been found in bats (see chapter Hantaviruses and their hosts). Infected rodents shed the virus approximately one week after infection in urine, faeces and saliva continuously, but in variable amounts up to 100 days post infection (see Figure 2) and (LEE et al. 1981; YANAGIHARA et al. 1985b; GLASS et al. 1988; HUTCHINSON et al. 1998; HJELLE and YATES 2001; EASTERBROOK and KLEIN 2008; HARDESTAM et al. 2008a).

In general, it is assumed, that the reservoir rodents are persistently infected and show no apparent signs of clinical illness (MEYER and SCHMALJOHN 2000). However, there are several reports suggesting effects of the infection on the natural reservoirs. Naturally PUUV infected bank voles *Myodes glareolus* have been shown to have a significantly lower overwinter survival rate than antibody negative animals (KALLIO et al. 2007). Moreover, SEOV seropositive wild brown rats *Rattus norvegicus* show slower growth rates compared to uninfected rats (CHILDS et al. 1989).

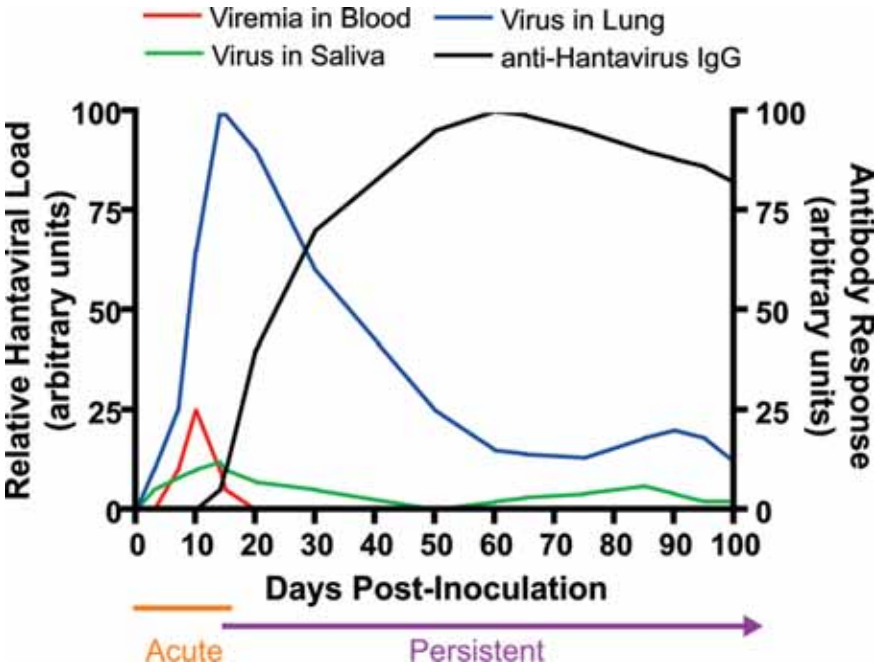

Figure 2 Kinetics of *Black Creek Canal, Hantaan, Seoul, Sin Nombre* and *Puumala virus* infection in their respective rodent reservoir species; taken from EASTERBROOK and KLEIN 2008, doi:10.1371/ journal.ppat.1000172.g001.

Furthermore, North American deer mice *Peromyscus maniculatus* naturally infected with SNV showed a decreased survival rate and gained less weight over the 1-month period following seroconversion (DOUGLASS et al. 2007; LUIS et al. 2012). In addition, naturally SNV-infected *P. maniculatus* revealed septal edema within lung tissue and mononuclear cell infiltrates in portal areas of the liver (NETSKI et al. 1999). Naturally NYV infected *P. maniculatus* demonstrated lymphohistocytic infiltrates in liver, slightly increased number of immunoblasts in splenic parenchyma (red pulp) and edematous alveolar septa with hyperplasia in the lungs (LYUBSKY et al. 1996).

Experimental infection of two reservoir host species with the corresponding hantavirus, *P. maniculatus* with SNV and *Sigmodon alstoni* with *Cano Delgadito virus* (CDGV), lead to the establishment of a persistent infection up to 90 days post infection, but animals showed no signs of clinical illness (BOTTEN et al. 2000; FULHORST et al. 2002). Experimental infection of a non-reservoir species, the Syrian hamster (*Mesocricetus auratus*), with South American ANDV and Maporal virus results in symptoms of rapidly progressing respiratory distress and high mortality rates (HOOPER et al. 2001; MILAZZO et al. 2002). In addition, suckling mice (*Mus musculus*) inoculated intracerebrally with HTNV elicit a neurologic disease (TSAI et al. 1982). In contrast to that, experimental infection of suckling Mongolian gerbils (*Meriones unguiculatus*) with PUUV caused in the development of subclinical persistent infections, similar to experimental infections observed in the reservoir host *Myodes glareolus* (YANAGIHARA et al. 1984; YANAGIHARA et al. 1985a).

Hantavirus antigen or viral RNA have been found in naturally and experimentally infected rodents in heart, lung, kidney, liver, brain, blood and brown adipose fat, but some tissue were found to be virus antigen and RNA negative (LEE et al. 1981; HUTCHINSON et al. 1998; BERNSHTEIN et al. 1999; MEYER and SCHMALJOHN 2000; MACKOW and GAVRILOVSKAYA 2001; BOTTEN et al. 2003; ESSBAUER et al. 2006; SCHMIDT-CHANASIT et al. 2010). Studies about the viral RNA load in tissues of naturally infected reservoir rodents are limited (KORVA et al. 2009). The measured viral RNA load ranges from 10^1-10^9 copies/ml for PUUV infected *Myodes glareolus* to 10^2-10^{11} copies/ml for DOBV/SAAV infected *Apodemus flavicollis*/*A. agrarius*, in different tissues. The highest concentrations were detected in lung, spleen, kidney and urinary bladder.

The main routes and mechanisms for hantavirus transmission in natural rodent communities are not fully understood. Hantaviruses are transmitted probably mainly indirectly by inhalation of virus-contaminated rodent excreta (LEE et al. 1978; MACKOW and GAVRILOVSKAYA 2001; HARDESTAM et al. 2008a; SCHONRICH et al. 2008). This indirect transmission is supposed to be the most common way of infection not only between the reservoir hosts, but also from the reservoir host to humans. Recent investigations support this indirect way of transmission, where a high stability of hantaviruses outside the host was confirmed (KALLIO et al. 2006; HARDESTAM et al. 2007). Biting represents an alternative, but rare mode of transmission to humans (DOURON et al. 1984; SCHULTZE et al. 2002) or between rodents (GLASS et al. 1988). In line, the possibility of hantavirus transmission through saliva was discussed (KLEIN et al. 2001; HARDESTAM et al. 2008a; HARDESTAM et al. 2008b; PETTERSSON et al. 2008).

Hantaviruses and their hosts

In general, each hantavirus is closely associated to a specific mammalian species or closely related species of the same genus, as its primary reservoir host. Usually the multiple detection of hantavirus sequences in a single reservoir host and their absence in sympatrically occurring other rodent or small mammal species is believed to be indicative for a reservoir host function (HJELLE and YATES 2001). Otherwise, spillover infections can also occur. Spillover infections are infections of rodent or small mammal species other than those identified as the predominant host for a particular hantavirus.

Different host adaptation mechanisms are supposed to be necessary for the establishment of a persistent infection in the reservoir host. Hantavirus cell attachment is currently believed to be the most important process associated with the host adaptation of hantaviruses. The hantavirus attachment is mediated via binding of the viral Gn to host cell integrins (MACKOW and GAVRILOVSKAYA 2001; SPIROPOULOU 2001; RAFTERY et al. 2002). Integrins consist of different α and β subunits in different composition. For rodent-borne human apathogenic or low pathogenic PHV and TULV β1 integrin and for human pathogenic HTNV, SEOV, NYV, SNV and PUUV β3 integrin usages were described (GAVRILOVSKAYA et al. 1999; MACKOW and GAVRILOVSKAYA 2001). As cells without β3 integrin expression can permit infections with HTNV, β3 may perhaps represent not the exclusive receptor for virus attachment or entry (SONG et al. 2005; MOU et al. 2006). The potentially human pathogenic Sangassou virus (SANGV) was demonstrated to use ß1 integrin rather than ß3 integrin. In addition, the decay accelerating factor DAF was identified as entry co-receptor for SANGV, DOBV, PUUV and HTNV in Vero cells (KRAUTKRAMER and ZEIER 2008; KLEMPA et al. 2012; POPUGAEVA et al. 2012). Furthermore, an unidentified 70kDa protein and a receptor for C1q (gC1qR/p32) were reported as additional receptors for HTNV (MOU et al. 2006; CHOI et al. 2008).

Since 2007 many novel hantavirus sequences were detected in mammals other than rodents e.g. in shrews (KLEMPA et al. 2007) and moles (KANG et al. 2009c). Recently single hantavirus sequences could be found in the hairy slit-faced bat *Nycteris hispida* (WEISS et al. 2012) and in the banana pipistrelle *Neoromicia nanus* (SUMIBCAY et al. 2012), both members of the mammalian order Chiroptera.

Rodent-borne hantaviruses

Although the first discovered hantavirus TPMV was found in a shrew, the majority of hantaviruses were historically described as rodent-borne viruses. The first comprehensive studies about hantaviruses and their hosts, were about rodent-borne hantaviruses within the rodent subfamilies Murinae, Arvicolinae (Table 2), Neotominae and Sigmodontinae (JONSSON et al. 2010; SIRONEN and PLYUSNIN 2011).

Table 2 Overview of different rodent-borne hantaviruses, their geographical distribution and initially described reservoir hosts within the Rodentia subfamilies Murinae and Arvicolinae.

Virus species* (Abbreviation)	**Reservoir host** **Subfamily,** Species	**Distribution**	**Reference**
	Murinae		
Dobrava-Belgrade virus			
(DOBV-Af)	*Apodemus flavicollis*	Europe	(AVSIC-ZUPANC et al. 1992)
(DOBV-Aa, SAAV)	*Apodemus agrarius*		(PLYUSNIN et al. 1997)
(DOBV-Ap)	*Apodemus ponticus*		(KLEMPA et al. 2008)
Hantaan virus (HTNV)	*Apodemus agrarius*	Asia	(LEE et al. 1978)
Amur/ Soochong virus (ASV)	*Apodemus peninsulae*		(LIANG et al. 1994)
Thailand virus (THAIV)	*Bandicota indica*		(ELWELL et al. 1985)
Da Bie Shan virus (DBSV)	*Niviventer confucianus*		(WANG et al. 2000)
Seoul virus (SEOV)	*Rattus norvegicus*		(LEE et al. 1982)
Serang virus (SERV)	*Rattus tanezumi*		(PLYUSNINA et al. 2009)
Sangassou virus (SANGV)	*Hylomyscus alleni* (*simus*)	Africa	(KLEMPA et al. 2006)
	Arvicolinae		
Tula virus (TULV)	*Microtus arvalis, M. levis*†	Europe	(PLYUSNIN et al. 1994; SIBOLD et al. 1995)
Khabarovsk virus (KHAV)	*Microtus fortis*	Asia	(HORLING et al. 1996)
Vladivostok virus (VLAV)	*Microtus fortis*		(KARIWA et al. 1999)
Yuanjiang virus (YUJV)	*Microtus fortis*		(ZOU et al. 2008c)
Prospect Hill virus (PHV)	*Microtus pennsylvanicus*	North America	(LEE et al. 1985)
Isla Vista virus (ISLAV)	*Microtus californicus*		(SONG et al. 1995)
Puumala virus (PUUV)	*Myodes glareolus*	Europe	(BRUMMER-KORVENKONTIO et al. 1980)
Muju virus (MUJV)	*Myodes regulus*	Asia	(SONG et al. 2007d)
Hokkaido virus (HOKV)	*Myodes rufocanus*		(KARIWA et al. 1995)
Topografov virus (TOPV)	*Lemmus sibiricus*		(PLYUSNIN et al. 1996b)

Table adapted from SIRONEN and PLYUSNIN 2011. *Hantavirus taxonomy according to KING et al 2011.The official hantavirus species are given in italics; others are tentative virus species. Rodentia taxonomy according to WILSON and REEDER 2005a. †formerly *M. rossiaemeridionalis*

For Europe, four rodent-borne hantaviruses were described so far, the murine-related DOBV, SEOV and the arvicoline-associated PUUV and TULV (Table 2).

DOBV is a hantavirus that have been proposed to appear in 3 distinct lineages, grouped according to their findings in different *Apodemus* species. The DOBV-Af lineage associated with the yellow-necked field mouse (*A. flavicollis*) was first described in formerly Yugoslavia and occurs in South-East Europe and Greece (AVSIC-ZUPANC et al. 1992; GLIGIC et al. 1992a; PAPA 2012). The Caucasus field mouse (*A. ponticus*) was found to carry another DOBV lineage (DOBV-Ap) in the

Southern part of European Russia (KLEMPA et al. 2008). In Central and Eastern Europe a DOBV-Aa lineage has been found, carried by *A. agrarius* (SIBOLD et al. 2001; KLEMPA et al. 2005; KLEMPA et al. 2008; SCHLEGEL et al. 2009). Another *A. agarius*–associated DOBV lineage, found on Saaremaa island in Estonia and called *Saaremaa virus* (SAAV), is proposed to be a distinct hantavirus species (PLYUSNIN et al. 1997; PLYUSNIN et al. 2011).

SEOV was first discovered in Asia (LEE et al. 1982), but is thought to be distributed worldwide, due to the fact that its natural host, the brown rat (*Rattus norvegicus*), is found all over the world (LIN et al. 2012). Most of the human SEOV infections are reported from China (ZHANG et al. 2010b). SEOV appears also in Europe, but no human infection is reported so far. SEOV-specific nucleic acid has been found in brown rats from France, (HEYMAN et al. 2004) and SEOV was isolated from rats and SEOV-specific antibodies were found in rat sera from Germany (PILASKI et al. 1991).

Four arvicoline-borne hantavirus species are distributed in Asia and Europe, TOPV, KHAV, PUUV and TULV. *Topografov virus* (TOPV) was detected in Siberian brown lemmings *Lemmus sibiricus* near the Topografov River in Siberia, (PLYUSNIN et al. 1996b) and *Khabarovsk virus* (KHAV) was first discovered in reed voles *Microtus fortis* from Far East Russia (HORLING et al. 1996). In addition, KHAV-sequences were also found in Maximowicz's voles *Microtus maximowiczii* from China (ZOU et al. 2008b). Both viruses are believed to have low or no pathogenicity for humans.

The most detailed investigated arvicoline-borne hantavirus is PUUV, which is responsible for the major part of human hantavirus infections in Europe (VAPALAHTI et al. 2003). PUUV was initially detected in bank voles *Myodes glareolus* from Finland (BRUMMER-KORVENKONTIO et al. 1980) and additionally in the gray red-backed vole *Myodes rufocanus* from Russia (DEKONENKO et al. 2003). However, numerous studies in other parts of Europe have pointed out, that this virus is associated with *Myodes glareolus* (BOWEN et al. 1997; ESCUTENAIRE et al. 2000a; SIRONEN et al. 2002; ESSBAUER et al. 2006).

Another important arvicoline-borne hantavirus in Central Europe is TULV. TULV has initially been found in the common vole *Microtus arvalis*, the East European vole *M. levis* (formerly *rossiaemeridionalis*), and subsequently in the field vole *M. agrestis*, common pine vole *M. (Terricola) subterraneus* and narrow-headed vole *M. (Stenocranius) gregalis* (PLYUSNIN et al. 1994;

SIBOLD et al. 1995; SCHARNINGHAUSEN et al. 2002; SONG et al. 2002; SCHMIDT-CHANASIT et al. 2010). Regarding these findings, the main reservoir host of TULV remains obscure. The human pathogenicity of this hantavirus, is also still unclear; there are some reports on serological detection of TULV-specific antibodies in humans (VAPALAHTI et al. 1996; SCHULTZE et al. 2002; CLEMENT et al. 2003; ULRICH et al. 2004). Interestingly, a serum derived from a patient with symptoms of HFRS from North-East Germany, revealed the presence of neutralizing antibodies against TULV in focus reduction neutralization assay (FRNT) (KLEMPA et al. 2003a). In addition, several sera of forestry workers from the federal state of Brandenburg showed exclusively TULV-reactive antibodies in enzyme-linked immunosorbent assay (ELISA); for one serum a TULV-specific antibody response was confirmed by FRNT (MERTENS et al. 2011b).

Insectivore-borne hantaviruses

In 1964, the first discovered insectivore-borne hantavirus TPMV, was identified in the shrew species *Suncus murinus* (order Soricomorpha) from India (CAREY et al. 1971). Approximately 40 years later, a rapidly increasing list of new soricomorph-related hantaviruses in shrews and moles were found (Table 3). Initially, a shrew-borne hantavirus, the Tanganya virus (TGNV), was discovered in the Therese's shrew *Crocidura theresae* from Africa (KLEMPA et al. 2007).

Table 3 Overview of different insectivore-borne hantaviruses, their geographical distribution and initially described reservoir hosts within the Soricomorpha subfamilies Crocidurinae, Soricinae, Talpinae and Scalopinae.

Virus species* (Abbreviation)	**Reservoir host** **Subfamily,** Species	**Distribution**	**Reference**
	Crocidurinae		
Tanganya virus (TGNV)	*Crocidura theresae*	Africa	(KLEMPA et al. 2007)
Azagny virus (AZGV)	*Crocidura obscurior*		(KANG et al. 2011c)
Imjin virus (MJNV)	*Crocidura lasiura*	Asia	(SONG et al. 2009a)
Thottapalayam virus (TPMV)	*Suncus murinus*		(CAREY et al. 1971)
	Soricinae		
Seewis virus (SWSV)	*Sorex araneus*	Eurasia	(SONG et al. 2007b)
Kenkeme virus (KKMV)	*Sorex roboratus*	Asia	(KANG et al. 2010)
Ash River virus (ARRV)	*Sorex cinereus*	North America	(ARAI et al. 2008a)
Jemez Springs virus (JMSV)	*Sorex monticolus*		
Camp Ripley virus (RPLV)	*Blarina brevicauda*		(ARAI et al. 2007)
Cao Bang virus (CBNV)	*Anourosorex squamipes*	Asia	(SONG et al. 2007c)
	Talpinae		
Asama virus (ASAV)	*Urotrichus talpoides*		(ARAI et al. 2008b)
Nova virus (NVAV)	*Talpa europaea*	Europe	(KANG et al. 2009c)
Oxbow virus (OXBV)	*Neurotrichus gibbsii*	North America	(KANG et al. 2009b)
	Scalopinae		
Rockport virus (RKPV)	*Scalopus aquaticus*		(KANG et al. 2011b)

Table adapted from SIRONEN and PLYUSNIN 2011. *Hantavirus taxonomy according to King et al. 2011. The official hantavirus species are given in italics; others are tentative virus species. Soricomorpha taxonomy according to WILSON and REEDER 2005b.

In the same year, two additional shrew-associated hantaviruses were found, the Camp Ripley virus (RPLV) detected in one of the few poisonous mammals, the Northern short-tailed shrew *Blarina brevicauda*, and Cao Bang virus (CBNV) in the Chinese mole shrew *Anourosorex squamipes* (ARAI et al. 2007; SONG et al. 2007c).

Shortly thereafter a new soricine-borne hantavirus Seewis virus (SWSV) was molecularly detected in common shrews *Sorex araneus* from Switzerland, Hungary and Finland, in Siberian large-toothed shrews *Sorex daphaenodon* and Tundra shrews *S. tundrensis* from Russia (SONG et al. 2007b; KANG et al. 2009a; YASHINA et al. 2010). Additional hantaviruses were detected in other shrew species of the subfamily Soricinae and Crocidurinae (Table 3).

During these investigations other hantaviruses were found in moles in the subfamilies Talpinae and Scalopinae (Table 3). For the mole-associated Asama virus (ASAV) and Oxbow virus (OXBV) phylogenetic relationships with soricid-borne and for Rockport virus (RKPV) with rodent-borne hantaviruses were reported, which suggest potential host-switch events in the past (ARAI et al. 2008b; KANG et al. 2009b; KANG et al. 2011b). The majority of these tentative hantavirus species were found in only one or a few animals originating from museum tissue samples. As a result of this limited data set, not for all hantaviruses, the “true” reservoir host has been verified. In particular, these discoveries of highly divergent hantaviruses, would suggest the existence of additional hantaviruses associated to other shrews and moles within the order Soricomorpha.

Hantavirus taxonomy, phylogeny and evolution

The International Committee on Taxonomy of Viruses (ICTV) has claimed guidelines for the definition of a particular hantavirus species. These guidelines include the following four criteria: a hantavirus species has to (i) be found in a unique primary reservoir species, (ii) have a 7% difference in the aa sequences of the complete N- and GPC proteins, (iii) show an at least 4-fold difference in a two-way cross-neutralization test, and (iv) be not undergone natural reassortment events with other species (KING et al. 2011). Several of the 23 hantavirus species, which are approved by the ICTV, do not fulfill all four criteria (MAES et al. 2009). Therefore, another scheme for the demarcation of hantavirus species (aa sequence distance >10% for N protein or >12% for

GPC) and hantavirus groups (aa sequence distance >24% for N protein or >32% for GPC) has been proposed (MAES et al. 2009).

Hantaviruses are thought to be relatively closely adapted to a single rodent species or closely related species of the same genus. This close relationship and the congruencies between rodent reservoir and hantavirus phylogenies (Figure 3) have lead to the hypothesis of a long-standing hantavirus–rodent host coevolution, since the common ancestor of the 4 rodent subfamilies, where hantaviruses were initially found, approximately ten million years ago (PLYUSNIN and MORZUNOV 2001).

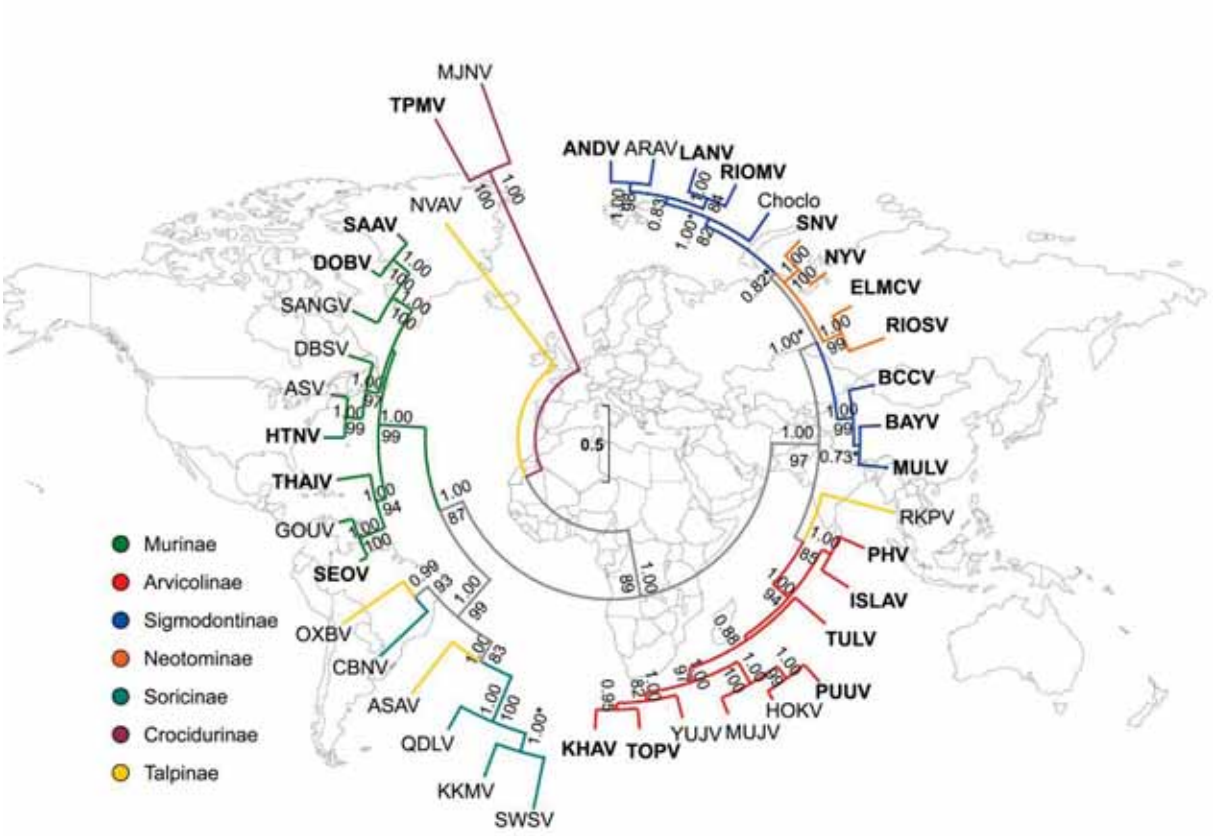

Figure 3 Bayesian phylogenetic tree of the complete N protein encoding ORF nucleotide sequences of different rodent- and insectivore-borne hantaviruses and their associations to the Rodentia and Soricomorpha subfamilies. Posterior probabilities for Bayesian analysis are given above the branches and bootstrap values for the corresponding Maximum Likelihood (ML) tree under the branches. Only values ≥0.7 and ≥70% are shown. * indicates differences in the topology between ML- and Bayesian-tree at this node. Official hantavirus species are given in bold. For virus abbreviations see Tables 2 and 3. Other abbreviations: ANDV, *Andes virus*; ARAV, Araraquara virus; BAYV, *Bayou virus*; BCCV, *Black Creek Canal virus*; ELMCV, *El Moro Canyon virus*; LANV, *Laguna Negra virus*; MULV, *Muleshoe virus*; NYV, *New York virus*; QDLV, Qiandao Lake virus; RIOMV, *Rio Mamore virus*; RIOSV, *Rio Segundo virus*; SNV, *Sin Nombre virus*.

In contrast to that, a recent investigation could ascertain no co-divergence between host and virus phylogenies and postulate an alternative scenario to the virus-host co-evolution, with geographical proximity influenced, host-specific adaptation processes and host switching (RAMSDEN et al. 2009). Such host switch events have been postulated for the evolutionary history of some rodent-borne hantaviruses i.e., TOPV (VAPALAHTI et al. 1999b), DOBV (NEMIROV et al. 2002), NYV and SNV (MORZUNOV et al. 1998) as well as for soricomorph-borne hantaviruses ASAV, Nova virus (NVAV) and OXBV (ARAI et al. 2008b; KANG et al. 2009b; KANG et al. 2009c). Recent investigations suggest that ancestral shrews or moles may have been the original hosts of ancient hantaviruses (KANG et al. 2009c; KANG et al. 2011b).

The calculated mutation rates reported for hantaviruses are contradictory. Molecular clock analysis of virus sequences from cell culture isolates and field studies showed that PUUV evolve at a calculated relatively slow rate of 0.7 x 10^{-7} to 2.2 x 10^{-6} nucleotide (nt) substitutions/site/year (SIRONEN et al. 2001). Higher substitution rates of 2.10 x 10^{-2} to 2.66 x 10^{-4} nt substitutions/site/year, were reported for Araraquara virus, DOBV, PUUV and TULV sequences derived from patients and rodents (RAMSDEN et al. 2008).

Genetic diversity in hantaviruses is thought to be caused by changes in the frequency of a gene sequence variant in a virus population (genetic drift), i.e., accumulation of nucleotide substitutions, deletions and insertions, reviewed in (SIRONEN and PLYUSNIN 2011). Therefore, selection acts on “clouds” of virus variants in the infected animal (quasispecies connected intra-host evolution). This quasispecies concept is commonly known for other RNA viruses (LAURING and ANDINO 2010) and has been experimentally analyzed also for hantaviruses *in vitro* as well as *in vivo* in animal models and in experimentally and naturally infected wild-rodent hosts (PLYUSNIN et al. 1995; PLYUSNIN et al. 1996a; LUNDKVIST et al. 1997; FEUER et al. 1999; CHUNG et al. 2007; SIRONEN et al. 2008). Additional rare processes for genetic diversity development are the reassortment, i.e. the complete exchange of a genome segment, and recombination events (SIRONEN and PLYUSNIN 2011). Genetic reassortments have been detected between different hantavirus lineages and strains (intra-species reassortment), and between different hantavirus species (inter-species reassortment). Intra-species DOBV reassortants were generated *in vitro* (KIRSANOVS et al. 2010) and were reported to occur *in vivo* (KLEMPA et al. 2003b). Additional reassortment events in nature were found for SNV (LI et al. 1995) and PUUV (RAZZAUTI et al. 2008; RAZZAUTI et al. 2009). Natural inter-species reassortment requires a double infection of an animal by two different hantavirus

species. This scenario requires a spillover infection of a non-reservoir animal and an accompanied infection of the same animal by the adapted host-specific virus. *In vitro* inter-species reassortment events have been reported for sigmodontine-borne *Black Creek Canal virus* (BCCV)/SNV and arvicoline-borne PUUV/PHV (RODRIGUEZ et al. 1998; HANDKE et al. 2010). A natural inter-species reassortment, between SEOV and HTNV, has been detected in *Rattus norvegicus* from China (ZOU et al. 2008a). Intragenomic recombination has been reported for TULV, PUUV, HTNV and ANDV *in vivo* (SIBOLD et al. 1999; SIRONEN et al. 2001; CHARE et al. 2003; MEDINA et al. 2009) and for TULV *in vitro* (PLYUSNIN et al. 2002).

Hantavirus diagnostics in reservoirs

Hantavirus detection can be done directly, i.e. by virus isolation, or indirectly, e.g. by detection of virus-specific antibodies. The isolation of hantaviruses has been tried by different approaches. Tissue samples from infected animals were most commonly used for *in vivo* or *in vitro* virus isolation. The prototype hantavirus HTNV could not be cultivated in several types of cell cultures nor in laboratory animals, but successfully propagated in the natural reservoir host *Apodemus agrarius* (LEE et al. 1978). In addition, SNV was initially passaged in *P. maniculatus* and TULV in laboratory-colonized *M. arvalis* and thereafter propagated in Vero E6 cells (CHIZHIKOV et al. 1995; VAPALAHTI et al. 1996). Similarly, laboratory rats were used to isolate SEOV from tissues of wild rats (LEE et al. 1982). In contrast to that, rodent-borne PUUV, DOBV, BCCV, CDGV, *Rio Mamore virus* (RIOMV), TOPV and shrew-borne TPMV have been cultivated and isolated in Vero cells using tissue samples from naturally infected animals (CAREY et al. 1971; YANAGIHARA et al. 1984; AVSIC-ZUPANC et al. 1992; ROLLIN et al. 1995; FULHORST et al. 1997; POWERS et al. 1999; VAPALAHTI et al. 1999b). In addition to hantavirus reservoir-derived isolates, DOBV, HTNV, PUUV and ANDV strains have been isolated from infected human patients (XIAO et al. 1994; GALENO et al. 2002). The characterization of the obtained virus isolates was performed by different direct immunofluorescence assay (IFA) and nucleic acid detection methods (see below).

Reverse transcription-polymerase chain reaction (RT-PCR) assays represent another direct method of virus detection. RT-PCR attempts, using S-, M- and L-segment genome specific primers, have been used not only for the detection of known hantaviruses, but also for the discovery of new hantaviruses (ELWELL et al. 1985; LEE et al. 1985; AVSIC-ZUPANC et al. 1992; PLYUSNIN et al. 1994; SIBOLD et al. 1995; SONG et al. 1995; JOHNSON et al. 1997; VAPALAHTI et al.

1999b). For a more sensitive hantavirus-specific nucleic acid detection in rodents and humans, DOBV-, HTNV-, SEOV- and PUUV-specific hantavirus quantitative real time RT-PCR assays (RT-qPCR) were developed (AITICHOU et al. 2005; HARDESTAM et al. 2008a; IBRAHIM et al. 2011). Further RT-qPCR assays were applied for reservoir host samples, which results in the detection of PUUV, DOBV and TULV positive rodents (WEIDMANN et al. 2005; KRAMSKI et al. 2007). These assays have limitations in detecting viruses, where the virus genome sequences are too variable and consequently the hybridization of the probe could fail, due to the use of hantavirus-specific DNA probes. A probe-independent RT-qPCR (SYBR-Green) assay has been only generated for detection of DOBV so far (JAKAB et al. 2007a; NEMETH et al. 2011). A broader, RT-nested-PCR approach was developed in 2006 and resulted in the discovery of three novel hantaviruses (KLEMPA et al. 2006; KLEMPA et al. 2007; WEISS et al. 2012). The multiple findings of other new hantaviruses in the previous years are realized by the establishment of large panels of different insectivore-borne hantavirus universal primers and touch-down PCR protocols (KANG et al. 2011a; KANG et al. 2011c; ARAI et al. 2012; SUMIBCAY et al. 2012).

The indirect detection of hantavirus infections is based on different serological assays using virus preparations, recombinant antigens produced in bacteria, yeast, insect and mammalian cells, and synthetic peptides (YOSHIMATSU et al. 1993a; KALLIO-KOKKO et al. 2000; KRUGER et al. 2001; RAZANSKIENE et al. 2004). Usually, indirect IFAs and immunoglobulin (Ig)G and IgM ELISA formats have been used for detection of hantavirus-specific antibodies in rodents (LEE et al. 1978; BRUMMER-KORVENKONTIO et al. 1980; LEE et al. 1982; BHARADWAJ et al. 1997; DEKONENKO et al. 2003; ROSA et al. 2005; VAHERI et al. 2008; HOLSOMBACK et al. 2009; BLASDELL et al. 2011; CHANDY et al. 2012; SANADA et al. 2012). Thus, the serological detection of PUUV, DOBV and TULV infections in rodent reservoirs is based on homologous antigens expressed in heterologous systems (ESSBAUER et al. 2006; SCHLEGEL et al. 2009; SCHMIDT-CHANASIT et al. 2010; MERTENS et al. 2011c). In rare cases, FRNTs were explored to more precisely identify the causative hantavirus species (HORLING et al. 1996; VAPALAHTI et al. 1996). In addition, a rapid field test was also developed for detection of anti-PUUV antibodies in rodents (SIROLA et al. 2004).

Immunological investigations in shrews and moles are limited by the lack of commercially available species-specific secondary anti-IgG antibodies. The only described serological system for detection of insectivore-borne hantavirus-specific antibodies in shrew sera is based on a mAb E5/G6 based capture ELISA, using a recombinant TPMV N fusion protein with an E5/G6 epitope

and HRP-conjugated protein A (OKUMURA et al. 2007). As almost all previous investigations in insectivores were mainly based on RT-PCR approaches, little is known about the humoral immune response against the new soricomorph-borne hantaviruses and its characteristics.

Objectives of the study

Recent reports on novel hantaviruses in shrews, moles and bats and the detection of spillover infections of rodent-borne hantaviruses in non-reservoir rodent species raise important questions on their host range and adaptation. Little is known about the distribution and frequency of hantavirus spillover infections in natural rodent and insectivore populations. In addition, for several hantaviruses, which have been detected in one single animal only, the “true” reservoir host still remains unknown.

Therefore, the objectives of the study were to characterize the host association and the presence, distribution and frequency of spillover infections for the rodent-borne TULV and the insectivore-borne SWSV in Central Europe.

For this purpose, novel laboratory techniques for molecular and serological detection of rodent- and insectivore-borne hantaviruses have to be developed. This includes the establishment of broad spectrum molecular assays for the identification of small mammal species, and known and new hantavirus species in Central Europe, the generation of shrew-associated hantavirus-specific monoclonal antibodies and anti-shrew IgG, as well as the development of an indirect ELISA to detect hantavirus-specific antibodies in shrews.

Paper I

Molecular identification of small mammal species using novel *cytochrome b* gene-derived degenerated primers

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NOTE

Molecular Identification of Small Mammal Species Using Novel *Cytochrome b* Gene-Derived Degenerated Primers

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Introduction

The close association and adaptation of several RNA and DNA viruses to their reservoir hosts are believed to be due to a long-lasting virus-host coevolution (Gottschling et al. 2007; Henttonen et al. 2008; Ehlers et al. 2008). Hantaviruses are examples of rodent- and insectivore-borne RNA viruses in which each virus species seems to be associated with a single reservoir species or closely related species of the same genus (Henttonen et al. 2008). There is increasing evidence, however, for hantavirus spillover infections from a reservoir host species to closely related sympatrically occurring rodent species (Schlegel et al. 2009; Schmidt-Chanasit et al. 2010). For these reasons a correct species annotation of animals infected by hantaviruses or other pathogens is urgently needed. A certain species identification based on morphological characters is sometimes difficult under field conditions or is hampered by the type and quality of the available sample, especially if only tail, ear, or blood samples are collected.

The *cytochrome b* (*cyt b*) gene has been used in many studies for species identification in various animal taxa (Irwin et al. 1991; Parson et al. 2000; Hsieh et al. 2001; Rajapaksha et al. 2002; Ono et al. 2007; Tsai et al. 2007). Some published broad-spectrum protocols were successfully applied for the determination

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of distantly related animal species (Kocher et al. 1989; Verma and Singh 2003); however, in an initial alignment of the primer pairs described in those papers with 18 different *cyt b* sequences originating from the rodent subfamilies Murinae and Arvicolinae and the soricomorph subfamilies Soricinae and Crocidurinae (representing the main target taxa of our zoonosis research), we found a large number of mismatches ($n = 30–69$; $n = 8–30$). Therefore, the objective of this study was the development of a simple, robust, and broad-spectrum molecular assay for identification of diverse small mammal species in Germany using novel degenerated primers.

Materials and Methods

Small mammal samples were taken from the tissue collection of the network "Rodent-borne pathogens" (Ulrich et al. 2008) stored at the Friedrich-Loeffler-Institut or were provided by additional collaborators. These samples represent 17 of the 23 genera and 26 of the 33 species of wild rodents and insectivores present in Germany. The morphological species determination was performed using common identification keys (Niethammer 1978, 1982; Görner and Hackethal 1988; Stresemann 1989).

For DNA preparation, about 50–100 mg frozen tissue was homogenized in 500 µl TNES-urea buffer (10 mM Tris–HCl, 0.3 M NaCl, 1% SDS, 10 mM EDTA, and 4 M urea, adjusted with HCl to pH 8.0) with stainless steel beads. Total DNA was extracted from the supernatant by a standard phenol–chloroform method. The DNA was dissolved in 100 µl H_2O and stored at 4°C for immediate use or frozen at –20°C and, for long-term storage, at –70°C.

The polymerase chain reaction (PCR) was carried out in a 50 µl reaction volume containing 10–50 ng template DNA, 25 mM each dNTP, 10 pmol each primer, 1.5 mM $MgCl_2$, 1 U Platinum *Taq* DNA polymerase (Invitrogen, Darmstadt, Germany), and 10× PCR buffer. The PCR amplification was performed with an initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 47°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The amplification of the expected products was proven in a 1.5% agarose gel stained with ethidium bromide. The PCR products were then purified and sequenced at least two times in both directions using the BigDye terminator sequencing kit (Perkin Elmer, Waltham, MA) on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The DNA sequences obtained were submitted to GenBank (Table 1).

Pairwise and multiple sequence alignments were generated using ClustalW (Thompson et al. 1994). The novel *cyt b* sequences were compared with GenBank sequences with the Blast algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic analysis was performed first using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) with Bayesian Metropolis–Hastings Markov chain Monte Carlo (MCMC) tree-sampling methods and then by calculating neighbor joining with Mega4 (Tamura et al. 2007). The optimal nucleotide substitution model was chosen using jModelTest (Posada 2008).

Table 1 New *cytochrome b* gene sequences generated from rodents and small mammals in this study

Order and family	Subfamily	Species	Geographic origin (coordinates of trapping site in Germany)	Accession no. (*cyt b* gene)
Rodentia				
Muridae	Murinae	*Apodemus agrarius* (Pallas, 1771)	54.141584, 13.221989	JF318967
		Apodemus flavicollis (Melchior, 1834)	54.166637, 13.353360	JF318968
		Apodemus sylvaticus (Linnaeus, 1758)	50.939644, 6.917732	JF318999
		Micromys minutus (Pallas, 1771)	54.196122, 13.285725	JF318978
		Mus musculus Linnaeus, 1758	48.804451, 9.205745	JF318982
		Rattus norvegicus (Berkenhout, 1769)	51.49562, 9.14669	JF318991
		Rattus rattus (Linnaeus, 1758)	51.544963, 12.703700	JF318992
Cricetidae	Arvicolinae	*Ondatra zibethicus* (Linnaeus, 1766)	52.143197, 14.042573	JF318989
		Arvicola amphibius (Linnaeus, 1758)	52.194065, 7.965814	JF318969
		Microtus arvalis (Pallas, 1778)	52.829181, 12.310338	JF318980
		Microtus agrestis (Linnaeus, 1761)	52.339241, 11.111183	JF318979
		Microtus subterraneus (de Selys-Longchamps, 1836)	51.999742, 7.335720	JF318998
		Microtus oeconomus (Pallas, 1776)	54.166738, 13.353360	JF318981
		Myodes glareolus (Schreber, 1780)	52.838717, 13.811817	JF318986
Caviidae	Caviinae	*Cavia porcellus*[a] (Linnaeus, 1758)	–	JF318970
Castoridae	–	*Castor fiber* Linnaeus, 1758	51.364956, 12.688515	JF318973
Myocastoridae	–	*Myocastor coypus* (Molina, 1782)	52.145836, 12.586098	JF318985
Gliridae	Glirinae	*Glis glis* (Linnaeus, 1766)	52.249542, 8.066066	JF318976
Sciuridae	Sciurinae	*Sciurus vulgaris* Linnaeus, 1758	50.593517, 12.650363	JF318993
Soricomorpha				
Soricidae	Soricinae	*Sorex araneus* Linnaeus, 1758	53.915021, 11.925902	JF318994
		Sorex minutus Linnaeus, 1766	51.895703, 11.291106	JF318995

Table 1 continued

Order and family	Subfamily	Species	Geographic origin (coordinates of trapping site in Germany)	Accession no. (*cyt b* gene)
		Neomys fodiens (Pennant, 1771)	50.541178, 8.903704	JF318987
	Crocidurinae	*Crocidura russula* (Hermann, 1780)	51.975249, 7.567366	JF318972
		Crocidura suaveolens (Pallas, 1811)	51.942116, 13.880683	JF318997
		Crocidura leucodon (Hermann, 1780)	52.990593, 12.65487	JF318971
Talpidae	Talpinae	*Talpa europaea* Linnaeus, 1758	54.183291, 13.363445	JF318996
Erinaceomorpha				
Erinaceidae	Erinaceinae	*Erinaceus europaeus* Linnaeus, 1758	54.096077, 13.399172	JF318975
Lagomorpha				
Leporidae	–	*Oryctolagus cuniculus*[a] (Linnaeus, 1758)	–	JF318990
		Lepus europaeus Pallas, 1778	50.89687, 14.786475	JF318977
Carnivora				
Mustelidae	Mustelinae	*Mustela nivalis* Linnaeus, 1766	51.246882, 12.704866	JF318983
		Mustela putorius Linnaeus, 1758	51.371543, 12.804433	JF318984
Chiroptera				
Vespertilionidae	–	*Eptesicus serotinus* (Schreber, 1774)	–	JF318974
		Nyctalus noctula (Schreber, 1774)	48.523577, 9.052041	JF318988

[a] Domesticated animal

Results and Discussion

On the basis of the multiple nucleotide sequence alignment of 18 complete *cyt b* gene sequences from rodent and other small mammal species, two conserved regions were identified at nucleotide positions 82–101 and 1009–1028 of the *cyt b* gene (numbering according to a *cyt b* gene sequence of *Mus musculus*, GenBank accession no. EF108332; Fig. 1). To overcome the sequence variations in these selected regions, we designed the following degenerated primers: CytB Uni fw 5′-TCATCMTGATGAAAYTTYGG-3′ and CytB Uni rev 5′-ACTGGYTGDCC BCCRATTCA-3′ (Fig. 1).

The performance of these novel primers in the *cyt b* PCR amplification was tested with tissue samples from 19 rodent and 14 other wild or domesticated small mammal species from Germany (Table 1). In all, 33 novel *cyt b* sequences from

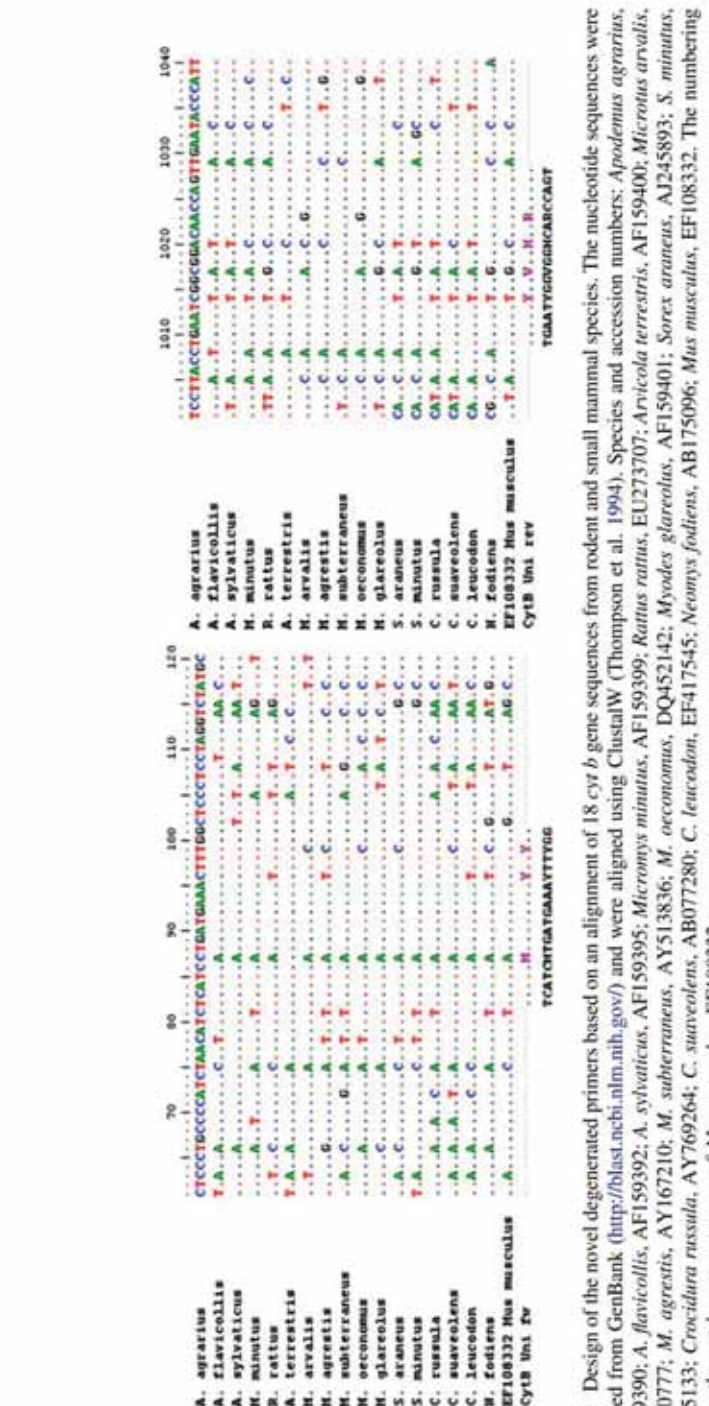

Fig. 1 Design of the novel degenerated primers based on an alignment of 18 *cyt b* gene sequences from rodent and small mammal species. The nucleotide sequences were obtained from GenBank (http://blast.ncbi.nlm.nih.gov/) and were aligned using ClustalW (Thompson et al. 1994). Species and accession numbers: *Apodemus agrarius*, AF159390; *A. flavicollis*, AF159392; *A. sylvaticus*, AF159395; *Microtus minutus*, AF159399; *Rattus rattus*, EU273707; *Arvicola terrestris*, AF159400; *Microtus arvalis*, AY220777; *M. agrestis*, AY167210; *M. subterraneus*, AY513836; *M. oeconomus*, DQ452142; *Myodes glareolus*, AF159401; *Sorex araneus*, AJ245893; *S. minutus*, AB175133; *Crocidura russula*, AY769264; *C. suaveolens*, AB077280; *C. leucodon*, EF417545; *Neomys fodiens*, AB175096; *Mus musculus*, EF108332. The numbering follows the *cyt b* gene sequence of *Mus musculus*, EF108332

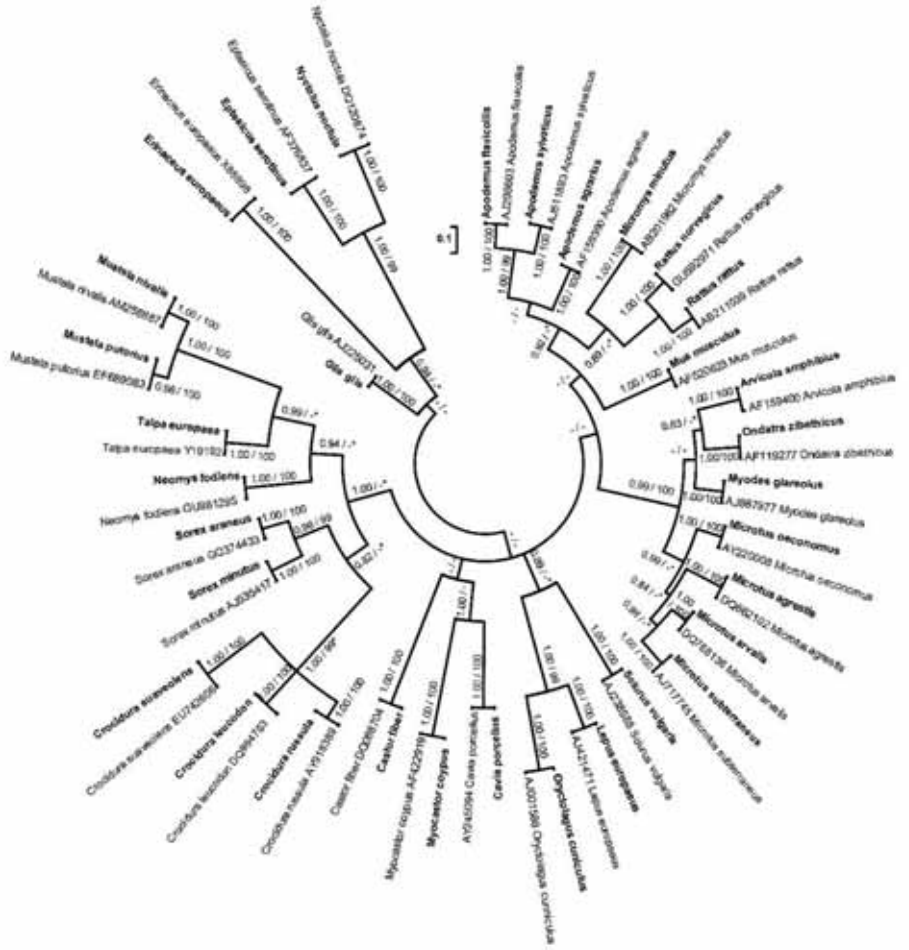

Fig. 2 Bayesian unrooted phylogenetic tree based on partial *cyt b* sequences of the analyzed specimens (in *bold*) and on corresponding GenBank sequences (given with their accession numbers). The tree is based on the region spanning nucleotides 169–795 (numbering according to *cyt b* sequence of *Mus musculus*, acc. no. EF108332). Values on branches are (*before the slash*) posterior probability for Bayesian analysis (≥0.7) based on two MCMC runs consisting of four chains of 2,000,000 generations with a burn-in of 25%, and (*after the slash*) bootstrap for the corresponding neighbor-joining analysis (≥70%, 100,000 replicates). *Scale bar* indicates number of nucleotide substitutions per site; an *asterisk* indicates topology differences between Bayesian and neighbor-joining tree at this node

various small mammal species prevalent in Germany were generated in this study. This panel includes not only representatives of the rodent families Muridae, Cricetidae, Caviidae, Castoridae, Myocastoridae, Gliridae, and Sciuridae and the soricomorph families Soricidae and Talpidae but also representatives of the orders Erinaceomorpha, Lagomorpha, Carnivora, and Chiroptera. An analysis of these sequences with the Blast algorithm resulted in the identification of all species based on the nearest matches with corresponding sequences in GenBank. The phylogenetic analysis illustrates the closest match for each novel sequence with GenBank

sequences already available (Fig. 2). The species identification accomplished by this approach was in line with that of the morphological species determination.

In conclusion, the described method is a very useful tool for identifying small mammal species in Germany and central Europe.

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Paper II

Novel serological tools for detection of *Thottapalayam virus*, a soricomorpha-borne hantavirus

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ORIGINAL ARTICLE

Novel serological tools for detection of Thottapalayam virus, a Soricomorpha-borne hantavirus

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Abstract We developed serological tools for the detection of hantavirus-specific antibodies and hantavirus antigens in shrews. The work was focussed to generate Thottapalayam virus (TPMV)-specific monoclonal antibodies (mAbs) and anti-shrew immunoglobulin G (IgG) antibodies. The mAbs against TPMV nucleocapsid (N) protein were produced after immunization of BALB/c mice with recombinant TPMV N proteins expressed in *Escherichia coli*, baculovirus and *Saccharomyces cerevisiae*-mediated expression systems. In total, six TPMV N-protein-specific mAbs were generated that showed a characteristic fluorescent pattern in indirect immunofluorescence assay (IFA) using TPMV-infected Vero cells. Out of the six mAbs tested, five showed no cross-reaction to rodent-associated hantaviruses (Hantaan, Seoul, Puumala, Tula, Dobrava-Belgrade and Sin Nombre viruses) in IFA and enzyme-linked immunosorbent assay (ELISA), although one mAb reacted to Sin Nombre virus in IFA. None of the mAbs cross-reacted with an amino-terminal segment of the shrew-borne Asama virus N protein. Anti-shrew-IgG sera were prepared after immunization of rabbits and BALB/c-mice with protein-G-purified shrew IgG. TPMV-N-protein-specific sera were raised by immunisation of Asian house shrews (*Suncus murinus*) with purified yeast-expressed TPMV N protein. Using these tools, an indirect ELISA was developed to detect TPMV-N-protein-specific antibodies in the sera of shrews. Using an established serological assay, high TPMV N protein specific antibody titres were measured in the sera of TPMV-N-protein-immunized and experimentally TPMV-infected shrews, whereas no cross-reactivity to other hantavirus N proteins was found. Therefore, the generated mAbs and the established ELISA system represent useful serological tools to detect TPMV, TPMV-related virus antigens or hantavirus-specific antibodies in hantavirus-infected shrews.

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Introduction

Hantaviruses, which are members of the family *Bunyaviridae*, genus *Hantavirus*, are negative-sense, single-stranded RNA viruses with three segments, designated large (L), medium (M), and small (S), which encode an RNA-dependent RNA polymerase, two envelope glycoproteins (G1/Gn and G2/Gc) and a nucleocapsid (N) protein [11]. Rodent-borne hantaviruses are the causative agents of two viral zoonoses: haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). In general, each hantavirus species is associated with a single predominant mammal species or a closely related species of the same genus that serves as its natural reservoir. This close relationship and the congruencies of phylogenies between hantaviruses and their hosts, could be explained by a virus-host co-evolution or, alternatively, by host-switching events followed by host adaptation processes [31, 32, 36].

Thottapalayam virus (TPMV), which was isolated from an Asian house shrew (order Soricomorpha, family Soricidae, *Suncus murinus*) that was captured during a survey for Japanese encephalitis virus in Tamil Nadu, India in 1964, for a long time represented the only exception of a non-rodent associated hantavirus [5]. Only recently was the complete genome of TPMV determined and the host association with *S. murinus* confirmed [38]. Recently, in addition to TPMV, numerous shrew- and mole-associated hantaviruses have been reported from America, Europe, Asia and Africa [1, 2, 12–14, 16, 37, 39, 40, 46]. These investigations were facilitated by the development of a broad-spectrum pan-L RT-PCR assay [15]. Previous investigations were mainly based on RT-PCR approaches. Therefore, little is known about the pathogenicity, the course of infection, or the humoral immune response in infected hosts. Moreover, the antigenic characteristics of TPMV and the new Soricomorpha-borne hantaviruses and their differences to those of rodent-borne hantaviruses have not yet been investigated. As evidenced by nucleotide (nt) and amino acid (aa) sequence analysis of the full-length S, M, and L segments and the encoded proteins, TPMV is the most genetically divergent from all other hantaviruses [38, 43]. In addition, either very low or no antigenic cross-reactivity has been observed between TPMV and other hantaviruses [6]. The current serological detection system for TPMV-specific antibodies in human and shrew sera is based on a monoclonal antibody (mAb) E5/G6 capture enzyme-linked immunosorbent assay (ELISA) using a recombinant TPMV N fusion protein with an E5/G6 epitope. Using this assay, a TPMV infection in a Laotian immigrant with a febrile illness was detected, and anti-TPMV antibodies in two out of 14 Asian house shrews captured in Indonesia were also detected [30].

For rodent-borne hantaviruses, a large panel of N- and glycoprotein-specific mAbs has been developed [3, 7, 8, 10, 17–24, 26, 34, 35, 41, 45, 49–51], but none are available for Soricomorpha-associated hantaviruses. In addition, immunological or serological investigations in shrews are also limited by the lack of commercially available species-specific secondary antibodies. For these reasons, our study was focussed on two areas: generation of TPMV-specific mAbs for detection of TPMV in infected cells and development of anti-shrew IgG antibodies applicable as species-specific secondary antibodies in immunological assays. The generation of the TPMV antigen using three different expression systems should prove to be valuable for generating TPMV-specific mAbs that are able to recognize the native antigen in virus-infected cells.

Materials and methods

Generation of recombinant full-length and truncated N proteins in *Escherichia coli* and insect cells

The full-length recombinant N protein of TPMV (accession no. AY526097) was expressed previously in both *E. coli* and insect cells using a baculovirus vector [30]. To construct plasmids expressing truncated N proteins spanning aa 1-80, 1-177, and 1-311 in *E. coli*, the corresponding coding regions were amplified by PCR using the following primers (restriction sites are given in capital letters): forward primer TPMVNATG#637, 5′-ttc aGA ATT Cga tga ctc aag gga aaa tga ctc ccg aag a; reverse primers TPMV290, 5′-ggC TCG Aga gca agc ata gct tgc ccg g, TPMV580, 5′-atC TCG AGg tcc tcc atg cat gag tca t; TPMV980, 5′-aaC TCG AGt ggg gtg gct tct gac tca a. To express a truncated N protein of shrew-borne Asama virus (ASAV; accession number EU929070), a region of the S segment encoding the amino-terminal 103 aa of the N protein was amplified by PCR using the primer pair ASAVs-ATG-EcoR I (5′-ata GAA TTC atg gac aac att gag gac atcc) and ASAVs-tr-NP-103-Xho I-TAG (5′-ata CTC GAG cta gat tgt att acc ata ccg c). The TPMV- and ASAV-derived PCR products that were obtained were ligated to the plasmid vector pET43.1b (Novagen, Merck, Darmstadt, Germany). The recombinant expression plasmids were used to transform competent BL21 cells (Invitrogen, Life Technologies, New York, USA). Synthesis of recombinant proteins was induced by the addition of isopropyl *β*-D-1-thiogalactopyranoside (IPTG: Wako, Osaka, Japan). Truncated N proteins fused to Nus-tag (491 amino acids) were extracted from *E. coli* and purified using a His-trap column according to the instructions of the manufacturer (Amersham, GE Healthcare Bio-Sciences, New York,

USA). An attempt to express the entire ASAV N protein in *E. coli* failed (data not shown).

Construction of a yeast expression plasmid and yeast expression of TPMV and other hantavirus N proteins in *Saccharomyces cerevisiae*

The entire open reading frame of the TPMV N protein was amplified by PCR using plasmid pFastBac–TPMV N [30] as a template with primers TPMV-5′ (5′-gat TCT AGA act caa ggg aaa atg act ccc gaa gag-3′) and TPMV 3′ (5′-gat TCT AGA tta cag ttt aat agg ctc ctg act tga-3′), which resulted in the addition of XbaI restriction sites to facilitate subsequent cloning. The XbaI-digested PCR product was cloned into XbaI-linearized yeast expression plasmid pFX7-His6 [33]. DNA sequence determination revealed a few nt exchanges compared to the published sequence (accession no. AY526097), which led to four aa exchanges (Q20K, A102T, G346S, G357A). For expression, *S. cerevisiae* strain gcn2 was transformed with a pFX7-derived expression plasmid encoding the TPMV N protein (pFX7-His6-TPMV N). Cultivation of yeast cells and expression and purification of TPMV, Tula virus (TULV), Puumala virus (PUUV) and Dobrava-Belgrade virus (DOBV) N proteins were performed as described previously [27, 28, 33].

Preparation of TPMV-N-protein specific monoclonal antibodies

Female BALB/c mice (Japan SLC, Inc, Hamamatsu, Japan and Taconic Europe, Ry, Denmark) were immunized four times with different recombinant TPMV N protein antigens (Table 1). Three days prior to fusion, the mice received a final immunization, and spleen cells from immunized mice were fused with myeloma P3X63Ag8U1 or SP2/0 cells using PEG1500 (Sigma-Aldrich, St. Louis, USA). Hybridoma supernatants were screened for TPMV-N-protein-specific antibodies in IFA and ELISA. Positive clones were re-cloned twice and TPMV-specific mAbs were further characterized as described below. The mAbs EB5, ED5, 1A3 and B5H9 were selected for subtyping in cell culture supernatant by ELISA using peroxidase-conjugated rabbit anti-mouse IgG-1, IgG-2a, IgG-2b, IgG-3, IgM, IgG (Zymed, Vienna, Austria), Lambda and Kappa (Caltag, Buckingham, UK) (1:5000). The mAbs 2H6 and 1F1 were subtyped using a Mouse Monoclonal Subtyping Kit (AbD Serotec, Kidlington, UK).

Indirect immunofluorescence assay (IFA)

In-house IFAs were performed essentially as described previously [47]. Acetone-fixed, TPMV, HTNV, SEOV, PUUV, TULV, or DOBV-infected Vero cells were used as antigens. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Zymed), was used as a secondary antibody. Commercial IFAs (Euroimmun, Lübeck, Germany) were performed according to the manufacturer's instructions using FITC-conjugated anti-mouse Ig (DakoCytomation, Glostrup, Denmark) as a secondary antibody.

Table 1 Characteristics of monoclonal antibodies produced in this study

Clone ID	Expression system used for generation of immunogen	Class/ subclass	ELISA detection with screening antigen
EB5	Baculovirus	IgM	*E. coli*-expressed TPMV N protein
ED5	Baculovirus	IgM	*E. coli*-expressed TPMV N protein
1A3	*Escherichia coli*	IgG1/kappa	*E. coli*-expressed TPMV N protein
B5H9	*Escherichia coli*	IgG*	*E. coli*-expressed TPMV N protein
2H6	*Saccharomyces cerevisiae*	IgG1/kappa	Yeast-expressed TPMV N protein
1F1	*Saccharomyces cerevisiae*	IgG1/kappa	Yeast-expressed TPMV N protein

Enzyme-linked immunosorbent assay, ELISA; Ig, immunoglobulin; N, nucleocapsid; TPMV, Thottapalayam virus

* The subclass and light chain of B5H9 were not defined

Enzyme-linked immunosorbent assay (ELISA)

Flat-bottomed 96-well plates were coated with purified *E. coli* or insect-cell-expressed TPMV and ASAV N proteins in phosphate-buffered saline (PBS), or with *S. cerevisiae* expressed TPMV, TULV, PUUV and DOBV N proteins in 0.05 M carbonate buffer and incubated overnight at 4 °C. PBS with 0.05 % Tween 20 containing 3 or 1 % bovine serum albumin (Sigma-Aldrich, St. Louis, USA) was added to block unsaturated binding sites, and the plates were incubated for 1 h at 37 °C or room temperature. After washing three times with 0.05 % PBS-Tween, hybridoma supernatants were added, and the plates were incubated for 1 h at 37 °C. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000) and *o*-phenylendiammonium dichloride substrate (Sigma-Aldrich, St. Louis, USA) or 3,3′,5,5′-tetramethylbenzidine (Bio-Rad, Hercules, California, USA). After incubating for 15 min at room temperature, the absorbance at 450 nm was measured.

Western blot test

The reactivity of the mAbs with full-length or truncated recombinant N proteins of TPMV and ASAV was tested in

a western blot test (WB) as described previously [48]. The purified yeast-expressed TPMV N protein was tested in the WB with mAbs raised against PUUV (2C6, 7A5, 5E11, 5C5, [52]; A1C5, [51]; 4C3, 2E12, 5A3, 1C12, [25]), SNV/ANDV (7G2, 4H3, [18]) and HTNV (E5/G6, ECO2, [49]; B5D9, [51]). The synthesis of the Nus fusion proteins was confirmed in the WB by staining with anti-NUS-tag mAb (Novagen).

Purification and biotinylation of the immunoglobulin fraction

The mAbs ED5 (IgM) and 1A3 (IgG) were purified using an IgM purification kit (Pierce, Thermo Scientific, Rockford, Illinois, USA) using protein-A Sepharose column chromatography and a MAPS II kit (Bio-Rad), respectively. The purified mAbs were biotinylated using a Biotin-AC5-OSu kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Competitive binding to *E. coli*-and insect-cell-expressed TPMV N proteins in ELISA

The relative binding avidity of mAbs to solid-phase recombinant antigen was evaluated by indirect ELISA. Flat-bottomed 96-well microtitre plates were coated with serial twofold dilutions of purified truncated N protein in PBS and then incubated overnight at 4 °C. Nonspecific binding was blocked with PBS containing 3 % bovine serum albumin for 1 h at room temperature. The plates were then washed three times with 0.05 % PBS-Tween. Dilutions of each biotin-labeled mAb were added and incubated for 1 h at room temperature. After additional washing, HRP conjugate (1:2000; Prozyme, Hayward, Canada) was added to the wells and incubated for 1 h at room temperature. Thereafter, *o*-phenylendiammonium dichloride substrate was added, and the plates were incubated for 30 min at room temperature. The relative binding avidity was defined as the amount of antibody required to yield an A450 value ranging from 0.4 to 1.4. Absorbance values at 405 nm were compared with those in the absence of a competitive antibody. For use in competitive binding assays, the serial fivefold dilutions of each unlabeled antibody were added to 96-well plates coated with antigen as described above. After a one-hour incubation at room temperature, the plates were washed, and binding of biotinylated mAbs was detected with avidin-peroxidase as described earlier.

Establishment of an ELISA to detect TPMV-specific antibodies in shrew sera

To establish this diagnostic tool, anti-shrew-IgG antisera were first prepared. Briefly, two BALB/c mice and two rabbits were immunized five times at four-week intervals with 200 µg protein-G-purified shrew IgG. To obtain shrew anti-TPMV-N sera, three Asian house shrews were immunized four times at four-week intervals with 50 µg of dialyzed TPMV N protein, and blood was taken after each immunization. In addition, two Asian house shrews were inoculated intraperitonially with 10^4 FFU of TPMV (Arikawa et al. unpublished data).

To test the shrew anti-TPMV-N-protein antisera, the anti-shrew-IgG, and the sera from experimentally TPMV-infected shrews, an indirect cross-titration ELISA was performed. Briefly, plates were coated as described earlier. The different shrew sera were tested on plates coated with TPMV, TULV, PUUV or DOBV N protein. As secondary antibodies, the anti-shrew-IgG antisera were cross-titrated. To determine the amount of antibody bound, an anti-mouse-IgG HRP-conjugate was used as a third antibody. All incubations were done for 1 h at 37 °C, and between the single incubation steps, the plates were washed three times as described above.

Results

For the generation of TPMV-N-protein-specific mAbs, the entire N protein of 435 aa residues was expressed using *E. coli* and baculovirus-mediated insect cell systems [30] and a yeast expression system. The high-level yeast expression of the entire His-tagged TPMV N protein resulted in a protein of the expected molecular weight (data not shown). This purified protein did not react in the immunoblot with any of the PUUV-specific mAbs (2C6, 7A5, 5E11, 5C5, A1C5, 4C3, 2E12, 5A3, 1C12), the SNV/ANDV-specific mAbs (7G2, 4H3) or the HTNV-specific mAbs E5/G6, ECO2, B5D9 (data not shown).

Six different mAbs were generated that recognized the TPMV N protein expressed in *E. coli*, insect cells and *S. cerevisiae*, and these were characterized by ELISA, IFA and WB (Tables 1, 2). The subtyping of the generated mAbs showed that the clones EB5 and ED5 were of the IgM class, whereas the remaining clones belong to the IgG class (Table 1). All six mAbs showed a characteristic cytoplasmic fluorescent pattern in IFA when using an acetone-fixed smear of TPMV-infected Vero cells and recombinant TPMV N protein expressed in insect High Five cells (Table 2). Their specificity was confirmed in ELISA and IFA against N proteins from rodent-associated hantaviruses (Hantaan virus, HTNV; Seoul virus, SEOV; PUUV; TULV; DOBV; Sin Nombre virus, SNV). Only the mAb ED5 was found to have cross-reactivity to SNV in IFA (Table 2). None of the TPMV-specific mAbs cross-reacted in the ELISA and WB with an amino-terminal segment of the shrew-borne ASAV N protein (Table 3).

Table 2 Cross-reactivities of monoclonal antibodies with different hantaviruses in indirect immunofluorescence assay (IFA) using hantavirus-infected Vero cells and insect cells expressing hantavirus nucleocapsid (N) protein

Clone ID	TPMV		HTNV	SEOV	PUUV	TULV	SNV*	DOBV
	Infected Vero cells	N protein expressed in High Five cells	Infected Vero cells					
EB5	+	+	−	−	−	−	−	−
ED5	+	+	−	−	−	−	+	−
1A3	+	+	−	−	−	−	−	−
B5H9	+	+	−	−	−	−	−	−
2H6	+	+	−	−	−	n.d.	−	−
1F1	+	+	−	−	−	n.d.	−	−

+ positive, − negative, n.d. not done, TPMV Thottapalayam virus, HTNV Hantaan virus, SEOV Seoul virus, PUUV Puumala virus, TULV Tula virus, SNV Sin Nombre virus, DOBV Dobrava-Belgrade virus

* Recombinant N protein of SNV was expressed by baculovirus vector in High Five insect cells

Table 3 Reactivities of monoclonal antibodies against *E. coli*-expressed full-length and truncated Thottapalayam virus (TPMV) and Asama virus (ASAV) nucleocapsid (N) proteins in ELISA and western blot test

Clone ID	ELISA					Western blot test				
	TPMV N (aa)				ASAV N (aa)	TPMV N (aa)				ASAV N (aa)
	1-80	1-177	1-311	1-435	1-103	1-80	1-177	1-311	1-435	1-103
EB5	−	−	−	+	−	−	n.d.	n.d.	−	−
ED5	+	+	+	+	−	+	+	+	+	−
1A3	+	+	+	+	−	+	+	+	+	−
B5H9	−	−	−	+	−	−	n.d.	n.d.	−	−
2H6	+	+	+	+	−	+	+	+	+	−
1F1	+	+	+	+	−	−	n.d.	n.d.	−	−

aa amino acid position, + positive, − negative, n.d., not done

Four of the six mAbs that were generated were reactive in ELISA with *E. coli*-derived truncated TPMV N proteins including the amino-terminal 80 aa (Table 3). Three of these four mAbs (1A3, ED5 and 2H6) showed an identical pattern of reactivity in ELISA and WB (see Fig. 1; Table 3), whereas mAb 1F1 failed to detect the full-length and truncated N proteins in the WB. The two remaining mAbs, EB5 and B5H9, reacted with the full-length TPMV N protein in the ELISA but not in the WB. The relative binding avidity of the biotin-labeled mAbs (1A3, ED5), tested by avidin-biotin indirect ELISA, showed different competitive effects when tested with unlabeled mAbs to recombinant TPMV N protein (Fig. 2). Based on the different levels of competition, two groups were defined, one comprising mAbs 1A3 and ED5, and the other comprising EB5 and B5H9.

Purified shrew IgG from *S. murinus* was used to produce anti-shrew-IgG sera in two BALB/c mice and two rabbits. TPMV-N-protein-specific shrew control sera were produced in *S. murinus* by immunization with the yeast-expressed TPMV N protein. The specific titres of these

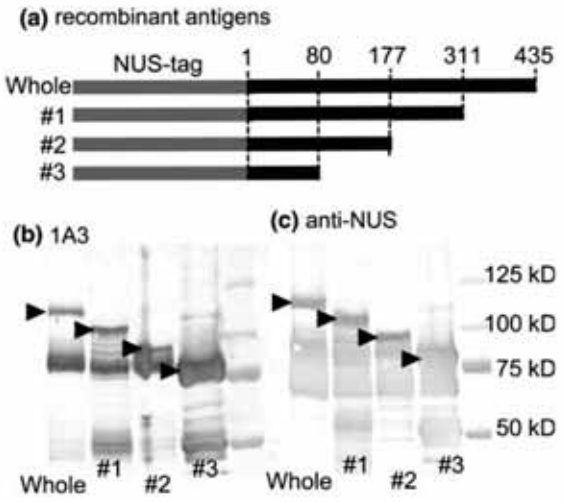

Fig. 1 Schematic representation of the structure (**a**) and western blot reactivity of full-length and truncated Thottapalayam virus nucleocapsid protein derivatives with TPMV-specific monoclonal antibody (mAb) 1A3 (**b**) and anti-NUS mAb (**c**)

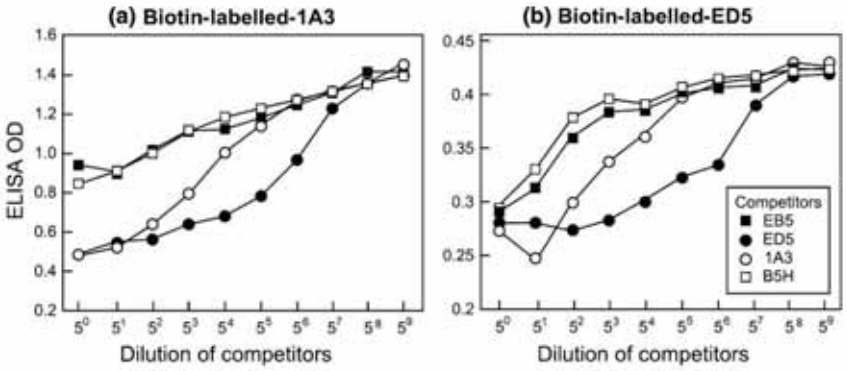

Fig. 2 Competitive binding assay with biotin-labeled mAbs 1A3 (**a**) and ED5 (**b**). Absorbance values (OD) at 405 nm are shown on the y-axis and are compared with those in the absence of competitive antibody. Dilutions of the competitive antibodies are shown on the x-axis. The relative binding avidity was defined as the amount of antibody required to yield an A450 of 0.4–1.4. Purified mAbs (1 mg/ml) were diluted from 1:5 to 1:510 and added as competitors

antisera and of the sera of experimentally TPMV-infected shrews were determined by ELISA. Using an initial cross-titration ELISA, the optimal dilution of the anti-shrew IgG for detection of TPMV-N-protein-specific antibodies in an immunized shrew was selected to be 1:32,000. By using the mouse anti-shrew-IgG at this selected dilution, strong TPMV-specific ELISA reactivities were detected in sera from all three immunized shrews and the two infected shrews (Fig. 3 and data not shown). In addition, in all three shrews, TPMV-N-protein-specific antibodies were already detected after the second immunization (data not shown). The TPMV-N-protein specific antibody titre in one of the immunized animals reached a level of 1:128,000 12 days after the final, fourth immunization. In general, no cross-reactivity to other hantavirus N proteins was found in any of the three TPMV-immunized shrews (data not shown). Only in one of the infected animals (shrew 1), 40 days postinfection, during the high IgG antibody response against TPMV, was a low cross-reactivity against DOBV detected (Fig. 3).

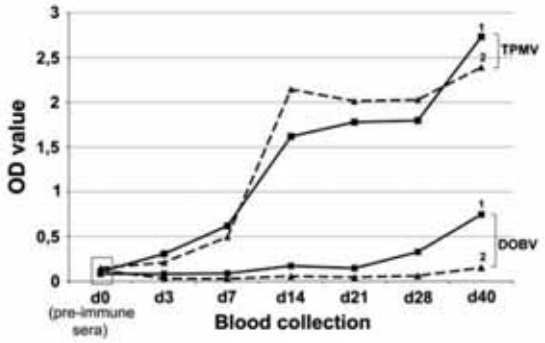

Fig. 3 ELISA reactivity of the sera from two experimentally TPMV-infected Asian house shrews against yeast-expressed TPMV and DOBV N proteins. Absorbance values (OD) of the ELISAs measured at 405 nm are shown on the y-axis. Time points after the experimental infection are given on the x-axis. Reactivities of non-immunizied shrew sera (pre-immune sera) are highlighted by a box. d, days postinfection

Discussion

Hantavirus-specific mAbs have been generated by various approaches [3, 10, 17, 19–24, 26, 34, 35, 41, 45, 49, 51]. Previously, yeast-expressed polyomavirus-derived virus-like particles harbouring 120 aa residues of the PUUV N protein and his-tagged N protein derivatives of SNV and ANDV were used for the generation of mAbs [18, 52]. These mAbs were found to react to the N antigens used for their generation, but also to a different extent to N proteins of other hantaviruses. Most importantly, these mAbs can be used to detect native viral antigen in virus-infected cell lines by IFA and in immunohistochemistry analysis of rodent and human tissue samples [18]. The TPMV-specific mAbs described here were also obtained by immunization with a His-tagged yeast-expressed TPMV N protein, confirming the value of this procedure. The generation of mAbs with similar properties using TPMV N protein from *E. coli* or baculovirus-mediated insect cell expression systems indicates the usefulness of all three different expression systems generating antigens for the production of mAbs. Moreover, the mAbs that were obtained, regardless of the expression system used for the generation of the antigen, were all found to detect native viral antigen in cell cultures.

The hantavirus N protein possesses immunodominant, linear and conformational cross-reactive epitopes within the first 100 aa of its N-terminus [9, 44, 49].

Mapping of the epitopes of N-protein-specific mAbs resulted in the identification of antigenic epitopes between aa 1-45 and 1-120 of PUUV, 166-175 of HTNV, 226-293 of TULV and 244-286 of ANDV and SNV [18, 24, 25, 42, 49]. In line with these observations, some of the TPMV-N-protein-specific mAbs described here are directed against the amino-terminal 80-aa region. The lack of reactivity of mAbs EB5 and B5H9 with the full-length N protein in the WB and with truncated N proteins in the WB and ELISA may suggest a conformation-dependent or discontinous epitope. Similarly, mAb 1F1 did not detect any TPMV N protein derivatives in the WB, suggesting that its binding site is at a conformational or discontinous epitope within the 80-aa amino-terminal region of the N protein. Future epitope mapping studies using linear synthetic peptides may allow localization of the linear epitopes of the mAbs ED5, 1A3 and 2H6 within the aa 1-80 region.

The lack of cross-reactivity of our mAbs to rodent-borne hantavirus N proteins (HTNV, SEOV, PUUV, TULV and DOBV) and ASAV N protein confirmed that TPMV is genetically and antigenetically highly divergent from all other known hantaviruses. In line with this observation, the yeast-expressed N protein of TPMV failed to react with a panel of mAbs raised against N proteins of PUUV, SNV/ANDV and HTNV. This particular feature of TPMV can be explained by the low aa sequence similarity of its N protein to those of other hantaviruses, which is also reflected in the isolated position of TPMV in phylogenetic trees [38, 43]. This lack of cross-reactivity is in contrast to the broad-spectrum cross-reactivity observed for rodent-borne hantavirus N-protein-specific mAbs with N proteins of these hantaviruses.

The mouse and rabbit anti-shrew-IgGs generated in this study are applicable in serological assays for the detection of IgG antibodies in sera of immunized and infected *S. murinus*, as demonstrated. Based on molecular data [29] and chromosome homology [4], *Suncus* represents a separated genus within the family Soricidae, which is closely associated with the genus *Crocidura*. The close relationship of these two genera, in spite of the detection of different new hantaviruses in members of the genus *Crocidura* [16, 40], may suggest that the application of the anti-shrew-IgG generated here is not limited to immunological approaches in *Suncus*. Indeed, an initial WB experiment demonstrated cross-reactivity of the rabbit anti-*Suncus* IgG with purified IgG of *Suncus*, *Crocidura* and *Sorex*, but a lack of reactivity with laboratory mouse IgG (data not shown). Additional efforts are needed to establish and validate serological assays to detect hantavirus-specific antibodies in these shrews by using defined negative and positive control sera. The efficiency of such serological assays could be improved by labelling the anti-shrew-IgGs with HRP, FITC or other fluorescent dyes.

In conclusion, the reactivity of the mAbs generated here in IFA using TPMV-infected cells indicates that these mAbs represent useful tools for detection of TPMV (and antigenically related hantaviruses) in cell culture and tissue of potentially infected animals or humans, where the shrew anti-TPMV-antisera could serve as a positive control. These serological tools will be helpful for discovering novel insectivore-associated hantaviruses and/or characterizing the humoral immune response and antigen expression in hantavirus-infected insectivores.

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Ethical statement Shrews, laboratory mice and rabbits were handled according to the Laboratory Animal Control Guidelines of the Hokkaido University Animal Research Committee in Japan and EU Council Directive 86/609/EEC for the protection of animals used for experiments in Germany.

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Paper III

Tula virus infection in the Eurasian water vole in Central Europe

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Tula Virus Infections in the Eurasian Water Vole in Central Europe

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Abstract

Recent reports of novel hantaviruses in shrews and moles and the detection of rodent-borne hantaviruses in different rodent species raise important questions about their host range and specificity, evolution, and host adaptation. Tula virus (TULV), a European hantavirus, is believed to be slightly or non-pathogenic in humans and was initially detected in the common vole *Microtus arvalis*, the East European vole *M. levis* (formerly *rossiaemeridionalis*), and subsequently in other *Microtus* species. Here we report the first multiple RT-PCR detection and sequence analyses of TULV in the Eurasian water vole *Arvicola amphibius* from different regions in Germany and Switzerland. Additional novel TULV S-, M-, and L-segment sequences were obtained from *M. arvalis* and *M. agrestis* trapped in Germany at sites close to trapping sites of TULV-RT-PCR-positive water voles. Serological investigations using a recombinant TULV nucleocapsid protein revealed the presence of TULV-reactive antibodies in RT-PCR-positive and a few RT-PCR-negative water voles. Phylogenetic analyses revealed a geographical clustering of the novel S-, M-, and L-segment sequences from *A. amphibius* with those of *M. arvalis*- and *M. agrestis*-derived TULV lineages, and may suggest multiple TULV spillover or a potential host switch to *A. amphibius*. Future longitudinal studies of sympatric *Microtus* and *Arvicola* populations and experimental infection studies have to prove the potential of *A. amphibius* as an additional TULV reservoir host.

Key Words: *Arvicola amphibius*—Central Europe—Hantavirus—Tula virus.

Introduction

HANTAVIRUSES REPRESENT A GENUS OF THE FAMILY Bunyaviridae with emerging human pathogenic representatives in Europe, Asia, and the Americas. Despite the problem of isolating and propagating these viruses, in the last three decades the knowledge of European and Asian hantaviruses has broadened significantly. Thus many new hantavirus species and strains, their host association, and prevalence and the frequency of human infection and disease have been characterized. Most importantly, besides the rodent-borne hantaviruses during recent years a large number of novel hantaviruses were detected in different shrew and mole species in Europe and Asia, with thus far unknown human pathogenicity (Song et al. 2007a,2007b,2007c,2009; Arai et al. 2008; Kang et al. 2009). Transmission of hantaviruses is believed to be mainly indirect by inhalation of virus-contaminated rodent-excreta derived aerosols, whereas bites seem to represent a very rare mode of transmission (Schönrich et al. 2008).

On the Eurasian continent hantaviruses may cause hemorrhagic fever with renal syndrome (HFRS) of different severity and case fatality rates of up to 10% (Krüger et al. 2011). One representative is the bank vole *Myodes glareolus*-transmitted Puumala virus (PUUV) distributed in almost all parts of Europe and causing a mild to moderate form of HFRS, designated nephropathia epidemica, with a case fatality rate of <0.1% (Brummer-Korvenkontio et al. 1999). Second, Dobrava-Belgrade virus (DOBV) genetic lineages DOBV-Af, DOBV-Ap, DOBV-Aa, and Saaremaa associated with the yellow-necked mouse *Apodemus flavicollis*, Caucasian wood

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mouse *A. ponticus*, or striped field mouse *A. agrarius* cause more severe, moderate, or mild/moderate cases of HFRS in certain parts of Europe, respectively (Avsic-Zupanc et al. 1999; Sibold et al. 2001; Vapalahti et al. 2003; Golovljova et al. 2007; Klempa et al. 2008; Krüger et al. 2011).

The knowledge of the human pathogenicity of the third hantavirus in Europe, the Tula virus (TULV), is sparse; there are only few reports of human TULV infections (Vapalahti et al. 1996; Schultze et al. 2002; Clement et al. 2003; Klempa et al. 2003; Mertens et al. 2011). This hantavirus has initially been found in the common vole *Microtus arvalis*, the East European vole *M. levis* (formerly *rossiaemeridionalis*), and subsequently in *M. agrestis*, *M. subterraneus*, and *M. gregalis*, and is broadly distributed in Central Europe (Plyusnin et al. 1994; Sibold et al. 1995; Song et al., 2002; Scharninghausen et al., 2002; Schmidt-Chanasit et al. 2010).

In general, the genome of hantaviruses is represented by three single-stranded RNA genome segments of negative polarity. The structural proteins of the hantaviruses, the nucleocapsid (N) protein, and the glycoproteins Gn (G1) and Gc (G2), are encoded by the small (S) segment of 1.6–2.0 kilobases (kb), and the medium (M) segment of 3.5–3.6 kb, respectively. The RNA-dependent RNA polymerase is encoded by the large (L) segment of approximately 6.5 kb.

The close association of a single hantavirus species with a single reservoir or closely related species of the same genus has been explained by a co-evolution hypothesis (Plyusnin and Morzunov 2001). However, the increasing number of hantavirus species, hantavirus studies on sympatrically-occurring rodent reservoir species (Schmidt-Chanasit et al. 2010), and the discovery of insectivore-borne hantaviruses in particular raises major questions on the evolution and host adaptation of hantavirus species (Henttonen et al. 2008). Alternatively to the virus-host co-evolution, recent studies have postulated a scenario of host switching and local host-specific adaptation for hantavirus/host evolution (Ramsden et al. 2008). Further, a host switch event in the distant past has been postulated for the ancestor of the Arvicolinae-associated Khabarovsk virus (Vapalahti et al. 1999).

The Eurasian water vole (*Arvicola amphibius*, formerly *A. terrestris*), like *Microtus*, is a member of the subfamily Arvicolinae, and is widely distributed in Europe. Interactions or sympatric occurrences of water voles with other arvicolines (e.g., *M. arvalis*) have been described, and their fluctuations in the population density sometimes correlate with the population density in *A. amphibius* (Wieland 1973). So far investigations of hantavirus infections in *A. amphibius* are sparse. Hantavirus antigen was detected in lung samples of *A. amphibius* from Russia using immunoglobulin G (IgG) antibodies directed against PUUV, Hantaan virus, and Vladivostok virus (Butenko et al. 1997). PUUV-reactive antibodies were demonstrated in 5.5% of 164 montane water voles (*Arvicola scherman*) trapped in France (Charbonnel et al. 2008). In this study, we report the first molecular evidence of multiple TULV infections in *A. amphibius* from Germany and Switzerland.

Materials and Methods

Rodent trapping and necropsy

During 2001 to 2009 a total of 424 *A. amphibius* were trapped at 20 different sites in Germany and at one locality in Switzerland. In addition, six *M. arvalis* and one *M. agrestis* trapped at three sites in Germany were included in this study (Fig. 1). The animals were necropsied according to standard protocols (Ulrich et al. 2008).

RT-PCR and sequencing

Lung samples were investigated by two L-, one S-, and one M-segment-specific RT-PCR assays. The initial screening occurred through a newly established SYBR-Green-based real-time reverse transcription-PCR (RT-qPCR) assay using the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) and novel degenerated primers (L2797F 5′ GAR GAR TAY ATH TCN TAT GGD GG-3′; L2951R 5′-HGG NGA CCA YTT NGT DGC AT-3′) targeting a conserved region in the L-segment (nt 2797-2819 and nt 2951-2970; positions according to NCBI reference sequence TULV, accession number: NC 005226) of different rodent- and insectivore-borne Old World hantaviruses. This assay was experimentally shown to detect DOBV-, PUUV-, and TULV-specific nucleic acid sequences in lungs from naturally-infected *Apodemus*, *Myodes*, and *Microtus* rodents from Germany (data not shown). Each water vole lung sample was determined as positive if the ct-value was <38 and by detection of a specific amplification product in a melting curve and in a 1.5% agarose gel. Samples with no ct-value and no specific amplification product were defined as negative, whereas those with a ct-value >38 and a typical melting curve were considered as equivocal. In addition, the samples from Baden-Wuerttemberg were tested in a nested RT-PCR assay using S-segment-specific primers (Sibold et al. 1999). All RT-qPCR positive and equivocal samples, as well as positive samples from the nested RT-PCR assay, were also tested in a One-Step RT-PCR assay using a Superscript III One Step RT-PCR Kit (Invitrogen, Darmstadt, Germany), and with primers targeting another region in the L-segment (Klempa et al. 2006). Thereafter, all L-segment-positive lung RNA samples were tested in S- and M-segment RT-PCR assays using primers specific for TULV and PUUV (Essbauer et al. 2006; Schmidt-Chanasit et al. 2010). The RT-PCR products were purified with a PCR Purification Kit (Qiagen), and sequenced using the BigDye terminator sequencing kit (Perkin-Elmer, Waltham, MA) on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

The phylogenetic analyses were performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with Bayesian Metropolis-Hastings Markov Chain Monte Carlo (MCMC) tree-sampling methods based on two MCMC runs consisting of four chains of 2,000,000 generations with a burn-in of 25%, and second by maximum-likelihood (ML) analysis calculated on a web server (http://www.phylo.org/sub_sections/portal). The optimal nucleotide substitution models according to jModeltest (Posada 2008) were the Transition Model 3 (TIM3) with a proportion of invariable sites (I), and a gamma-shaped distribution (G) for the L-segment, the Hasegawa, Kishino, and Yano Model (HKY) + I + G for the M-segment, and the Transition Model 2 (TIM2) + G for the S-segment sequences. All partial L-, S-, and M-segment sequences were screened for recombination using the program RDP3 (Martin et al. 2010), which comprises six recombination detection approaches (Bootscan, Chimeric, GENECONV, MaxChi, RDP, and SiScan) with their default parameters.

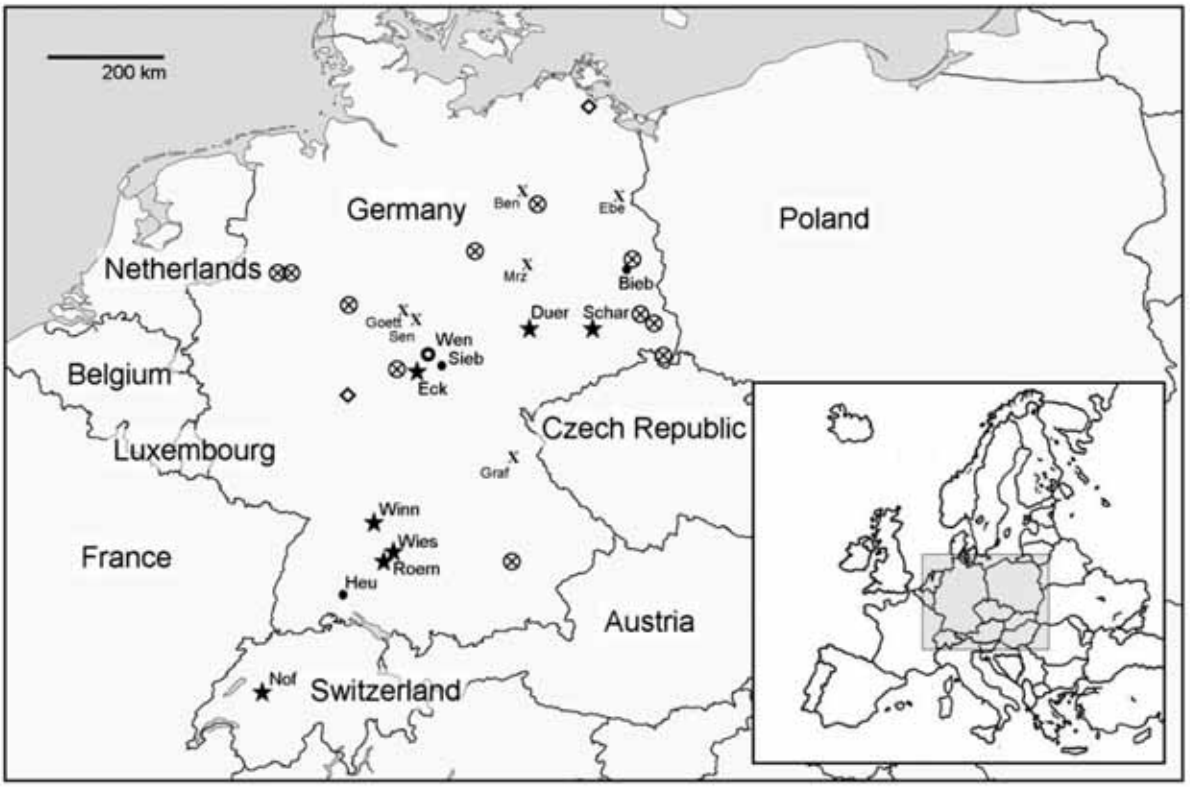

FIG. 1. Map of trapping sites with RT-PCR/serologically positive and negative *Arvicola amphibius*, *Microtus arvalis*, and *M. agrestis* (stars indicate trapping sites with seroreactive and RT-PCR positive/equivocal *A. amphibius*; circles with white centers indicate trapping sites with seroreactive but RT-PCR-negative *A. amphibius*; diamonds indicate trapping sites with RT-PCR equivocal and negative *A. amphibius*; circles with crosses in the center indicate trapping sites with RT-PCR negative *A. amphibius*; solid circles indicate trapping sites with Tula virus (TULV)-RT-PCR-positive *M. arvalis* and *M. agrestis*; "x" indicates the origin of published TULV sequences; Schmidt-Chanasit, et al. 2010). Trapping sites: Ben, Bendelin; Ebe, Eberswalde; Mrz, Marzehns; Bieb, Biebersdorf; Duer, Dürrweitzschen; Schar, Scharfenberg; Goett, Göettingen; Sen, Sennickerode; Wen, Wenigenlupnitz; Sieb, Siebleben; Eck, Eckhardtshausen; Graf, Grafenwöhr; Winn, Winnenden; Wies, Wiesensteig; Roem, Römerstein; Heu, Heuberg; Nof, Noflen.

Serology

Serological screening of phosphate-buffered saline (PBS) diluted chest cavity fluid (CCF) was done by an in-house IgG ELISA using a yeast-expressed TULV nucleocapsid protein (Mertens et al. 2011), and by following a previously published protocol (Essbauer et al. 2006). The CCFs were diluted 1:10 in 0.5% bovine serum albumin/0.05% Tween-20. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA) was used as secondary antibody. Detection of specific antibodies was accomplished by addition of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Bio-Rad). The reaction was stopped by the addition of 50 μL of 1 M H_2SO_4 (10 min).

Cytochrome b PCR

For all TULV-RT-PCR-positive *A. amphibius* from the seven trapping sites and one additional non-infected individual from each trapping site the morphological species determination was confirmed by a mitochondrial *cytochrome b* (*cyt b*) gene-specific PCR (Schlegel et al. 2011), and a subsequent BLAST search-mediated comparison of the novel *cyt b* sequences with sequences available in GenBank (http://www.ncbi.nlm.nih.gov). Phylogenetic analyses of *cyt b* sequences were performed as described for sequences derived from TULV. The optimal nucleotide substitution model was HKY + G. Sequences from the species *Arvicola sapidus*, as well as additional sequences from *A. amphibius* available in GenBank were included in the analysis. The phylogenetic tree was rooted with a sequence from *Myodes glareolus* as an outgroup.

Results

An initial screening of all 424 *Arvicola* lung samples with the new SYBR-Green RT-qPCR assay identified 4 positive and 32 equivocal samples from eight different trapping sites in Germany and Switzerland (Table 1 and Fig. 1). The subsequent analysis of all 36 RT-qPCR-positive and equivocal samples and 2 RT-qPCR-negative, but previously positive screened samples (GER127 and GER129) in a One-Step RT-PCR assay with primers targeting another region in the L-segment (Klempa et al. 2006) revealed a total of 8 samples

TABLE 1. LIST OF RT-PCR- AND/OR SEROLOGICALLY-POSITIVE *ARVICOLA AMPHIBIUS*

			Rodent			RT-PCR			
Country and region	*District*	*Trapping site*	*Number*	*Sex*	*TULV-IgG ELISA*[b]	*SYBR-Green real-time L-segment (ct-value)*	*One step RT-PCR L-segment*	*One step RT-PCR S-segment*	*One step RT-PCR M-segment*
Switzerland									
Canton of Bern	Bern-Mittelland	Nof	CH/09/1026	F	+ + +	28.48	Positive	Positive	Positive
Germany									
Saxony	Meißen	Schar	GER/08/712	F	+ + +	33.76	Positive	Positive	Positive
			GER/08/725	F	(+)	Neg	Neg	ND	ND
			GER/08/738	F	+	Neg	Neg	ND	ND
	Leipzig	Duer	GER/09/815	M	+ + +	37.10	Positive	Positive	Positive
Germany									
Thuringia	Wartburgkreis	Wen	GER/09/21-00,30,35,36[a]	M	(+)	Neg	Neg	ND	ND
			GER/09/21-08,23[a]	F	(+)	Neg	Neg	ND	ND
			GER/09/21-02,19,20,26[a]	F	+	Neg	Neg	ND	ND
		Eck	GER/09/2155	M	+ + +	37.09	Positive	Positive	Positive
Germany									
Baden-Wuerttemberg	Reutlingen	Roem	GER/109	F	ND	38.02	Positive	Positive	Positive[c]
	Göeppingen	Wies	GER/127	M	ND	Neg	Positive	Positive	Positive
			GER/139	F	ND	Neg	Positive[c]	Neg	Positive
	Rems-Murr	Winn	GER/152	M	ND	40.81	Positive	Positive	Positive

[a]Samples with the same ELISA reactivity and RT-PCR results.
[b]Optical density values: + + +, ≥2.8; +, 0.5–0.3; (+), 0.2–0.13; neg, <lower cut-off (on average 0.041).
[c]Agarose gel analysis showed a weak amplification product of the expected size; sequencing approaches remained unsuccessful.
F, female; M, male; ND, not done; Neg, negative; TULV, Tula virus; Duer, Dürrweitzschen; Wen, Wenigenlupnitz; Roem, Römerstein; Winn, Winnenden; Wies, Wiesensteig; Schar, Scharfenberg; Nof, Noflen; Eck, Eckhardtshausen.

showing the expected amplification product in the agarose gel, but only for 7 samples could a sequence be obtained (Table 1). These sequences represent a 336-bp-long part of the L-segment (nt 2962–3297 encoding aa 988–1099 of the RNA polymerase).

An initial BLAST search demonstrated the strongest similarity of these *Arvicola*-derived L-segment sequences to corresponding TULV sequences from *M. arvalis* and *M. agrestis* (data not shown). Therefore, we determined additional novel TULV partial L-segment as well as partial S- and M-segment sequences from *M. arvalis* and *M. agrestis* trapped in Baden-Wuerttemberg (Heu), Brandenburg (Bieb), and Thuringia (Sieb) near sites where positive *A. amphibius* have been previously identified (Fig. 1). A pair-wise comparison of the *Arvicola*-derived TULV L-segment and deduced RNA polymerase sequences revealed nucleotide and amino acid sequence differences of 14.6–21% and 0–5.5%, respectively. All 8 L-segment positive lung RNA samples were then tested in S- and M-segment RT-PCR assays using TULV- and PUUV-specific primers. The obtained RT-PCR products were sequenced and found to represent a 378-bp-long part of the M-segment (nt 2365–2742 encoding aa 789–914 of the glycoprotein precursor), and a 549-bp-long part of the S-segment (nt 379–927 encoding aa 127–305 of the N protein).

The phylogenetic analyses of these partial L-, S-, and M-segment sequences showed that the novel *A. amphibius*-derived sequences cluster geographically with TULV sequences derived from *M. arvalis* and *M. agrestis* in Germany and Europe, but are clearly separated from sequences of *Microtus*-borne Prospect Hill virus and Isla Vista virus and other Arvicolinae-derived hantaviruses (Fig. 2A, B, and C). All TULV S- and M-segment sequences from *Arvicola* are part of two well-supported groups "Germany I" and "Germany III/Switzerland." For both segments, the first group contains sequences derived from *A. amphibius* from the federal states Thuringia and Saxony (trapping sites Eck, Schar, and Duer), clustering together with TULV sequences from *M. arvalis* (Marv) and *M. agrestis* (Magr) from Lower Saxony (trapping sites Sen and Goett), Thuringia (trapping site Sieb), and Brandenburg (trapping sites Bieb, Mrz, Ben, and Ebe) (Fig. 2B and C). The group "Germany III/Switzerland" comprises water vole-derived TULV sequences from Baden-Wuerttemberg (trapping sites Roem, Winn, and Wies), and Switzerland (trapping site Nof), as well as *Microtus*-derived sequences from Baden-Wuerttemberg (trapping site Heu), and Bavaria (trapping site Graf) (Fig. 2B and C). Pair-wise comparisons of the novel partial *Arvicola*-derived TULV S- and M-segment nucleotide sequences (and deduced amino acid sequences) showed divergences of 0.2–20.4% (0–8.4%) and 0.3–19.9% (0–1.6%), respectively (data not shown). The screening for recombination did not detect any putative recombinant regions.

The serological screening of 286 *A. amphibius* from 5 different trapping sites where CCF was available identified 16 reactive samples with prevalences of 2.3–27% (Fig. 1 and Table 1).

Phylogenetic analysis of *cyt b* sequences did not support evolutionary distinctness of the novel *A. amphibius* sequences from Germany and Switzerland, but rather showed relatively high similarity with other *A. amphibius* sequences from Switzerland and Finland (Fig. 3). The *cyt b* sequences derived from Spanish southern water voles *A. sapidus* formed an own cluster.

Discussion

In this study we report the first multiple molecular evidence of *Microtus*-associated TULV infections in a representative of another genus of the subfamily Arvicolinae (i.e., in the Eurasian water vole). This finding is based on sequences from all three TULV genome segments from a large panel of water vole samples from different geographic regions in Germany and Switzerland. The observed frequent spillover (or host switch) is highly unexpected, as paleozoological and molecular investigations suggest a last common ancestor of *Microtus* and *Arvicola* more than three million years ago (Chaline and Graf 1988; Conroy and Cook 1999; Abramson et al. 2009; Fink et al. 2010). Such transmissions of a hantavirus from the original reservoir host to distantly related species other than humans have been reported only very rarely in Europe, as for example a single house mouse infected by *Apodemus flavicollis*-associated Dobrava-Belgrade virus (Weidmann et al. 2005). Spillover infections have been documented in numerous studies in the New World, with detection of *Microtus*-borne viruses in *Peromyscus* and *Sigmodon* species, *Peromyscus*-borne viruses in *Reithrodontomys* species, chipmunks and *Mus* species, *Reithrodontomys*-borne viruses in voles and wood rats, and *Oryzomys*-borne viruses in *Sigmodon* species (Ulrich et al. 2002).

The geographical clustering of the *Arvicola*-borne sequences with *Microtus*-derived TULV sequences from different parts of Germany and Europe suggests multiple spillover infections of TULV from *M. arvalis* or *M. agrestis* to *A. amphibius*. Although an opposite direction of the spillover infections seems possible, the frequency of molecular detection of TULV in *Microtus* species, however, suggests that members of this genus represent the reservoir host (Plyusnin et al. 1994; Sibold et al. 1995; Schmidt-Chanasit et al. 2010). In addition, the detection of TULV-reactive antibodies without detection of TULV-specific RNA in the water vole may also support this conclusion. Although the route of TULV transmission from *Microtus* to *Arvicola* is not known, both species are sometimes found in the same habitats and may even use the same burrows (G Heckel, R Wolf, personal communication).

Alternatively, the multiple molecular detection of TULV infections in water voles from several sites and different geographic regions in Germany and Switzerland indicates the potential of this rodent species as an additional reservoir host of this particular virus. Thus our findings also raise more general questions on the definition of a reservoir host for a given hantavirus and the role of hantavirus/rodent cospeciation in their molecular evolution. Usually the multiple detection of nucleic acid sequences in a single reservoir host and their absence in sympatrically-occurring other rodent or small mammal species is believed to be indicative of a reservoir host function (Hjelle and Yates 2001). Thus, *M. arvalis* has initially been identified as the most likely reservoir host of TULV (Plyusnin et al. 1994; Sibold et al. 1995). However, TULV was molecularly detected in additional *Microtus* species (i.e., *M. levis*, formerly *M. rossiaemeridionalis*), *M. agrestis*, *M. subterraneus*, and *M. gregalis* (Plyusnin et al. 1994; Sibold et al. 1995; Schmidt-Chanasit et al. 2010), and as here described in *A. amphibius*. Similarly, a TULV infection has been recorded in *Lagurus lagurus*, a representative from another genus of the Arvicolinae subfamily (GenBank accession numbers AF442619 and AF442618; Dekonenko and

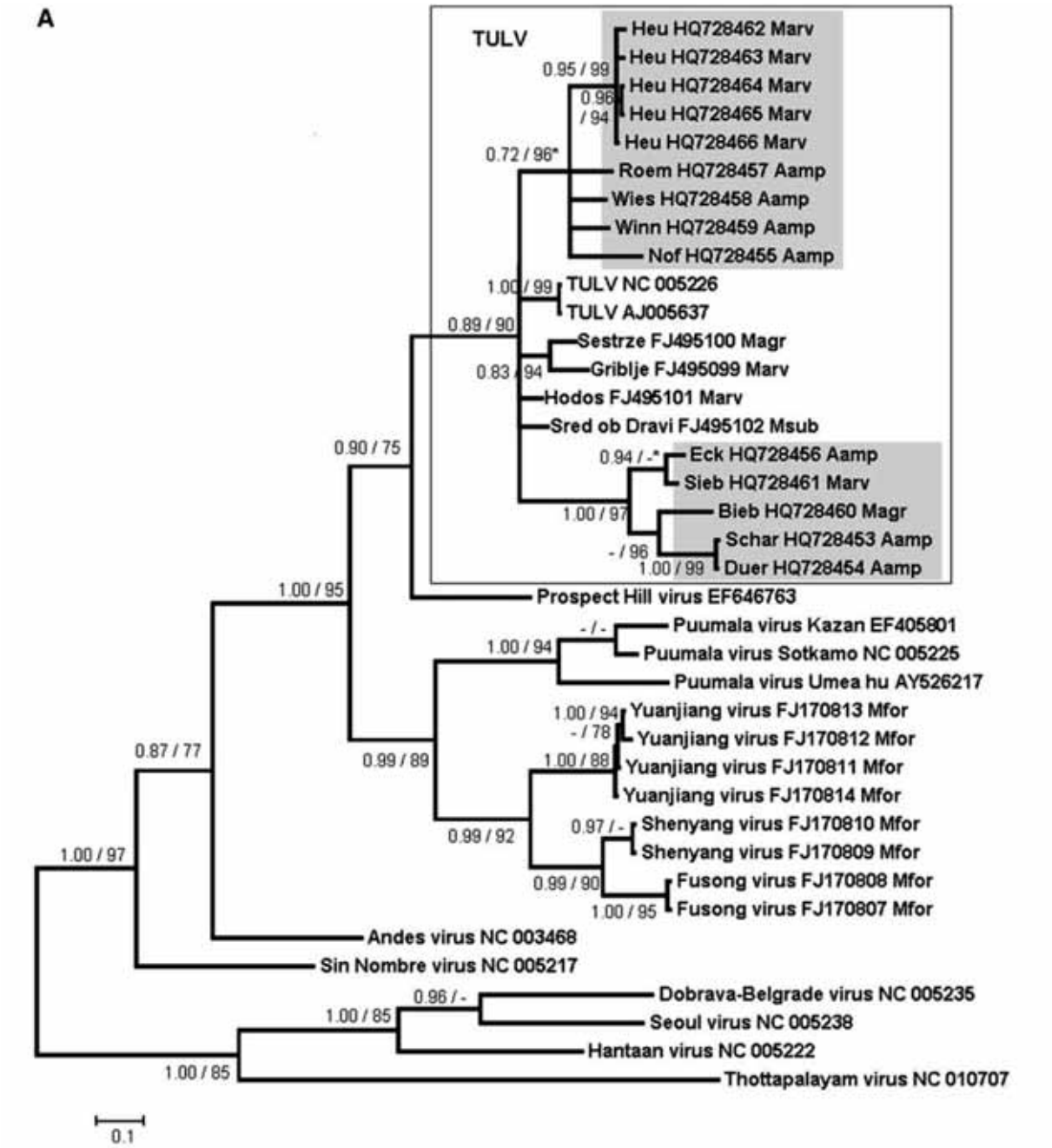

FIG. 2. Bayesian trees based on partial L-segment (336 nucleotides) (**A**), S-segment (549 nucleotides) (**B**), and M-segment (378 nucleotides) (**C**) nucleotide sequences of the novel *Arvicola*- and *Microtus*-associated Tula virus (TULV) sequences, *Microtus*-associated Prospect Hill virus and Isla Vista virus sequences, and other hantavirus sequences. Posterior probabilities for Bayesian analysis are given before the slashes, and aLRT (approximate Likelihood-Ratio Test) values for branches for maximum likelihood (ML) analysis are shown after the slashes. Only values ≥0.7% and ≥70% are shown (values ≤0.7% and ≤70% are indicated by hyphens; scale bar indicates the number of nucleotide substitutions per site; asterisks indicate differences in the topology between ML- and Bayesian-tree at this node). Novel TULV sequences derived from *A. amphibius*, *M. arvalis*, and *M. agrestis* are highlighted by gray boxes. Outgroup clusters of closely-related sequences were collapsed (**B**). Outgroup cluster accession numbers: Isla Vista virus (U31535, U31534, and U19302); Puumala virus (NC_005224, Z84204, and AY526219); Khabarovsk virus (EU360900, EU360897, and EU360898); Yakeshi virus (EU072484 and EU072482); Yuanjiang virus (FJ170795, FJ170793, FJ170794, and FJ170792); Shenyang virus (FJ170797 and FJ170796); Fusong/Vladivostok virus (EU072481, AB011630, and EU072480); Marv, *Microtus arvalis*; Magr, *Microtus agrestis*; Aamp, *Arvicola amphibius*; Llag, *Lagurus lagurus*; Mgre, *Microtus gregalis*; Mros, *Microtus levis* (formerly *rossiaemeridionalis*); Msub, *Microtus subterraneus*, Mmax, *Microtus maximowiczii*; Mfor, *Microtus fortis*; V, virus cell culture isolate.

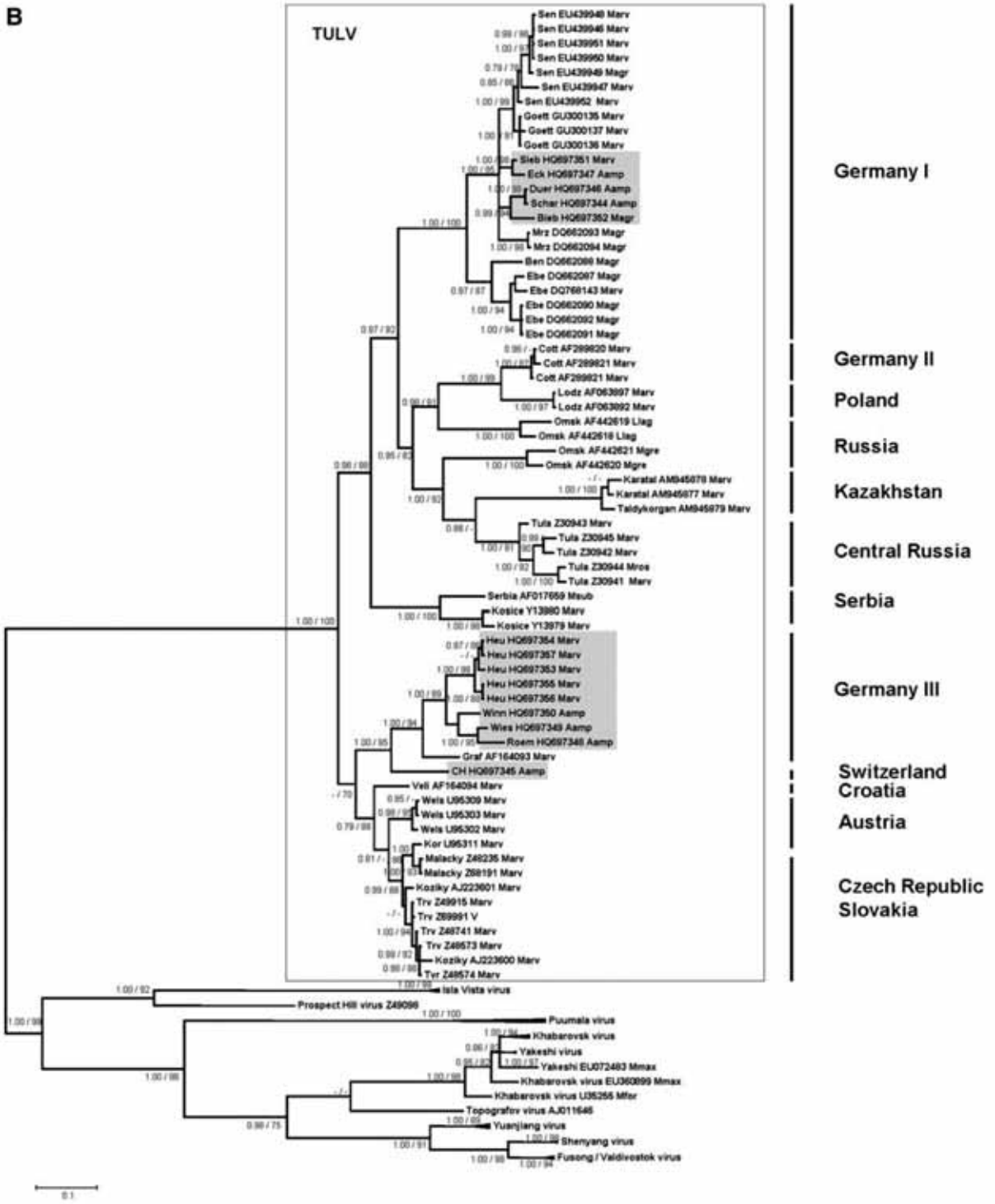

FIG. 2. (Continued).

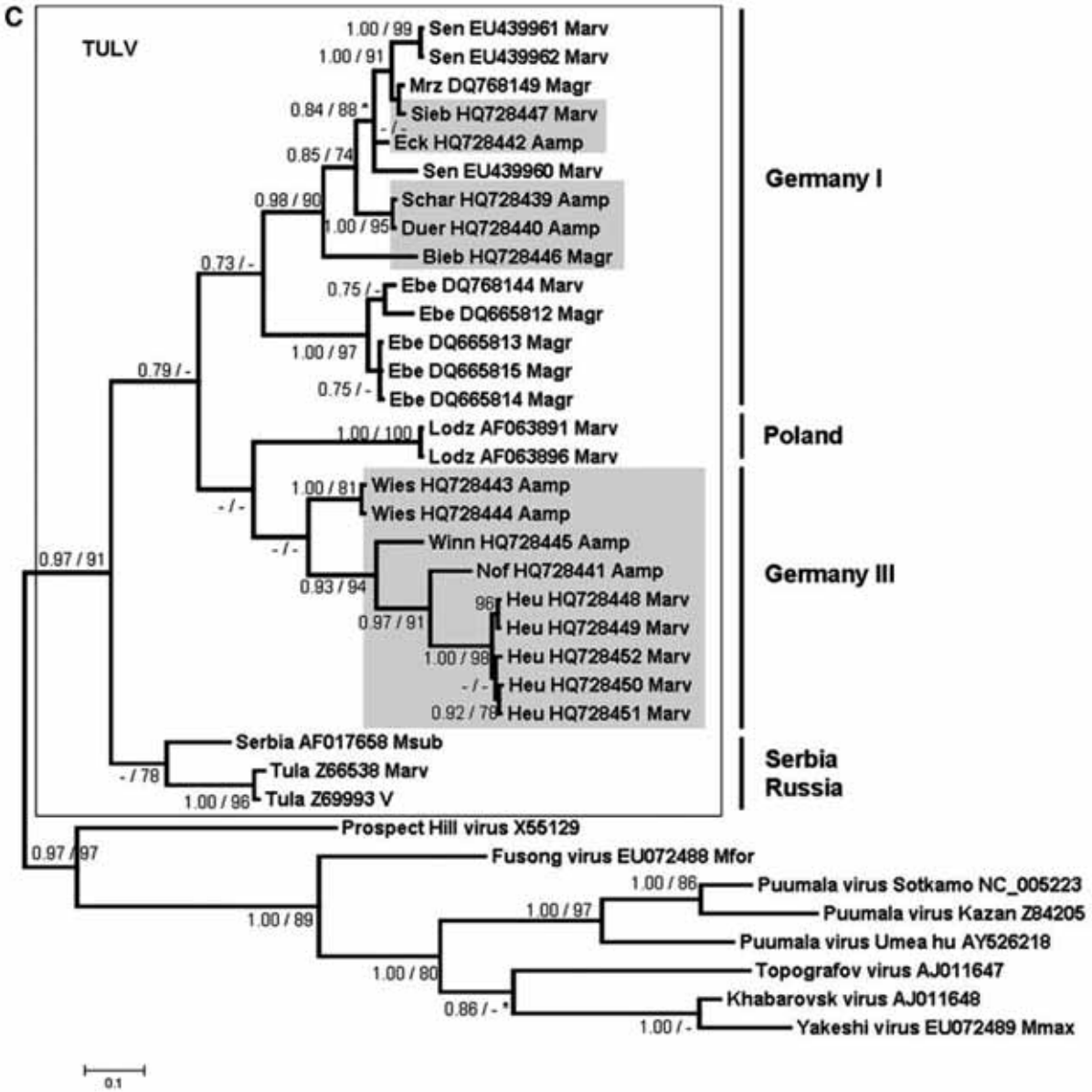

FIG. 2. (Continued).

Yakimenko, unpublished data). Moreover, a previous study suggested an already established isolated replication and transmission cycle of TULV in *M. agrestis* (Schmidt-Chanasit et al. 2010). Finally, these findings are in contrast to the usually assumed co-evolution hypothesis, according to which one would expect different TULV lineages in *Arvicola* and *Microtus* reservoirs.

TULV has previously been detected in reservoir hosts from several parts of Germany and from Russia, Slovakia, Croatia, the Czech Republic, Austria, Poland, Belgium, France, Hungary, The Netherlands, and Slovenia (Schmidt-Chanasit et al. 2010). The herein described detection of TULV sequences in *A. amphibius*, *M. arvalis*, and *M. agrestis* from Thuringia and Baden-Wuerttemberg, southeast and southwest Germany, thus enlarges our knowledge of the geographical distribution of this virus. Obviously, this virus has a broad, likely Germany-wide, distribution. Moreover, the detection of a TULV sequence in *Arvicola* from Switzerland is the first molecular detection of this virus in Switzerland, as previously only a clinical case caused by TULV was reported, using focus reduction neutralization assay analysis (Schultze et al. 2002).

This broad geographical distribution of TULV in the reservoir host also raises questions about the reasons for the currently very low number of reported human TULV infections, with only two case reports of potential TULV-induced disease in humans (Schultze et al. 2002; Clement et al. 2003;

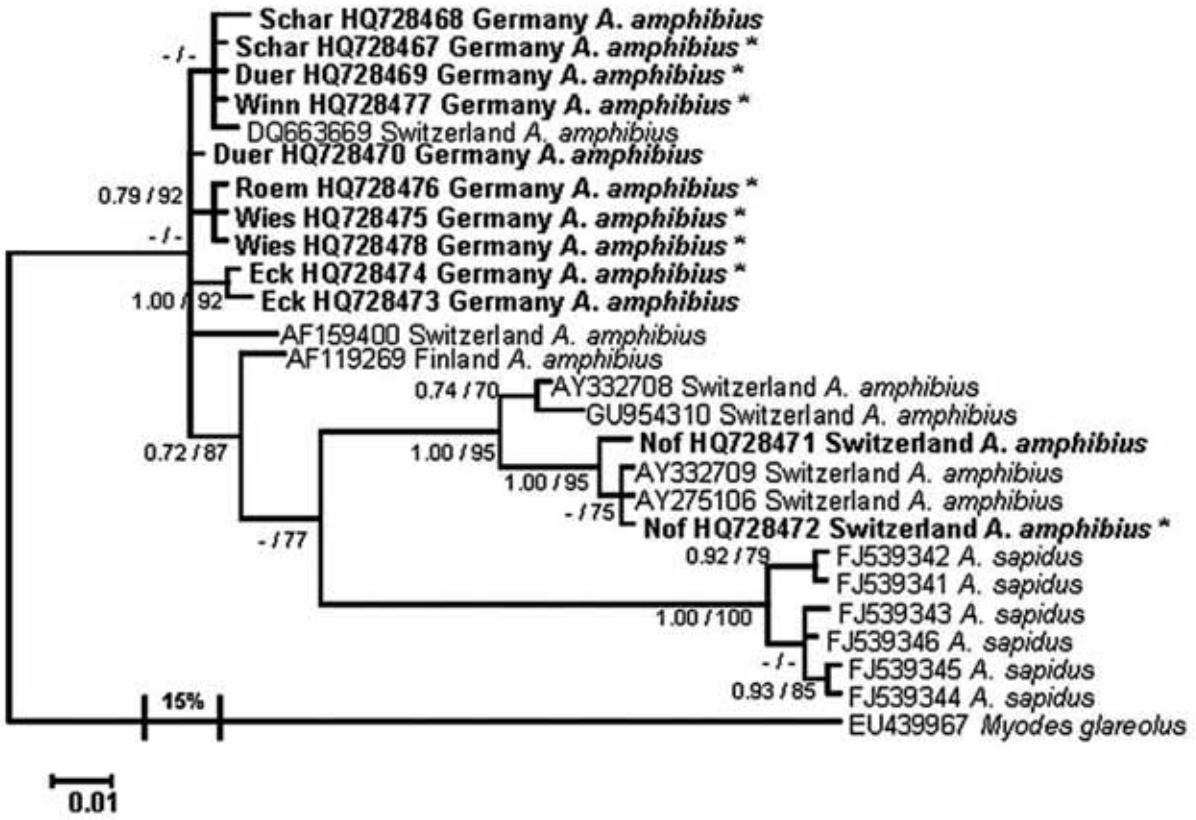

FIG. 3. Bayesian tree based on partial *cytochrome b* sequences (608 bp) of Tula virus-RT-PCR-positive (*) and negative *Arvicola amphibius* from Germany and Switzerland. Posterior probabilities for Bayesian analysis are given before the slashes, and aLRT (approximate Likelihood-Ratio Test) values for branches for maximum likelihood analysis after the slashes. Only values ≥0.7% and ≥70% are shown; values ≤0.7% and ≤70% are indicated by hyphens. Scale bar indicates the number of nucleotide substitutions per site. The outgroup branch is condensed and the divergence to *Arvicola* spp. is given in percentages at the branches in the tree. The novel sequences are shown in bold. Sequences from the sister species *A. sapidus*, and additional sequences from *A. amphibius* available from GenBank were included, and their accession numbers are displayed in the tree. *Myodes glareolus* was used as an outgroup to root the phylogenetic tree.

Klempa et al. 2003). TULV-related *Microtus*-associated hantaviruses in the New World (i.e., Prospect Hill virus, Bloodland Lake virus, and Isla Vista virus) have not been shown to cause significant disease in humans (Lee et al. 1985; Hjelle et al. 1995; Song et al. 1995). Similarly, TULV and another Eurasian hantavirus species (Khabarovsk virus hosted by the reed vole *Microtus fortis*) (Hörling et al. 1996), are believed to have low or no pathogenicity for humans. The low frequency of the detection of human TULV infections might be explained by its low pathogenic potential as determined in cell culture experiments (Kraus et al. 2004). On the other hand, a recent study in a forestry worker risk group in a region where TULV has been detected in *Microtus* reservoirs demonstrated frequent serological detection of TULV-reactive antibodies in an ELISA test using the homologous antigen (Mertens et al. 2011). Therefore, more extended serological investigations should determine if TULV represents a neglected human pathogenic hantavirus that is currently overlooked in human infections due to the use of insufficient diagnostic tools.

In conclusion, multiple molecular detections of TULV infections in water voles underline the unique potential of this virus to infect distantly-related rodents of two different genera. Future studies of wild water voles and other *Arvicola* species from different regions in Europe are needed to verify the frequency of spillover and/or host-switch events for TULV, and may thus allow the definition of the host range of TULV. These studies should be accompanied by experimental infection studies addressing the pathogenic consequences of TULV infections in different hosts, and the viral and host factors determining the host range, transmission pathways, and human pathogenicity of this virus.

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Author Disclosure Statement

No competing financial interests exist.

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Paper IV

Broad geographical distribution and high genetic diversity of shrew-borne Seewis hantavirus in Central Europe

Virus Genes
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Broad geographical distribution and high genetic diversity of shrew-borne Seewis hantavirus in Central Europe

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Abstract For a long time hantaviruses were believed to be exclusively rodent-borne pathogens. Recent findings of numerous shrew- and mole-borne hantaviruses raise important questions on their phylogenetic origin. The objective of our study was to prove the presence and distribution of shrew-associated Seewis virus (SWSV) in different *Sorex* species in Central Europe. Therefore, a total of 353 *Sorex araneus*, 59 *S. minutus*, 27 *S. coronatus*, and one *S. alpinus* were collected in Germany, the Czech Republic, and Slovakia. Screening by hantavirus-specific L-segment RT-PCR revealed specific amplification products in tissues of 49 out of 353 *S. araneus* and four out of 59 *S. minutus*. S-segment sequences were obtained for 45 of the L-segment positive *S. araneus* and all four L-segment positive *S. minutus*. Phylogenetic investigation of these sequences from Germany, the Czech Republic, and Slovakia demonstrated their similarity to SWSV sequences from Hungary, Finland, Austria, and other sites in Germany. The low intra-cluster sequence variability and the high inter-cluster divergence suggest a long-term SWSV evolution in isolated *Sorex* populations. In 28 of the 49 SWSV S-segment sequences, an additional putative open reading frame (ORF) on the opposite strand to the nucleocapsid protein-encoding ORF was identified. This is the

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first comprehensive sequence analysis of SWSV strains from Germany, the Czech Republic, and Slovakia, indicating its broad geographical distribution and high genetic divergence. Future studies have to prove whether both *S. araneus* and *S. minutus* represent SWSV reservoir hosts or spillover infections are responsible for the parallel molecular detection of SWSV in both species.

Keywords Hantavirus · Seewis virus · Shrew · *Sorex araneus* · Central Europe · Spillover

Although historically, the first hantavirus, i.e., *Thottapalayam virus* (TPMV), was isolated from a shrew, the Asian house shrew *Suncus murinus*, in India [1], it took more than 30 years until this virus was completely sequenced and confirmed to be a shrew-borne hantavirus [2]. Development of a broad-spectrum RT-PCR assay allowed the discovery of a second shrew-borne hantavirus, Tanganya virus, from *Crocidura theresae* in 2007 [3]. Further studies on shrew-borne hantaviruses were encouraged, resulting in the identification of similar viruses in other members of the genus *Crocidura*, i.e., *Crocidura lasiura* and *Crocidura obscurior* [4, 5]. Hantaviruses were detected in other shrew species of the subfamily Soricinae too, i.e., *Anourosorex squamipes*, *Blarina brevicauda*, *Sorex cinereus*, *S. monticolus*, and *S. roboratus* [6–9]. In addition, novel hantavirus sequences were found in *S. caecutiens* (GenBank Acc. Nos. EU424339–40) and *S. cylindricauda* (GenBank Acc. Nos. GU566021–23). Moreover, hantaviruses were found in moles, within the subfamily Talpinae, i.e., *Urotrichus talpoides*, *Neurotrichus gibbsii*, and *Talpa europaea* [10–12]. The finding of a novel hantavirus in the Eastern mole (*Scalopus aquaticus*), closely related to Cricetidae-borne hantaviruses, underlines the complex evolutionary history of hantaviruses [13]. Recently, hantaviruses were molecularly detected in bats [14, 15].

All novel insectivore-borne hantaviruses have the same genome organisation as the rodent-borne hantaviruses [12]. The single stranded RNA genome of negative polarity consists of a small (S)-segment coding for a nucleocapsid (N) protein, medium (M)-segment coding for a glycoprotein precursor, and large (L)-segment coding for a RNA-dependent RNA polymerase.

Previously, only rodent-borne hantavirus species were known in Central Europe: the Arvicolinae-borne *Puumala virus* and *Tula virus* (TULV) and the Murinae-borne *Dobrava-Belgrade virus* (DOBV) [16]. In 2007, a Soricinae-borne hantavirus, Seewis virus (SWSV), was detected in the common shrew *Sorex araneus* from Switzerland [17]. Shortly thereafter, this virus was also found in Hungary, Finland, and Russia [18, 19]. Here we describe a comprehensive investigation of the presence, distribution, and genetic variability of SWSV in different *Sorex* species in three countries of Central Europe.

A total of 353 common shrews *S. araneus*, 59 Eurasian pygmy shrews *S. minutus*, 27 Millet's shrews *S. coronatus*, and one Alpine shrew *S. alpinus* were collected at 68 trapping sites in Germany, 12 trapping sites in the Czech Republic, and four trapping sites in Slovakia (Table 1). For hantavirus-specific RT-PCR, total RNA was extracted from lung, liver, or kidney, reverse transcribed, and amplified using primers targeting the L-segment [20]. Thereafter, all L-segment positive samples were additionally tested in S-segment RT-PCR assays using previously published primers SW-S-AF2-404 and SW-S-AR2-1249 [19] and newly designed primers SWSV-22-fw (5′-GCA TAC TAC GAA ACA GAG AGC-3′), SWSV-391-rev (5′-CCT TRA GCA AGA TGG TRT CTA C-3′), S750F (5′-AGY CCW GTN ATG RGD GTN RT-3′), S750R (5′-AYN ACH CYC ATN ACW GGR CT-3′), SW-S-962F (5′-TGG GTM TTY GCR GGW GCA CCT GA-3′), SWSV-1384-rev (5′-AGT GAG TGC GAA GCA GCA G-3′), SW-S-1590R (5′-GTG TTT GAG GTA KTG GAG TG-3′), SW-S-3′end (5′-TAG TAG TAK RCT CCY TRA ARA G-3′).

The L-segment amplicons of about 390 bp were directly sequenced, and the obtained sequence information was analyzed as previously described [21]. In total, in 49 out of 353 *S. araneus* and in four out of 59 *S. minutus* hantavirus L-segment sequences were found. S-segment sequences were obtained for 45 of the 49 L-segment positive *S. araneus* and for all four L-segment positive *S. minutus*. The RT-PCR positive samples originated from five sites in Germany, seven sites in the Czech Republic, and one site in Slovakia (Table 1; Fig. 1a). None of the investigated *S. coronatus* and *S. alpinus* revealed specific RT-PCR products. An initial phylogenetic analysis of all 53 *Sorex*-derived L-segment sequences demonstrated their similarity with SWSV sequences from Hungary, Finland, Austria, and other sites in Germany (Fig. 1b). An additional well-separated cluster is formed by sequences from Far-East Russia. The phylogenetic tree showed evidence for a geographical clustering of SWSV sequences, e.g., different clusters from the Czech Republic (Drahany, Raĭkovský les/Nosislav, Beskydy/Raĭkov). A pairwise comparison of the novel partial SWSV L-segment nucleotide sequences and deduced amino acid sequences (values shown in brackets) from sites Drahany and sites Beskydy/Raĭkov showed intra-cluster divergences of 0–3.2 % (0–2.2 %). The highest sequence divergences of the novel sequences to other SWSV sequences were found to be 24.9 % (5.4 %). The comparison between the novel sequences and other *Sorex*-associated hantavirus sequences showed higher divergences of 16–29.5 % (1.1–19.2 %) (data not shown).

Owing to the higher conservation and limited length of the analyzed L-segment sequences and the resulting lower

Fig. 1 Map with the geographical origin of Seewis virus (SWSV) RT-PCR positive *Sorex araneus* and *S. minutus* (**a**), phylogenetic Bayesian trees based on partial L-segment (**b**) and S-segment (**c**) sequences of the novel SWSV, *Sorex*-associated as well as other shrew-, mole- and rodent-borne hantavirus sequences and localization of the putative second ORF on the S-segment (**d**). **a** *Five pointed star* trapping site where SWSV was originally detected [17]; *black circle* trapping sites with SWSV RT-PCR positive *S. araneus* or *S. minutus*; *open circle* trapping sites with previously reported findings of SWSV (GenBank Acc. Nos. EU418604-16 and [18]); *asterisk* Site Raĭkovský les is located close to trapping site Raĭkov. **b**, **c** The phylogenetic trees were initially generated using MrBayes 3.1.2 [35] as previously described [21] and by Maximum-Likelihood (ML) analysis with 1,000 bootstrap pseudoreplicates calculated with MEGA5 [36]. Partial L-segment (nucleotide positions 3,011–3,292; numbering according GenBank Acc. No. NC005235) and S-segment sequences (nucleotide positions 431-1,114; numbering according GenBank Acc. No. EF636024) were used. The optimal nucleotide substitution model, determined by jModeltest [37], was the GTR+I+G model for both, the L- and S-segments. The screening of the novel SWSV L- and S-segment sequences for recombination, did not reveal any putative recombinant regions supported by more than three programs implemented in the RDP3 program [38]. All identical sequences were excluded from the alignments for tree reconstruction including L-segment sequences from trapping site Drahany (JQ425319, JQ425324) and Beskydy (JQ425337) and S-segment sequences from České Budejovice (JQ425268), Drahany (JQ425274, JQ425283, JQ425284), Nosislav (JQ425292) and Beskydy (JQ425294-96, JQ425298, JQ425305, JQ425308). Novel SWSV sequences are given in *bold* and *labeled* with Acc.No./trapping site/trapping year/species. Posterior probabilities for Bayesian analysis are given before slashes and bootstrap values for ML analysis after slashes, respectively; only values ≥0.7 and ≥70 % are shown. Scale bar indicates number of nucleotide substitutions per site. *Asterisk* indicates differences in the topology between Bayesian- and ML-tree at this node. ANDV, *Andes virus*; ARRV, Ash River virus; ASAV, Asama virus; AZGV, Azagny virus; CBNV, Cao Bang virus; DOBV, *Dobrava-Belgrade virus*; HTNV, *Hantaan virus*; JMSV, Jemez Springs virus; KKMV, Kenkeme virus; MJNV, Imjin virus; NVAV, Nova virus; OXBV, Oxbow virus; PUUV, *Puumala virus*; RKPV, Rockport virus; RPLV, Camp Ripley virus; SEOV, *Seoul virus*; SNV, *Sin Nombre virus*; TGNV, Tanganya virus; TPMV, *Thottapalayam virus*; TULV, *Tula virus*; Na, *Neomys anomalus*; Sa, *Sorex araneus*; Sd, *Sorex daphe-odon*; Sm, *Sorex minutus*; St, *Sorex tundrensis*. (**d**) The genetic map illustrates the localization and reading direction of the N ORF on the S-segment (numbering of nucleotide positions according to GenBank Acc. No. EF636024) and the localization of the putative ORF2 on the opposite strand found in 28 SWSV S-segment sequences from Germany and the Czech Republic

support of the Maximum Likelihood tree, a deeper phylogenetic analysis of the novel SWSV strains was based on S-segment sequences of 699 nt in length. This analysis demonstrated a clear separation of all SWSV sequences from other *Sorex*-borne hantaviruses including Kenkeme virus (Fig. 1c). The S-segment sequences showed a complex geographical clustering. Thus, SWSV sequences from the Czech Republic formed in correlation to their trapping sites five clusters (Ia, Ib, II, III, IV). The three sequences from Gotha formed a cluster with a sequence from Ködnitz

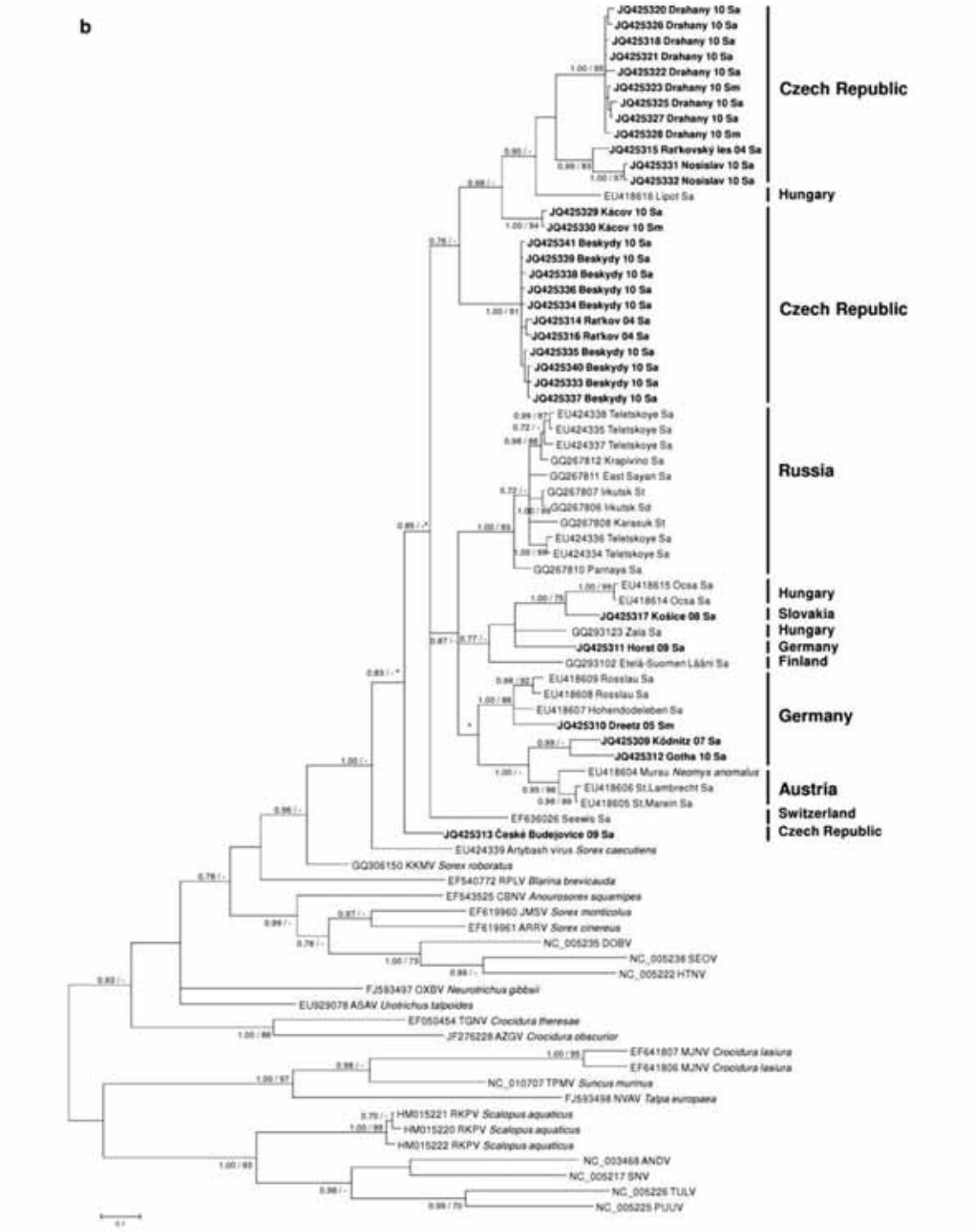

Fig. 1 continued

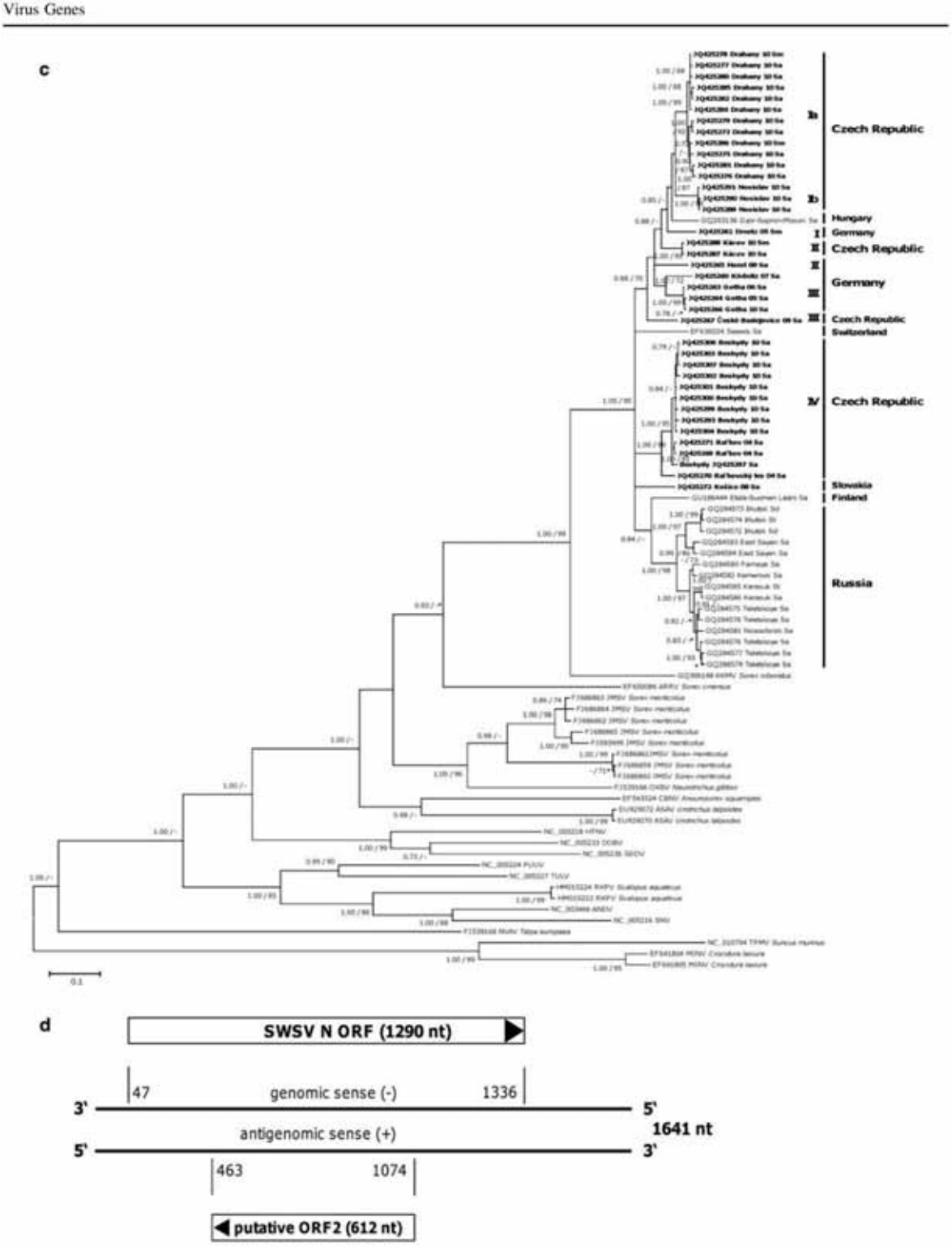

Fig. 1 continued

Table 1 Hantavirus RT-PCR results for *Sorex araneus* and other *Sorex* spp. from trapping sites in Germany, the Czech Republic and Slovakia

Country	Trapping site[a]	Trapping year (s)	Number of RT-PCR positive/total number of tested animals		
			S. araneus	*S. minutus*	*S. coronatus*
Germany	Ködnitz	2007	1/1	0	0
	Dreetz	2005, 2008	0/12	1/3	0
	Mücke-Merlau	2007	1/1[b]	0/1	0
	Horst	2008–2010	1/108	0/7	0/2
	Gotha	2005–2007, 2010	3/22	0	0
	Additional 63 sites	1983–1985, 2002–2009	0/95	0/29	0/25
	Total		6/239	1/40	0/27
Czech Republic	Kácov	2010	1/2	1/3	0
	Beskydy[c]	2010	18/34	0	0
	Drahany	2010	14/39	2/14	0
	České Budejovice	2009	2/4	0/1	0
	Nosislav	2010	4/5	0	0
	Raĭkov	2004	2/6	0	0
	Raĭkovský les	2004	1/3	0	0
	Additional five sites	2003, 2006–2008, 2010	0/11	0/1	0
	Total		42/104	3/19	0
Slovakia	Košice	2008	1/1	0	0
	Additional three sites	2009–2011	0/9	0	0
	Total		1/10	0	0

[a] For location of the trapping sites see Fig. 1a

[b] Novel SWSV L- and S-segment sequences (Acc. No. JQ425262) from this sample were not shown in the trees, because of their restricted length (135 nucleotides, and 393 nucleotides, respectively). They showed the highest nucleotide sequence identity to a sequence from Ködnitz (JQ425309 Sa) and a sequence from Drahany (JQ425275 Sa), respectively

[c] One additional RT-PCR negative *Sorex alpinus* was trapped here

(Germany III) which is apart from all other sequences from Germany. The sequence from Slovakia is also well separated from all other SWSV sequences. The S-segment sequences showed intra-cluster nucleotide sequence divergences of 0–5.4 % (amino acid sequence divergences 0–0.9 %) for clusters Czech Republic Ia (Drahany), Czech Republic Ib (Nosislav), Czech Republic IV (Beskydy/Raĭkov/Raĭkovský les) and Germany III (Gotha). Two sequences from *S. araneus* trapped at the same site (Gotha) in different years (2006, 2010) showed two silent transitions (C-T), both on the third codon position, in the open reading frame (ORF) of the N protein encoding sequence. The sequence divergences between the novel and known SWSV S-segment sequences reached 17.3 % (3 %). The comparison with other *Sorex*-associated hantavirus sequences showed divergences of 14.1–33.9 % (0.5–26.9 %) (data not shown).

Our study demonstrated a broad geographical distribution of SWSV in *Sorex araneus* in Central Europe. Together with the initial detection of SWSV in Switzerland and the previous detection of SWSV in Hungary, Finland, and Russia, this virus shows a very broad geographical distribution in Europe and Far-East-Russia. A more in-depth phylogenetic analysis demonstrated a high genetic divergence of the SWSV strains from different parts of Europe. This finding might be explained by the phylogeography of the reservoir host, *S. araneus*, and an independent long-term evolution of the SWSV strains in isolated local *Sorex* populations. The recolonization of Europe from three potential main refugia in Portugal–Spain, Italy, and the Balkans has been dated to the last glaciation period 20,000–18,000 years ago when the ice shield left Europe [22]. Based on *cytochrome b* (*cyt b*) and *cytochrome oxidase I* gene data, the three major Western European phylogroups of *S. araneus* differentiated during the Middle Pleistocene from 0.47 to 0.27 million years ago [23]. A phylogenetic analysis of *cyt b* gene sequences generated by a recently described protocol [24] of *S. araneus*, *S. minutus*, and *S. alpinus* samples from all investigated trapping sites demonstrated a clear separation of the *cyt b* sequences from the three species and a high genetic diversity, but not a geographical clustering of the *cyt b* sequences from *S. araneus* (data not shown). Future investigations have to prove the possible association of the phylogeographical history of *S. araneus* and the evolution and host association of SWSV in Europe during these calculated timescales.

The geographical clustering of *S. minutus*-borne SWSV sequences with *S. araneus*-derived sequences and the more frequent detection of SWSV in *S. araneus* may indicate that *S. araneus* represents the reservoir host of SWSV and the detection in *S. minutus* is due to spillover infections. Similarly, the molecular detection of SWSV in other *Sorex* species, i.e., *S. daphaenodon*, *S. tundrensis* [19], and in one case in *Neomys anomalus* (GenBank Acc. No. EU418604) might also be explained by spillover infections. The exact route of those SWSV spillover transmissions is unclear; but *S. araneus*, *S. minutus,* and *Neomys anomalus* could occur sympatrically in the same habitats [25–27]. Previously spillover infections have been reported, e.g., for European rodent-borne hantaviruses TULV and DOBV [21, 28–30]. On the other hand, it cannot be excluded that SWSV has a broader host range consisting of several *Sorex* species (and even species of other shrew genera). These and similar findings for TULV [21, 28] underline the current problems in the identification of hantavirus reservoir hosts [31]. Spillover infections may represent a first step in the development of new virus-host associations. Host-switch and subsequent host adaptation has been discussed as an alternative evolution process for hantaviruses, besides the general host-virus codivergence [28, 32]. Interestingly, the *S. araneus*-borne SWSV S- and L-sequences from Raĭkovský les were found at different positions of the corresponding trees. Further studies have to prove if this finding can be explained by a reassortment process.

In addition, we found a second putative ORF overlapping with the N protein coding ORF in 28 of the analyzed S-segment sequences from two trapping sites in Germany (Ködnitz, Gotha) and four sites in the Czech Republic (Raĭkov, Raĭkovský les, Beskydy, Nosislav). This ORF was predicted to be encoded by the antigenomic strand in a -2 frame and encodes a putative 203 amino acid-long protein (nucleotide positions 1,028–417; numbering according to GenBank Acc. No. EF636024; see Fig. 1d). A protein BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and an analysis with ScanProsite (http://prosite.expasy.org/scanprosite) did not detect any conserved or functional domains within this putative protein (data not shown). Although for Arvicolinae-borne PUUV and TULV a NSs ORF was found in the same reading direction as the N ORF on the genomic strand [33, 34], such an ORF as observed here on the antigenomic strand has never been found in other shrew-, mole-, or rodent-borne hantaviruses. Future studies are needed to prove if this putative ORF has any function in the replication cycle of SWSV and if an ambisense coding strategy exists for these strains, as known for some other bunyaviruses and for arenaviruses.

In conclusion, our study demonstrated a broad geographical distribution of SWSV in Central Europe. In line with the high divergence of its host *S. araneus,* the SWSV L- and S-segment sequences revealed a strong genetic differentiation of local strains. The detection of SWSV sequences in *S. minutus* can be explained by spillover infections. Alternatively, *S. minutus* might represent an additional reservoir. The broad geographical distribution of SWSV strongly encourages future studies on the zoonotic potential of SWSV and its pathogenicity for humans in particular. In addition, a more detailed analysis of the molecular evolution of SWSV may shed more light on the complex evolution and host adaptation processes of hantaviruses in general.

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Summarized Results and Discussion

Development of diagnostic tools

Molecular small mammal species identification

Mammals are distributed worldwide and have conquered nearly all biotopes on earth, except the deep sea regions and the South Pole. The three largest mammalian orders are Rodentia with 33 families, 481 genera, 2,277 species, Chiroptera with 18 families, 202 genera, 1,116 species, and Soricomorpha with four families, 45 genera and 428 species (WILSON and REEDER 2005b; WILSON and REEDER 2005a). Several species within these orders are very small and can only be identified by zoological experts. For example, species within the rodent genera *Microtus* and *Apodemus* are morphologically, based on skull morphometric and teeth characteristics, or biochemically difficult to distinguish (MARKOVA 1986; REUTTER et al. 1999). Similarly, members of the Soricomorpha genera *Crocidura* and *Sorex* can only be identified by the use of teeth formulas or the examination of cranial characteristics (RICHTER 1962; MYS et al. 1985). As described above (see Tables 2 and 3), all hantaviruses were identified in small mammals.

Currently, small mammals are collected by several partners of the network "Rodent-borne pathogens" (ULRICH et al. 2008). The morphological species identification of these samples is sometimes hampered by the quality and type of the available sample, especially if only tail, ear, or blood samples are collected. For these reasons a robust and broad-spectrum molecular assay for identification of rodent and other small mammal species was developed (**Paper I**). This novel assay is based on PCR amplification using degenerated primers targeting the *cytochrome b* (*cyt b*) gene, nucleotide sequence analysis of the amplified *cyt b* gene portion followed by pairwise sequence comparison to published sequences using the BLAST function of GenBank. In total 33 novel *cyt b* sequences from different small mammal species in Germany were generated by this approach, including not only representatives of the Rodentia families Muridae, Cricetidae, Caviidae, Castoridae, Gliridae, Sciuridae and the Soricomorpha families Soricidae and Talpidae, but also for all investigated representatives of the orders Erinaceomorpha, Lagomorpha, Carnivora and Chiroptera (**Paper I, Table**). The molecular species identification accomplished by this approach was in line with the morphological species determination of 33 investigated small mammal species and revealed a nt sequence identity to the corresponding GenBank entries of 100% (**data not shown, Paper I**). A phylogenetic analysis of all 33 novel sequences with already available GenBank sequences confirmed the classification based on the sequence identity comparison (**Paper I, Figure 2**).

The described method was demonstrated to represent a very useful tool for identification of different small mammal species from Central Europe (GUENTHER et al. 2010; ACHAZI et al. 2011; MAYER-SCHOLL et al. 2011) and (**Paper I, III, IV**). In addition, the method was initially used to determine small mammal species from Africa and Asia (unpublished data, see Table 4). These generated partial *cyt b* sequences, showed lower nt identities, to corresponding GenBank sequences, than observed for samples from Central Europe. Therefore, additional parallel morphological and molecular species determination approaches are needed to validate the *cyt b* assay for samples from these regions.

Table 4 Additional small mammal species which were determined by the described *cyt b* PCR

Order*	Subfamily	Geographical origin	Species	Nucleotide identity
Rodentia	Murinae	Africa	*Mastomys natalensis*	96%
			Mastomys erythroleucus	98%
			Arvicanthis niloticus	95%
	Gerbillinae	Africa	*Gerbilliscus kempi*	89%
	Cricetinae	Asia	*Cricetulus migratorius*	97-98%
Soricomorpha	Crocidurinae	Asia	*Suncus etruscus*	98%

*Taxonomy according to WILSON and REEDER 2005a, b. (Sheikh Ali et al.; Schlegel et al. unpublished data).

The *cyt b* gene has been shown to be sufficient for species identification in many studies and for various animal taxa (**for references see Paper I**). By the use of mitochondrial (mt) DNA PCR assays a co-amplification of nuclear mt pseudogenes (numts) may occur. These numts are non-functional copies of mtDNA in the nucleus that have been found in eukaryotic organisms and could be co-amplified especially when using universal primers (SONG et al. 2008). Using the assay described in **Paper I**, this problem has been solved by amplification of an approximately 900 nt long *cyt b* sequence fragment as most eukaryotic numts are shorter than 700 nt (RICHLY and LEISTER 2004).

Shortly after **Paper I** was published, novel universal primers for complete *cyt b* gene sequencing in mammals were described (NAIDU et al. 2012). These primers showed amplification of non-target fragments *in silico* and *in vitro* in 30% of the 40 tested species. Therefore a gel extraction of the amplicon was required in 70% of the samples, and in one case the PCR failed to amplify the target sequence. In our investigations, in 94% of all samples a direct purification of the PCR product was sufficient **(Paper I)**. Only for two species of the orders Carnivora and Chiroptera (*Mustela putorius*, *Nyctalus noctula*), from the 33 tested species (6%), a co-amplification of smaller

non-target products was observed (**Paper I, data not shown**). Additionally, this assay requires no expensive and time-consuming DNA extraction methods. *Cyt b* sequence fragments could be simply amplified directly by PCR from small mouse tail samples treated with lysis buffer and proteinase K (**Paper I, data not shown**).

Limitations for the use of the novel assay arise from the availability of corresponding *cyt b* sequences in GenBank. In the case of investigations with samples coming from geographical regions and species, where molecular data are lacking so far, this limitation could be complicate (see Table 4). In addition, another problem could be mtDNA introgression and recombination, which could lead to inaccurate species identifications in animal taxa (RUBINOFF et al. 2006). The possibility and consequences of recombination in mtDNA are controversially discussed (ROKAS et al. 2003; PIGANEAU et al. 2004; TSAOUSIS et al. 2005). Therefore, mtDNA-based species identification can fail to distinguish, in case of the use of a single mtDNA gene, members of closely related species groups and morphologically highly similar species (RUBINOFF et al. 2006). Thus, *cyt b* sequences of *Crocidura suaveolens* and *C. sibirica* from Asia were found to be 99-100% identical to each other (Schlegel et al. unpublished data). In these cases, correct species identification is problematic, and other discrimination methods have to be used, e.g. karyotype, skull morphometric or comprehensive genomic DNA analysis.

Finally, the described *cyt b* assay was demonstrated to be a useful tool for identification of small mammal species from Central Europe. Moreover, the developed assay could be used for the generation of mtDNA sequences of the reservoir host, which are useful for the characterisation of the evolutionary history of the hantavirus-host-relationship. The usability of this assay should be checked and, if needed, adopted in future studies for further small mammal specimens.

Generation of Thottapalayam virus-specific monoclonal antibodies

For rodent-borne hantaviruses a large panel of mAbs has been developed by various approaches (**for references see Paper II**), but none for soricomorph-associated hantaviruses. In addition, only for TPMV Gc protein cross-reactivity's with rodent-borne HTNV-specific mAbs has been reported so far (ARIKAWA et al. 1989; OKUMURA et al. 2007). In line, a large panel of rodent-borne hantavirus N protein-specific mAbs failed to react with recombinant TPMV N protein (**Paper II**).

Therefore, in **Paper II** TPMV-specific mAbs for the detection of TPMV (and antigenically related hantaviruses) in cell culture and in tissue of potentially infected animals, were developed. The TPMV-specific mAbs were generated using recombinant TPMV N proteins produced in *Escherichia coli*-, baculovirus- and *Saccharomyces cerevisiae*-mediated expression systems (**Paper II, Table 1**). As demonstrated, all three expression systems resulted in the generation of mAbs that recognize the native viral protein. The TPMV N protein-specific mAbs showed a characteristic fluorescent pattern in IFA with TPMV infected cells. In general, no crossreactions to rodent-associated hantaviruses in IFA and ELISA were observed, except for one mAb which reacted in IFA with SNV (**Paper II, Table 2**).

The N-terminus of the N protein of rodent-borne hantaviruses was demonstrated to represent an immunodominant region as evidenced by the reactivity with polyclonal sera and mAbs (**for references see Paper II** and (ELGH et al. 1996; GELDMACHER et al. 2004). In line with these investigations, three TPMV-specific mAbs were reactive with all truncated N proteins and entire N protein in ELISA and WB suggesting an epitope localization of these mAbs within the N-terminal aa 1-80 region **(Paper II, Table 3)**. In contrast to that, two mAbs were reactive only with the entire N protein in ELISA but not in WB, which might be explained by a conformation-dependent nature of the epitope for these mAbs. The missing cross-reactivity of the mAbs to rodent-borne hantavirus N proteins (HTNV, SEOV, PUUV, TULV and DOBV) confirmed the conclusion that TPMV is antigenically and genetically highly divergent to all other known hantaviruses. None of the TPMV mAbs cross-react with the N-terminal segment of shrew-borne ASAV N protein (**Paper II, Table 3**). This lacking cross-reactivity is in contrast to the broad-spectrum cross-reactivity observed for rodent-borne hantavirus N protein-specific mAbs with N proteins of other rodent-borne hantaviruses (SJOLANDER and LUNDKVIST 1999; LUNDKVIST et al. 2002; TISCHLER et al. 2008; KUCINSKAITE-KODZE et al. 2011; SAASA et al. 2012). Although the secondary structure of the N protein of different rodent- and soricomorph-borne hantaviruses is quite similar, comparisons of aa sequences showed relatively low identities and therefore may explain the missing reactivity's with ASAV N protein (ARAI et al. 2008b; KANG et al. 2009b; KANG et al. 2009c).

In conclusion, the reactivity of the mAbs in IFA, using TPMV-infected cells, indicates that the mAbs represent useful tools for detection and characterization of TPMV. The cross-reactivity of the TPMV-specific mAbs with additional soricomorph-borne hantavirus N proteins should be tested in future investigations.

Development of serological tools for detection of hantavirus-specific antibodies in shrews

Regarding the multiple detection of novel hantaviruses in soricomorphs, the development of tools for serological investigations, is another challenge in hantavirus research. Immunological or serological investigations in shrews are limited by the lack of commercially available species-specific secondary antibodies. Therefore, little is known about the humoral immune response, the antigenic and immunological characteristics and the occurrence of human infections of soricomorph-borne hantaviruses. Only one report is available, where a serological evidence of TPMV infection in a patient, suggesting a human infection with a shrew-associated hantavirus (OKUMURA et al. 2007).

Therefore, anti-shrew IgG antibodies were produced in BALB/c-mice and rabbits which can be used for the detection of IgG antibodies in sera of infected Asian house shrews (*Suncus murinus*) or other genetically related shrews (**Paper II**). For this purpose, first anti-*Suncus* IgG sera were prepared after immunization of BALB/c-mice with protein G purified *Suncus* IgG. TPMV N protein-specific positive control sera were generated by immunisation of *S. murinus* with purified yeast-expressed TPMV N protein. Using these tools, an indirect ELISA for detection of TPMV N protein specific antibodies in sera of *S. murinus was* developed. This assay was successfully applied for the detection of TPMV N protein specific antibodies in sera of TPMV N protein immunized and experimentally TPMV infected *S. murinus* (**Paper II, Figure 3 and data not shown**). In parallel, no cross-reactivity of TPMV N protein-specific *Suncus* IgG to other rodent-borne hantavirus N proteins were found (**Paper II, data not shown**). In addition, two rabbits were immunized and a rabbit-anti-*Suncus* IgG was prepared. A WB analysis with the rabbit-anti-*Suncus* IgG revealed the strongest reactivity to the homologous IgG. The anti-*Suncus* IgG detected also purified IgG from *Sorex* spp. and *Crocidura* spp., but at a much lower intensity, whereas the *Mus musculus* IgG failed to react with the anti-*Suncus* IgG (Schlegel, Koellner et al. unpublished data). A comparison of nucleotide sequences of Ig heavy chain constant *(C)* μ genes between house mouse, human and *S. murinus* indicated, that the evolutionary distance between human and *S.murinus* is lower than that between *S. murinus* and house mouse (ISHIGURO et al. 1989). This may explain the missing reactivity of *Mus musculus* IgG with anti-*Suncus* IgG in WB and demonstrates the importance to use homologous anti-IgG antibodies in immunological assays.

In conclusion, the established TPMV IgG ELISA represent a useful serological tool to detect TPMV or TPMV-related virus specific antibodies in infected shrews, where the generated shrew anti-TPMV-antisera could serve as a positive control. This serological assay will be helpful for discovering novel insectivore-associated hantaviruses and characterizing the humoral immune response in hantavirus-infected shrews. To further enhance the sensitivity of the detection of hantavirus-specific IgG antibodies in shrew sera, other shrew species-specific IgGs should be prepared.

Development of a Pan-Hantavirus RT-PCR screening assay

A Pan-Hantavirus RT-nested-PCR assay was recently shown to have the capacity to detect novel rodent- and insectivore-borne hantaviruses (KLEMPA et al. 2006; KLEMPA et al. 2007). Broad spectrum RT-nested-PCR approaches are commonly used in the detection of different RNA viruses (ZAAYMAN et al. 2009; JOHNE et al. 2010; ZHANG et al. 2010a; DREXLER et al. 2012), but might be affected by cross-contaminations. To avoid possible contaminations and resulting false positive results, the outer primers of the RT-nested-PCR assay, described in KLEMPA et al. 2006, were selected for use in a One Step RT-PCR assay (**Papers III and IV**). In addition, a newly generated RT-qPCR assay, using SYBR-Green as detection reagent, was established (**Paper III**). SYBR-Green based Pan-Hantavirus RT-qPCR assays have never been used before for the search for novel hantaviruses. Only hantavirus S-segment-specific SYBR-Green based assays have been used for detection of DOBV (JAKAB et al. 2007a; JAKAB et al. 2007b; NEMETH et al. 2011). For the SYBR-Green Pan-Hantavirus RT-qPCR assay, novel L-segment-specific degenerated primers were designed based on an alignment of different rodent- and insectivore-borne Old World hantavirus sequences.

This SYBR-Green based RT-qPCR assay was experimentally shown to detect DOBV-, PUUV-, and TULV-specific RNA in lungs from naturally-infected *Apodemus agrarius*, *Myodes glareolus* and *Microtus arvalis* from Germany (**Paper III, data not shown**). To evaluate the usability of the SYBR-Green and One Step Pan-Hantavirus RT-PCR assays for identification of novel hantavirus sequences in rodents and shrews, they were applied in parallel for studies in the Eurasian water vole *Arvicola amphibius* and different *Sorex* species. Both assays were able to detect TULV in *A. amphibius*, *Microtus arvalis* and *M. agrestis* (**Paper III**), but the SYBR-Green

Pan-Hantavirus RT-qPCR assay failed to detect SWSV in shrews (**Paper IV, data not shown**). Therefore, only the Pan-Hantavirus One Step RT-PCR assay was used as screening assay for SWSV (**Paper IV)**.

In conclusion, the established SYBR-Green Pan-Hantavirus RT-qPCR assay represents a useful tool for the identification of already known and perhaps novel rodent-borne hantaviruses. The usability of this assay for the detection of new hantavirus species should be comprehensively proved in future investigations and, if needed, improved by, modification of the selected primers, selection of other genome regions or the use of different primer combinations.

Host association of TULV

TULV has been detected exclusively in arvicoline rodents ("voles"), in *Microtus arvalis*, *M. levis*, *M. subterraneus*, *M. gregalis* (PLYUSNIN et al. 1994; SIBOLD et al. 1995; SCHARNINGHAUSEN et al. 2002; SONG et al. 2002) and additionally in the steppe vole *Lagurus lagurus*, also a representative of the Arvicolinae subfamily (GenBank accession numbers AF442619 and AF442618; Dekonenko and Yakimenko, unpublished data). In Germany TULV has been demonstrated to be broadly distributed and associated with *M. arvalis* and *M. agrestis*. A recent study suggests an already established isolated replication and transmission cycle of TULV in *M. agrestis* from a site in Germany (SCHMIDT-CHANASIT et al. 2010). Three additional microtine species are present in Germany. *M. subterraneus* and *M. oeconomus* were found to harbour TULV and Vladivostok virus (VLAV) in the Balkan and Far-East Russia (SONG et al. 2002; PLYUSNINA et al. 2008), but for Germany no data are available so far. The presence of hantavirus infections in the Bavarian pine vole *Microtus (Terricola) bavaricus* has not been studied to date, due to its restricted distribution range (HARING et al. 2000). For additional arvicolines in Germany initial data on the occurrence of hantaviruses have been reported. Thus, in six common muskrats *Ondatra zibethicus* from the eastern part of Germany PUUV infections were described (VAHLENKAMP et al. 1998). In addition, hantavirus antigen or hantavirus-specific antibodies were detected in the Eurasian water vole *Arvicola amphibius* (formerly *terrestris*) trapped in Russia, respectively (BUTENKO et al. 1997).Therefore, the objective of the study was to prove the relevance of *A. amphibius* as a potential hantavirus host in Central Europe. Lung samples and chest cavity fluids from *A. amphibius* originating from Germany and Switzerland were investigated for hantavirus-specific RNA, with the above described assays, and for TULV-reactive antibodies according to a previously developed assay (SCHMIDT-CHANASIT et al. 2010; MERTENS et al. 2011b).

The initial screening of all *A. amphibius* lung samples with the new SYBR-Green RT-qPCR assay and the subsequent analysis with the One Step RT-PCR assay identified eight positive samples from eight different trapping sites in Germany and Switzerland (**Paper III, Table 1**). An initial analysis demonstrated the strongest similarity of these *Arvicola*-derived sequences to corresponding TULV sequences from *M. arvalis* and *M. agrestis*. Therefore, we determined additional novel TULV S, M- and L-segment sequences from *M. arvalis* and *M. agrestis* near sites where TULV-positive *A. amphibius* have been identified (**Paper III, Figure 1**). In parallel, TULV S- and M-segment sequences were generated from all eight L-segment RT-PCR positive *A. amphibius*. Maximum-Likelihood- and Bayesian statistics-based phylogenetic analyses were performed for *A. amphibius* and *Microtus*-derived TULV sequences from all three genome segments and *A. amphibius cyt b* sequences (**Paper III**). These analyses revealed a geographical clustering of the novel TULV sequences from *A. amphibius* with those of *M. arvalis*- and *M. agrestis*-derived TULV lineages (**Paper III, Figure 2A, B and C**). This observation may suggest multiple TULV spillover or a potential host switch from *M. arvalis* or *M. agrestis* to *A. amphibius*. In addition, the detection of TULV-reactive antibodies in five RT-PCR-negative *A. amphibius* may support the assumption of spillover infection events (**Paper III, Figure 1 and Table 1**). The phylogenetic analysis of the *cyt b* sequences of *A. amphibius* from Germany and Switzerland, which were generated using the assay described in **Paper I**, showed relatively high similarity with other *A. amphibius* sequences from Switzerland and Finland, but a clear separation from *A. sapidus* sequences (**Paper III**, **Figure 3**).

Obviously the partial hantavirus S-, M- and L-segment sequences, generated in this study, belong to different TULV lineages distributed mainly in *Microtus* species from Central Europe. The high nucleotide sequence identities (91-98%) between *Arvicola*- and *Microtus*-derived sequences from the same region and the non-separated positions of the *Arvicola*-derived sequences in the trees of all three segments stand against the hypothesis of an *Arvicola*-adapted TULV (**Paper III**). Paleozoological and molecular investigations suggest a last common ancestor of *Microtus* and *Arvicola* more than three million years ago (**for references see Paper III**). During these time scales the count of substitutions on one nt position should be high enough to see specific differences between *Arvicola*- and *Microtus*-associated TULV. Future investigations should prove on complete TULV genome sequences, potential ongoing processes of a host adaptation of TULV in *Microtus* and *Arvicola*.

The route of hantavirus transmission between *Microtus* and *Arvicola* (**Paper III**) is not known. Water voles show a high plasticity to different habitat types and ecological conditions (LAMBIN et al. 2004). Interactions and sympatric occurrences of *A. amphibius* with other arvicolines (e.g., *M. arvalis*) have been reported (WIELAND 1973 and G. Heckel, R. Wolf, personal communication). Water voles are capable to disperse several kilometres between different rivers and watersheds and dispersal distances were calculated with an average of about 2 km (AARS et al. 2006). As already known for other arvicoline rodents, *A. amphibius* tend to cyclic changes in their population densities. Fluctuations in *A. amphibius* population density sometimes correlate with changes in the population density in *M. arvalis* (WIELAND 1973). It is reported that rodent outbreaks do not occur everywhere at the same time, but could spread with a propagation speed of over 10 km per year (BERTHIER et al. 2005). Such outbreaks are reported for hantavirus reservoir species, as *Myodes glareolus* and *M. arvalis*, and could be important in the spread of a particular hantavirus and enhance the possibility of hantavirus transmission to other rodents, other susceptible species and humans (ESCUTENAIRE et al. 2000a; MADHAV et al. 2007; HEYMAN et al. 2009; PALO 2009; WALTEE et al. 2009; FABER et al. 2010; LUIS et al. 2010; CARVER et al. 2011). Similarly, the frequency of infections with the parasite *Echinococcus multilocularis* is strongly influenced by the densities of *A. amphibius* (VIEL et al. 1999). Although the human pathogenicity of TULV and the role of *A. amphibius* as potential reservoir host is still not clear, changes in the densities of the various hosts susceptible for TULV infection and therewith the virus prevalence should be monitored in future longitudinal studies.

In conclusion, this is the first multiple molecular evidence and the first comprehensive study about TULV infections in a representative of another genus of the subfamily Arvicolinae. The molecular detection of TULV infections in *A. amphibius* indicates the potential of this rodent species as an additional reservoir host of this particular virus. Furthermore, the role of *A. amphibius* or the other microtine rodents as the “real” reservoir hosts for TULV in Central Europe could only be clarified through additional field investigations, cell culture experiments (using *Arvicola* and *Microtus*-derived cell lines) and animal infection studies. These studies should compromise the cellular receptor usage, e.g., integrins, and the innate and adaptive immune responses in different rodent species. Importantly, it has to be proved if *A. amphibius* is able to shed the virus and transmit it to other rodent species.

Host association of Seewis virus

SWSV has been previously described in different *Sorex* species in Switzerland, Hungary, Finland and Far East Russia (SONG et al. 2007b; KANG et al. 2009a; YASHINA et al. 2010). These findings raise questions about the host association, presence and distribution of SWSV in additional countries of Europe and in Central Europe in particular. Therefore, common shrews *S. araneus*, Eurasian pygmy shrews *S. minutus*, Millet's shrews *S. coronatus* and Alpine shrews *S. alpinus* from Germany, the Czech Republic and Slovakia were analysed with different RT-PCR assays (**Paper IV**). The initial screening was done using an L-segment Pan-Hantavirus RT-PCR assay (KLEMPA et al. 2006). All L-segment positive samples were additionally tested in S-segment RT-PCR assays using previously published (YASHINA et al. 2010) and newly designed primers (**Paper IV**). The L-segment-specific screening revealed amplification products in lung, liver or kidney tissues of 49 out of 353 *S. araneus* and four out of 59 *S. minutus*. Almost complete N protein-encoding S-segment sequences were obtained for 45 of the L-segment positive *S. araneus* and all four L-segment positive *S. minutus* from Germany, Czech Republic and Slovakia (**Paper IV, Figure 1a and Table 1**). Afterwards all non-identical L- and S-segment sequences were phylogenetically analysed by Maximum-Likelihood and Bayesian analysis. The phylogenetic investigations of these novel sequences from Central Europe demonstrated their similarity to SWSV sequences from Hungary, Finland, Austria and other sites in Germany, but also confirmed a high genetic divergence of the SWSV strains (**Paper IV, Figure 1b, and c**). The low intra-cluster nt (and aa) sequence differences of 0-3.2% (0-2.2%) and 0-5.4% (0-0.9%) as well as the high inter-cluster divergence of 24.9% (5.4%) and 17.3% (3%) in L- (RdRp) and S- (N protein) segment suggest a long-term SWSV evolution in local *Sorex* populations.

The common shrew *Sorex araneus* is one of the most widely distributed mammals in Europe and is a fascinating example for chromosomal evolution in mammals. Until now, more than 60 chromosomal races have been described in *S. araneus* (WOJCIK et al. 2003). This chromosomal variability is mainly due to chromosome translocations, between two acrocentric chromosomes (Robertsonian rearrangements), accompanied by telomere–centromere tandem translocations, centromere shifts and pericentric inversions (YANNIC et al. 2008). These chromosomal differences within the *S. araneus* group are probably a result of geographical isolation during the last glacial episode, which led to genetic divergence, influenced the postglacial recolonization and the local diversification (TABERLET et al. 1998). Moreover, the recolonization of Europe had been dated to the

last glaciation period 20,000 – 18,000 years ago when the ice shield left Europe and the three major Western European phylogroups of *S. araneus* differentiated during the Middle Pleistocene from 0.47 to 0.27 million years ago (TABERLET et al. 1998; YANNIC et al. 2008). Therefore, the evolution and host association of SWSV in Europe is likely associated with the phylogeographical history of *S. araneus*. However, comparisons of mtDNA and Y-chromosome phylogenies suggest, that the genetic and chromosomal evolution in the *S. araneus* group are disconnected processes (ANDERSSON et al. 2005; YANNIC et al. 2008). A phylogenetic analysis of *cyt b* gene sequences of *S. araneus*, *S. minutus* and *S. alpinus* from all investigated trapping sites, using the recently published protocol (**Paper I**), demonstrated a species-specific separation of the *cyt b* sequences, but not a geographical clustering of the *S. araneus cyt b* sequences (**Paper IV, data not shown**). The relevance of the chromosomal races of *S. araneus* in the molecular evolution of SWSV is unclear and needs to be clarified in the future.

Interestingly, in 28 of the 49 SWSV S-segment sequences an additional putative ORF on the opposite strand to the N protein-encoding ORF was identified. This putative ORF was predicted to be localized on the antigenomic strand in a -2 frame and to encode a putative 203 aa-long protein (**Paper IV, Figure 1d**). Such a second ORF on the S-segment has never been reported for any other hantavirus. However, on the S-segment of arvicoline-, sigmodontine- and neotomine-associated hantaviruses a second ORF was identified, but on the same coding strand as the N-ORF, but with a frameshift of +1 (PLYUSNIN and MORZUNOV 2001; JAASKELAINEN et al. 2007). On the S-segment of other bunyaviruses an additional ORF, encoding a non-structural protein (NSs), exist and in phleboviruses (family *Bunyaviridae*) an amibisense coding strategy for the S segment is described (PLYUSNIN et al. 2011). The detection of the second ORF in SWSV-sequences from six different trapping sites in Germany and the Czech Republic could be an evidence for an adaptation process of the virus within a specific chromosomal race. This hypothesis has to be approved in the future by karyotype analyses of the host.

In conclusion, **Paper IV** describes the first comprehensive study of SWSV strains from Germany, the Czech Republic and Slovakia, indicating its broad geographical distribution and high genetic divergence. The detection of SWSV sequences in *S. minutus* might be explained by spillover infections, or alternatively, *S. minutus* could represent an additional reservoir host for SWSV. A more detailed analysis, of the molecular evolution of SWSV, may shed more light on the host adaptation of SWSV. The broad geographical distribution of SWSV strongly encourages future studies on the zoonotic potential of SWSV and its pathogenicity for humans in particular. For this, studies are ongoing to generate a recombinant N protein of SWSV, which can be used for the

detection of SWSV-specific antibodies in humans. In addition, this recombinant N protein could be used as antigen, in combination with the generated shrew IgG-specific antibodies (**Paper II**), for the serological detection of SWSV in the reservoir host.

Hantavirus host association and spillover infections

Although each hantavirus seems to be associated with a specific mammal species, there are few reports of the detection of hantavirus-specific antibodies in other mammal species not known to harbor a hantavirus, i.e. other small mammals, carnivores, ruminants and non-human primates (BENNETT et al. 1990; AHLM et al. 2000; ESCUTENAIRE et al. 2000b; MERTENS et al. 2011a). In addition, in several reports hantavirus-specific antibodies have been detected in rodent species, which are not the predominant carrier of a particular hantavirus, e.g. *Mus msuculus, Apodemus sylvaticus, Mus caroli*, *Maxomys surifer* (GLIGIC et al. 1992b; HJELLE and YATES 2001; KLINGSTROM et al. 2002; HEYMAN et al. 2009; BLASDELL et al. 2011). In particular, classical serological methods are not able to discriminate infections by different hantavirus species (KRUGER et al. 2001). Moreover, even the use of a FRNT approach is limited based on the used hantaviruses (ULRICH et al. 2004). Therefore, for the identification of the causative agent of a spillover infection, RT-PCR investigations and subsequent nucleic acid sequence determination, as shown in **Paper III and IV,** are needed.

Natural spillover infections are believed to be rare, but may have crucial importance in the evolution of hantaviruses. In **Paper IV**, parallel to the more frequent findings of SWSV in *S. araneus*, four infected *S. minutus* from different trapping sites have been detected. This is in accordance with other investigations, where SWSV was molecularly detected in other *Sorex* species, i.e. *S. daphaenodon*, *S. tundrensis* (YASHINA et al. 2010) and in one case in *Neomys anomalus* (GenBank Acc. No. EU418604). These results and the molecular detection of TULV infections in *M. agrestis*, *M. levis*, *M. subterraneus*, *M. gregalis*, *Lagurus lagurus* (**for references see Paper III**) and in *A. amphibius* (**Paper III)** could be probably interpreted as spillover infections.

Additional spillover infections have been reported for a large number of different rodent species (Table 5). Spillover events can occur between mammal species of the same genus (intra-genus spillover), between species of different genera of the same subfamily (inter-genus spillover) and between species of different mammal subfamilies or orders (Table 5).

Table 5 Reported nucleic acid detection of insectivore-borne SWSV and different rodent-borne hantaviruses in rodent and insectivore species, which are not postulated or identified as the predominant host for this particular hantavirus.

Virus species*	Reservoir host species	Natural spillover infections Species	Reference**
Hantaan virus	*Apodemus agrarius*	*Apodemus peninsulae*	(ZHANG et al. 2007)
		Rattus norvegicus	(ZOU et al. 2008a)
Seoul virus	*Rattus norvegicus*	*Rattus flavipectus*	Zhang et al. 2006, GenBank Acc. No. EF210133***
		Rattus tanezumi	GenBank Acc. No. HQ992814
		Rattus losea	(SHI et al. 2003)
		Mus musculus	GenBank Acc. No. GU592939
Dobrava-Belgrade virus	*Apodemus agrarius*	*Apodemus flavicollis*	**(Schlegel et al. 2009)**
	Apodemus flavicollis	*Apodemus sylvaticus, Mus musculus*	(WEIDMANN et al. 2005; ZHURAVLEV et al. 2008; GARANINA et al. 2009)
		Sorex araneus, Microtus arvalis, Myodes glareolus, Meriones tamariscinus	(ZHURAVLEV et al. 2008; GARANINA et al. 2009)
Puumala virus	*Myodes glareolus*	*Apodemus flavicollis, Microtus agrestis*	**Schlegel et al. unpublished data**
Tula virus	*Microtus arvalis*	*Microtus levis*	(PLYUSNIN et al. 1994)
		Microtus agrestis	(SCHARNINGHAUSEN et al. 2002; SCHMIDT-CHANASIT et al. 2010), **Paper III**
		Microtus subterranus	(SONG et al. 2002)
		Microtus gregalis	GenBank Acc. No. AF442620-1
		Lagurus lagurus,	GenBank Acc. No. AF 442618-9
		Arvicola amphibius	**Paper III**
		Myodes glareolus	**Schlegel et al. unpublished data**
Isla Vista virus	*Microtus californicus*	*Peromyscus maniculatus, Peromyscus californicus*	(SONG et al. 1995)
Sin Nombre virus	*Peromyscus maniculatus*	*Rheitrodontomys megalotis, Peromyscus leucopus*	(HJELLE et al. 1995; RAWLINGS et al. 1996)
El Moro Canyon virus	*Reithrodontomys megalotis*	*Peromyscus maniculatus*	(RAWLINGS et al. 1996)
Andes virus	*Oligoryzomys longicaudatus*	*Rattus rattus, Abrothrix longipilis, Loxodontomys micropus, Oligoryzomys flavescens*	(MEDINA et al. 2009)
		Oligoryzomys chacoensis	(GONZALEZ DELLA VALLE et al. 2002)
Bayou virus	*Oryzomys palustris*	*Sigmodon hispidus*	(TORREZ-MARTINEZ et al. 1998)
Laguna Negra virus	*Calomys laucha*	*Calomys callosus, Akodon simulator*	(LEVIS et al. 2004)
Rio Mamore virus	*Oligoryzomys microtis*	*Holochilus sciureus, Oligoryzomys fornesi*	(ROSA et al. 2005)
Seewis virus	*Sorex araneus*	*Sorex daphaenodon, Sorex tundrensis*	(YASHINA et al. 2010)
		Sorex minutus	**Paper IV**
		Neomys anomalus	GenBank Acc. No. EU418604

*Hantavirus taxonomy according to KING et al. 2011. **Additional GenBank entries of *Rattus flavipectus*: FJ803201-04, FJ803209, FJ803213, FJ884369, FJ884391, FJ884401, GU592943. Own investigations are highlighted in bold.

In **Paper III and IV** and several other studies intra-genus spillover infections were reported for rodent-borne HTNV, SEOV, DOBV, TULV, SNV, ANDV, Laguna Negra virus (LANV), RIOMV, shrew-borne SWSV and inter-genus spillover infections were described for HTNV, SEOV, DOBV, PUUV, TULV, SNV, El Moro Canyon virus (ELMCV), ANDV, Bayou virus (BAYV), LANV and RIOMV (Table 5). Only few data about natural spillover infections between species of different rodent subfamilies and one between different mammal orders are available. The arvicoline-associated PUUV has been molecularly detected in a single *Apodemus flavicollis* (subfamily Murinae) from Germany (Schlegel et al. unpublished data) and an infection with another arvicoline-borne hantavirus ISLAV has been reported in *Peromyscus califonicus* (subfamily Neotominae) from North America (SONG et al. 1995). The sigmodontine-borne ANDV was found in *Rattus rattus* (subfamily Murinae) from Chile (MEDINA et al. 2009). Murine-related DOBV sequences were found in *Meriones tamariscinus* (subfamily Gerbillinae) in Russia (ZHURAVLEV et al. 2008; GARANINA et al. 2009). Only a single case of a molecularly detected spillover infection of a rodent-borne hantavirus (DOBV) in a shrew (*S. araneus*) from Russia has been reported (GARANINA et al. 2009).

The exact route of natural spillover transmissions is often unclear. In case of genetically closely related host species, which share the same habitats, a spillover transmission seems to be very likely. For *A. amphibius* interactions with *Microtus* rodents have been described and *S. araneus*, *S. minutus* and *Neomys anomalus* could occur sympatrically in the same habitats (**for references see Paper III and IV**).

Sympatric occurrences have been reported for *Myodes glareolus*, *M. agrestis*, *Apodemus agrarius* in Southern Poland (CHELKOWSKA et al. 1985), *A. sylvaticus*, *Mus musculus* in Central Italy (BOITANI et al. 1985) and *A. flavicollis*, *A. sylvaticus*, *Myodes glareolus* in Denmark (GEBCZYNSKI et al. 1986). In North America *Peromyscus* and *Microtus* species, which are carrying different hantaviruses, have the same geographical distribution, but are ecologically separated (M'CLOSKEY and FIELDWICK 1975).

Species diversity in a particular habitat has been shown to decrease the prevalence of SNV ("dilution effect") and influences the spread of the virus by reducing encounters between the reservoir host and non-reservoir species (CLAY et al. 2009a; CLAY et al. 2009b). A reduced biodiversity due to experimental removal of non-reservoir species could increase the hantavirus prevalence and density of the reservoir-host population in a specific habitat (SUZAN et al. 2009). In contrast to that, species diversity may support the transmission of a hantavirus from the original

reservoir host to distantly related, but susceptible species, as observed for TULV and SWSV in **Paper III and IV**. In addition, environmental changes could result in a greater habitat overlap of different mammal species, which results in more encounters, and may lead to hantavirus spillover infections or host switch events (ALLEN et al. 2009). Moreover, non-reservoir species could also play a role as temporary carriers for hantaviruses and as mediators for virus transmission into new habitats, as postulated for *A. sylvaticus* (HEYMAN et al. 2009).

The host association of hantaviruses is influenced by multiple extrinsic and intrinsic biological parameters, e.g., habitat factors, reservoir host distribution, population density and biodiversity, immune response and cellular receptor gene polymorphisms. The association of a hantavirus with its host seems to be genetically defined. Mammal species, which are genetically closely related with the reservoir-host species, seems to have a higher susceptibility for infections with the particular hantavirus than more distantly related species. For instance, in animal infection experiments, *M. agrestis* and *Lemmus sibiricus* could be infected with arvicoline-borne PUUV, but not Syrian hamsters or BALB/c mice. In addition, *A. flavicollis*, *A. agrarius* and *Mus musculus* (BALB/c and NMRi mice) could be infected by murine-associated DOBV, but not *Myodes glareolus*. In contrast to that, neither *Myodes glareolus* nor *M. agrestis* could be infected by arvicoline-related, but cell-culture adapted TULV strain (KLINGSTROM et al. 2002). Interestingly, the cell-culture adaptation of PUUV strain Kazan also resulted in a virus variant which is not infectious for *Myodes glareolus* (LUNDKVIST et al. 1997). A sequence analysis identified regions, at nt positions 26, 1577 and 1580, on the S- and at 2053 on the L-segment, to be responsible for this phenotype (LUNDKVIST et al. 1997; NEMIROV et al. 2003). Therefore, future infection studies should use wild-type or reservoir-host passaged hantavirus strains to identify its host association. Interestingly, during the acute phase naturally spillover-infected animals seems to have a high viral load and might therefore represent good candidates for virus isolation approaches (POPUGAEVA et al. 2012).

Spillover infections could represent a first step in the development of a new virus-host association and are a crucial prerequisite for host switch and genetic reassortment. Furthermore, the multiple findings of spillover transmissions underline the current problem of the reservoir host definition. Only a detailed analysis of the frequency of infection by a particular hantavirus in sympatrically occurring small mammal species within a geographic region may allow to identify the "true" reservoir host for this virus (HJELLE and YATES 2001). Therefore, comprehensive future studies in wild living mammal communities will be helpful to evaluate the transmission cycles of hantaviruses, to identify their "true" reservoir hosts and to estimate the susceptibility of sympatrically occurring small mammal species. For definition of a reservoir host function, it has to

be proved if a particular mammal species is persistently infected, able to shed and transmit the virus. To prove the main factors of virus cell entry and persistence establishment and maintenance, field studies should be accompanied by *in vitro* investigations in cell culture and *in vivo* studies in a feasible animal model. Such investigations could give a more detailed knowledge about the biological processes during hantavirus infections and will help to establish novel ways for prevention of human infections.

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Own contribution to publications

Own contribution to Paper I

For this study, I designed the primers, established the *cytochrome b* PCR and was participated in the acquisition of the samples. I determined and analysed the derived sequences, performed the phylogenetic analyses and wrote the major part of the manuscript.

Own contribution to Paper II

In this paper, I was involved in the preparing of the monoclonal antibodies directed against yeast-expressed TPMV N protein, the immunization of the Asian house shrews and the production of the anti-shrew IgG antibodies. In addition, I performed the respective part of the ELISA and IFA investigations and wrote the major part of the manuscript.

Own contribution to Paper III

In this publication, I designed the primers, established the SYBR-Green RT-PCR assay, and performed the RNA extraction and the major part of the RT-PCR analyses. Additionally, I carried out the sequencing, prepared the sequences, conducted the phylogenetic analyses and wrote the major part of the manuscript.

Own contribution to Paper IV

For this publication, I was responsible for the RNA extraction and RT-PCR analyses of the *Sorex* samples from Germany. The RNA extraction and RT-PCR analyses of the samples from Czech Republic and Slovakia was done by our colleagues in Slovakia and Berlin. I designed the respective part of the used primers, performed the phylogenetic analyses of the sequences and wrote the major part of the manuscript.

In agreement:

…….

Signature PD Dr. Rainer G. Ulrich

List of publications

Overview

Publication	Number of publications	
	First / Senior author	Coauthor
Original article	5	7
Abstract publications	2	4
Miscellaneous	2	3
Poster (Conference)	10	4
Oral presentations	4	-

Publications which are part of this thesis are highlighted in gray.

1. Original article

1.1. First author

1. **Schlegel, M**[1]., Klempa, B[1]., Auste, B., Bemmann, M., Schmidt-Chanasit, J., Büchner, T., Groschup, M.H., Meier, M., Buschmann, A., Zoller, H., Krüger, D.H., Ulrich, R.G. (2009). Multiple *Dobrava-Belgrade virus* spillover infections, Germany. Emerg. Infect. Dis. 15(12):2017-2020. [1]both authors contributed equally to this article
2. **Schlegel, M.**, Sheikh Ali, H., Stieger, N., Groschup, M.H., Wolf, R., Ulrich, R.G. (2011). Molecular identification of small mammal species using novel *cytochrome b* gene-derived degenerated primers. Biochem. Genetics. 50(5-6):440-447.
3. **Schlegel, M**., Kindler, E., Essbauer, S.S., Wolf, R., Thiel, J., Groschup, M.H., Heckel, G., Oehme, R.M., Ulrich R.G. (2011). *Tula virus* infections in the Eurasian water vole in Central Europe. Vector-borne Zoonotic Dis. 12(6):503-513.
4. **Schlegel, M**., Radosa, L., Rosenfeld, U.M., Schmidt, S., Triebenbacher, C., Löhr, P.W., Fuchs, D., Heroldová, M., Jánová, E., Stanko, M., Mošanský, L., Fričová, J., Pejčoch, M., Suchomel, J., Purchart, L., Groschup, M.H., Krüger, D.H., Klempa, B., Ulrich, R.G. (2012). Broad geographical distribution and high genetic diversity of shrew-borne Seewis hantavirus in Central Europe. Virus Genes. 45(1):48-55.
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1.2. Coauthor

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2. Abstract publications

2.1. First author

1. **Schlegel, M**. Essbauer, S.S., Mertens, M., Groschup, M.H., Schmidt-Chanasit, J., Freise, J., Wegener, W., Ulrich, R.G. (2009). Longitudinal studies on *Puumala virus* prevalence in bank voles from two endemic regions in Germany. Mammalian Biology. 74: 22-23.
2. **Schlegel, M**., Thiel, J., Triebenbacher, C., Löhr, P.-W., Groschup, M.H., Ulrich, R.G. (2010). Novel lineage of Seewis virus in *Sorex araneus* in Germany. Mammalian Biology. 75: 23.

2.2. Coauthor

1. Ulrich, R.G., **Schlegel, M**., Schmidt-Chanasit, J., Klempa, B., Mertens, M., Masur, D., Büchner, T., Sevke, K., Freise, J., Jacob, J., Krüger, D.H., Oehme, R., Brockmann, S.O., Heckel, G., Essbauer, S.S. (2008). First Germany-wide epidemiology of hantavirus infections in rodent reservoir hosts. Mammalian Biology. 73:42-43.

2. Maaz, D., **Schlegel, M**., Gerwin, W., Ansorge, H., Ulrich, R.G. (2010). Recolonization of a post-mining landscape in Germany by small mammals and their associated hantaviruses. Mammalian Biology. 75:17-18.
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4. Ulrich, R.G., **Schlegel, M**., Baumann, K., Breithaupt, A., Binder, A., Schotte, U., Ruhl, S., Krohmann, C., Essbauer, S., Frangoulidis, D., Kayßer, P., Meyer, H., Riehm, J., Faulde, M., Lewitzki, J., Sauer, S., Teifke, J.P. (2012). Searching for zoonotic pathogens in small mammals from Afghanistan. Mammalian Biology. 77:21.

3. Miscellaneous

3.1. First author

1. **Schlegel, M**., Groschup, M.H., Ulrich, R.G. (2009). Reservoirwirt des *Dobrava-Belgrad-Virus* identifiziert. Der Loeffler. 6(2):6.
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3.2. Coauthor

1. Sombke, A., **Schlegel, M**. (2007). Orthoptera and Mantodea of Istria and the Croatian Island Šipan. Rostocker Meeresbiologische Beiträge. 18:131-137.
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3. Ulrich, R.G., **Schlegel, M**., Mertens, M., Groschup, M.H., Schmidt-Chanasit,. J., Plenge-Bönig, A., Jacob, J., Pelz, H.-J., Freise, J., Wenk, M., Thiel, J., Triebenbacher, C., Schmolz, E., Kurth, A., Krüger, F., Rühe, F., Kiffner, C., Ansorge, H., Gerwin, W., Wegener, W., Müller, J., Bemmann, M., Wolf, R., Otto, L.-F., Oehme, R., Pfeffer, M., Heckel, G., Schex, S., Essbauer, S.S. (2009). Netzwerk „Nagetier-übertragene Pathogene": Monitoring von Hantavirus-Infektionen in Deutschland. Beiträge zur Jagd- und Wildforschung. 34:229-250.

4. Poster (Abstracts)

4.1. First and Senior author

1. **Schlegel, M**., Schmidt-Chanasit, J., Masur, D., Büchner, T., Sevke, K., Wegener, W., Essbauer, S.S., Brockmannn, S.O., Mertens, M., Ulrich, R.G. and members of the network „Rodent-borne pathogens“. Serological detection of *Puumala virus*-specific antibodies in bank voles from different regions in Germany. Medical Biodefense Conference, 17-18. October 2007, Munich, Germany.
2. **Schlegel, M**. Essbauer, S.S., Mertens, M., Groschup, M.H., Schmidt-Chanasit, J., Freise, J., Wegener, W., Ulrich, R.G. Longitudinal studies on *Puumala virus* prevalence in bank voles from two endemic regions in Germany. 83th Annual Meeting of the German Society of Mammalogy, 13-17. September 2009, Dresden, Germany.
3. **Schlegel, M**., Thiel, J., Triebenbacher, C., Löhr, P.W., Groschup, M.H., Ulrich, R.G. Novel lineage of Seewis virus in *Sorex araneus* in Germany. 84th Annual Meeting of the German Society of Mammalogy, 12-16. September 2010, Berlin, Germany.
4. **Schlegel, M**., Thiel, J., Triebenbacher, C., Löhr, P.W., Groschup, M.H., Ulrich, R.G. Network „Rodent-borne pathogens“: Detection of a shrew-borne hantavirus in Germany. National Symposium on Zoonoses Research, 7-8. October 2010, Berlin, Germany.
5. **Schlegel, M**., Hammerschmidt, B., Yoshimatsu, K., Groschup, M.H., Arikawa, J., Friedrich, R., Petraityte, R., Sasnauskas, K., Heidemanns, K., Siniza, S., Giere, P., Ulrich, R.G., Koellner, B. Novel tools for hantavirus diagnostics in shrews. 8th International Conference on HFRS, HPS & Hantaviruses, 20-22 May 2010, Athens, Greece.
6. **Schlegel, M**., Thiel, J., Triebenbacher, C., Löhr, P.W., Groschup, M.H., Ulrich, R.G. Novel lineage of Seewis virus in *Sorex araneus* in Germany. 8th International Conference on HFRS, HPS & Hantaviruses, 20-22. May 2010, Athens, Greece.
7. **Schlegel, M**., Maaz, D., Kindler, E., Essbauer, S.S., Wolf, R., Thiel, J., Groschup, M.H., Heckel, G., Ansorge, H., Oehme, R.M., Ulrich, R.G. Host range and genetic lineages of *Tula virus* in Central Europe. 21st Annual Meeting of the GfV (Society for Virology), 23–26. March 2011, Freiburg, Germany.
8. **Schlegel, M**., Thiel, J., Triebenbacher, C., Löhr, P., Groschup, M.H., Ulrich, R.G. Detection of a shrew-borne hantavirus in Germany. 21st Annual Meeting of the GfV (Society for Virology), 23-26. March 2011, Freiburg, Germany.
9. **Schlegel, M**., Hammerschmidt, B., Yoshimatsu, K., Groschup, M.H., Arikawa, J., Friedrich, R., Petraityte, R., Sasnauskas, K., Heidemanns, K., Siniza, S., Giere, P., Ulrich, R.G., Koellner, B. Novel tools for hantavirus diagnostics in shrews. 21st Annual Meeting of the GfV (Society for Virology), 23–26. March 2011, Freiburg, Germany.
10. **Schlegel, M**., Radosa, L., Rosenfeld, U.M., Schmidt, S., Thiel, J., Triebenbacher, C., Groschup, M.H., Heroldová, M., Jánová, E., Stanko, M., Pejčoch, M., Suchomel, J., Purchart, L., Krüger, D.H., Klempa, B., Ulrich, R.G. Novel lineages of Seewis virus in *Sorex araneus* and *Sorex minutus* from Central Europe. Medical Biodefense Conference, 25-28. October 2011, Munich, Germany.

4.2. Coauthor

1. Ulrich, R.G., **Schlegel, M**., Schmidt-Chanasit, J., Klempa, B., Mertens, M., Masur, D., Büchner, T., Sevke, K., Freise, J., Jacob, J., Krüger, D.H., Oehme, R., Brockmann, S.O., Heckel, G., Essbauer, S.S. First Germany-wide epidemiology of hantavirus infections in

rodent reservoir hosts. 82th Annual Meeting of the German Society of Mammalogy, 14-17. September 2008, Vienna, Austria.

2. Popugaeva, E., **Schlegel, M**., Ulrich, R.G., Hofmann, J., Krüger, D.H., Klempa, B. Isolation of *Dobrava-Belgrade* hantavirus from an endemic area of haemorrhagic fever with renal syndrome in North-East Germany. 19st Annual Meeting of the GfV (Society for Virology), 18-21. March 2009, Leipzig, Germany.
3. Maaz, D., **Schlegel, M**., Gerwin, W., Ansorge, H., Ulrich, R.G. Recolonization of a post-mining landscape in Germany by small mammals and their associated hantaviruses. 84th Annual Meeting of the German Society of Mammalogy, 12-16. September 2010, Berlin, Germany.
4. Radosa, L., **Schlegel, M**., Rosenfeld, U.M., Schmidt, S., Thiel, J., Triebenbacher, C., Groschup, M.H., Heroldová, M., Jánová, E., Stanko, M., Pejčoch, M., Suchomel, J., Purchart, L., Krüger, D.H., Klempa, B., Ulrich, R.G. Shrew-borne Seewis hantavirus in Central Europe: high genetic diversity and putative spillover infections. 22st Annual Meeting of the GfV (Society for Virology), 14-17. March 2012, Essen, Germany.

5. Oral presentations

1. **Schlegel, M**. First molecular identification of *Apodemus agarius*-borne *Dobrava-Belgrade virus* in different *Apodemus* species in North-East Germany. 19st Annual Meeting of the GfV (Society for Virology), 18-21. March 2009, Leipzig, Germany.
2. **Schlegel, M**., Ulrich, R. Novel tools for serological and nucleic acid diagnostics. Hantavirus Workshop, 2010, Liverpool, United Kingdom, organized by the Rabies and Wildlife Zoonoses Group, Veterinary Laboratories Agency, National Centre for Zoonosis Research.
3. **Schlegel, M**., Ulrich, R. Hantaviruses in Germany: Novel virus lineages and spillover infections. Workshop “The Math of Flu”, 2011, Greifswald, Germany.
4. **Schlegel, M**., Sheikh Ali, H. (2011). Molecular evolution of *Puumala* hantavirus in an endemic region in Lower Saxony and Host spectrum and spillover infections of rodent- and insectivore-borne hantaviruses. Medical Biodefense Conference, 25-28. October 2011, Munich, Germany.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

01.10.2012, Greifswald Unterschrift des Promovenden

Lebenslauf

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