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Sindbis Virus Strains of Divergent Origin Isolated from Humans and Mosquitoes During a Recent Outbreak in Finland

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Abstract

Sindbis virus (SINV) is a mosquito-borne avian hosted virus that is widely distributed in Europe, Africa, Asia, and Oceania. Disease in humans is documented mainly from Northern Europe and South Africa and associated with genotype I. In 2018 under extremely warm climatic conditions, a small outbreak of 71 diagnosed SINV infections was recorded in Finland. We screened 52 mosquito pools (570 mosquitoes) and 223 human sera for SINV with real-time RT-PCR and the positive samples with virus isolation. One SINV strain was isolated from a pool ($n = 13$) of genus *Ochlerotatus* mosquitoes and three strains from patient serum samples. Complete genome analysis suggested all the isolates to be divergent from one another and related to previous Finnish, Swedish, and German strains. The study provides evidence of SINV strain transfer within Europe across regions with different epidemiological characteristics. Whether these are influenced by different mosquito genera involved in the transmission remains to be studied.

Keywords: Sindbis virus, mosquito-borne virus, alphavirus, virus isolation, *Ochlerotatus*, Finland

Introduction

Sindbis virus (SINV) (Genus *Alphavirus*) is a mosquito-borne virus with a life cycle involving amplifying avian host (Hubalek 2008). The distribution of the six genotypes of SINV covers Europe, Africa, Asia, and Oceania. Human cases are mainly detected from Northern Europe and South Africa where genotype I is circulating. The recent comprehensive phylogenetic analysis suggests that SINV was imported from Africa to Northern Europe, possibly first to Sweden, by migratory birds (Ling et al. 2019).

In Finland SINV infections are diagnosed annually in late summer (Brummer-Korvenkontio et al. 2002). The seroprevalence for SINV is highest in Eastern Finland (16%) and increases with age (Kurkela et al. 2008). The disease caused by SINV, known as Pogosta disease in Finland, manifests after a median incubation period of 4 days with arthralgia, arthritis, myalgia, rash, and fever (Kurkela et al. 2005). The symptoms are usually self-limiting and clear spontaneously within a few weeks, but in 25% of patients the symptoms persist at least 3 years and have an impact in the quality of life (Kurkela et al. 2008).

In Finland the larger SINV outbreaks of hundreds of cases occur approximately once in a decade. The latest larger epidemic was observed in 2012 with 189 cases (''National Infectious Disease Registry maintained by the Finnish Institute for Health and Welfare'' 2019, Brummer-Korvenkontio et al. 2002). Environmental factors known to promote SINV transmission in Northern Europe include high summer temperature and precipitation and thick snow layer during the spring (Jalava et al. 2013). Summer 2018 was unusually warm and dry in all Europe, including Finland. Average summer temperature between June and August was 2° higher than usual, and precipitation was half of normal in Finland (FMI 2019).

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In Southern and Eastern Europe, exceptionally high numbers of infections caused by another mosquito-borne and bird hosted virus, West Nile virus, were observed (ECDC 2018). In Finland a small outbreak of SINV with 71 diagnoses was observed (National Infectious Disease Registry maintained by the Finnish Institute for Health and Welfare 2019).

Materials and Methods

To study SINV during this exceptional season, mosquitoes were collected in known endemic region at three locations in August 2018 (Fig. 1) using Prokopack (The John W. Hock Company, Gainesville) and Mosquito Magnet® Pioneer (Woodstream Corporation, Lancaster) trap with Octenol attractant. Collections were done during daytime and evenings in forests, bogs, and gardens, and the specimens were kept in empty tubes or in Virocult medium (Medical Wire, England) on dry ice while being transported to −80°C storage. Out of 570 female mosquitoes 52 pools consisting of 1–30 individuals were constructed based on morphologically identified genus (Becker et al. 2010), collection site, and blood feeding status.

In Finland, 2018, altogether 71 SINV clinical cases were documented in late summer/early autumn, which is more than in a few preceding years (Fig. 2). The patient serum samples for this study were obtained from Helsinki University Hospital (HUSLAB) where the samples had been sent for serological testing in 2018 from different health care facilities throughout Finland (project TYH2018322, Research permit HUS/32/2018). The serodiagnosis of the patients was based in IgM and IgG detection using methodology by Manni et al. (2008). The samples selected for this study included acute phase serum samples from diagnosed Sindbis patients ($n = 31$) for whom also a consecutive sample was available with IgM and IgG seroconversion. In addition, samples of 192 patients suspected for SINV infection, but without the serological diagnosis, were included in the study.

For screening mosquitoes were processed in pools. The mosquito pools were homogenized to Dulbecco's phosphate-buffered saline supplemented with 0.2% bovine serum albumin using Qiagen TissueLyser. Nucleic acids were extracted from 140 μL mosquito homogenate and human serum samples and eluted in 50 μL of elution buffer using QIAamp Viral

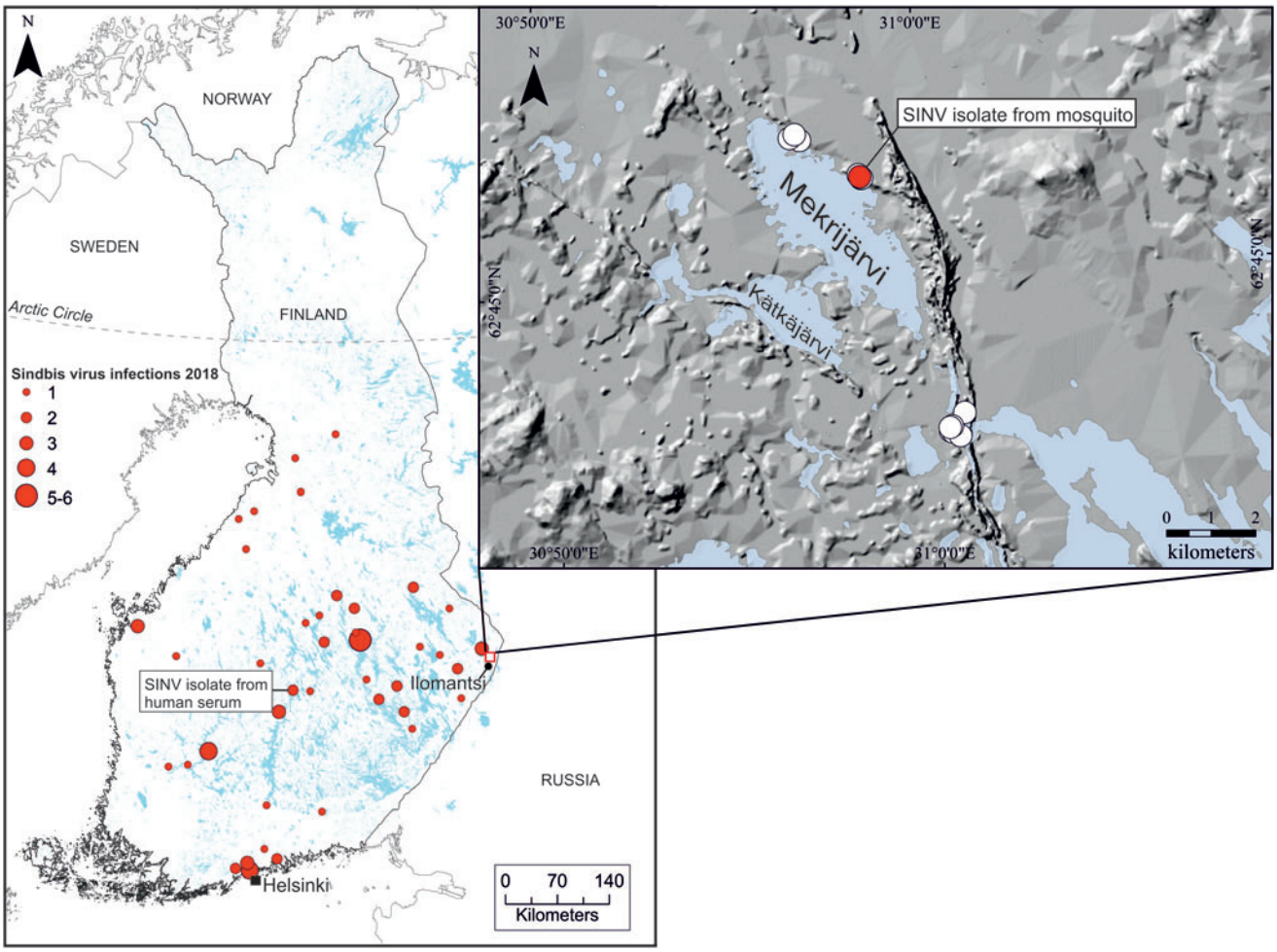

FIG. 1. Map of Finland showing the locations of diagnosed SINV infections in 2018 and origin of virus isolation positive patient sample. The collection sites (indicated with *white* and *red circles*) of the mosquito pools are shown in zoomed map of Mekrijärvi. The virus isolation positive mosquito pool (P1) was collected on 13th of August from a garden (location indicated with a *red circle*). SINV, Sindbis virus. Map was created with ESRI ArcGIS (version 10.3.1) by using open source GIS data (Finnish Environment Institute 2018; NLS of Finland 2000). Hillshade was created in ArcGIS from a digital elevation model (DEM) with 25-m resolution (NLS of Finland 2000). Color images are available online.

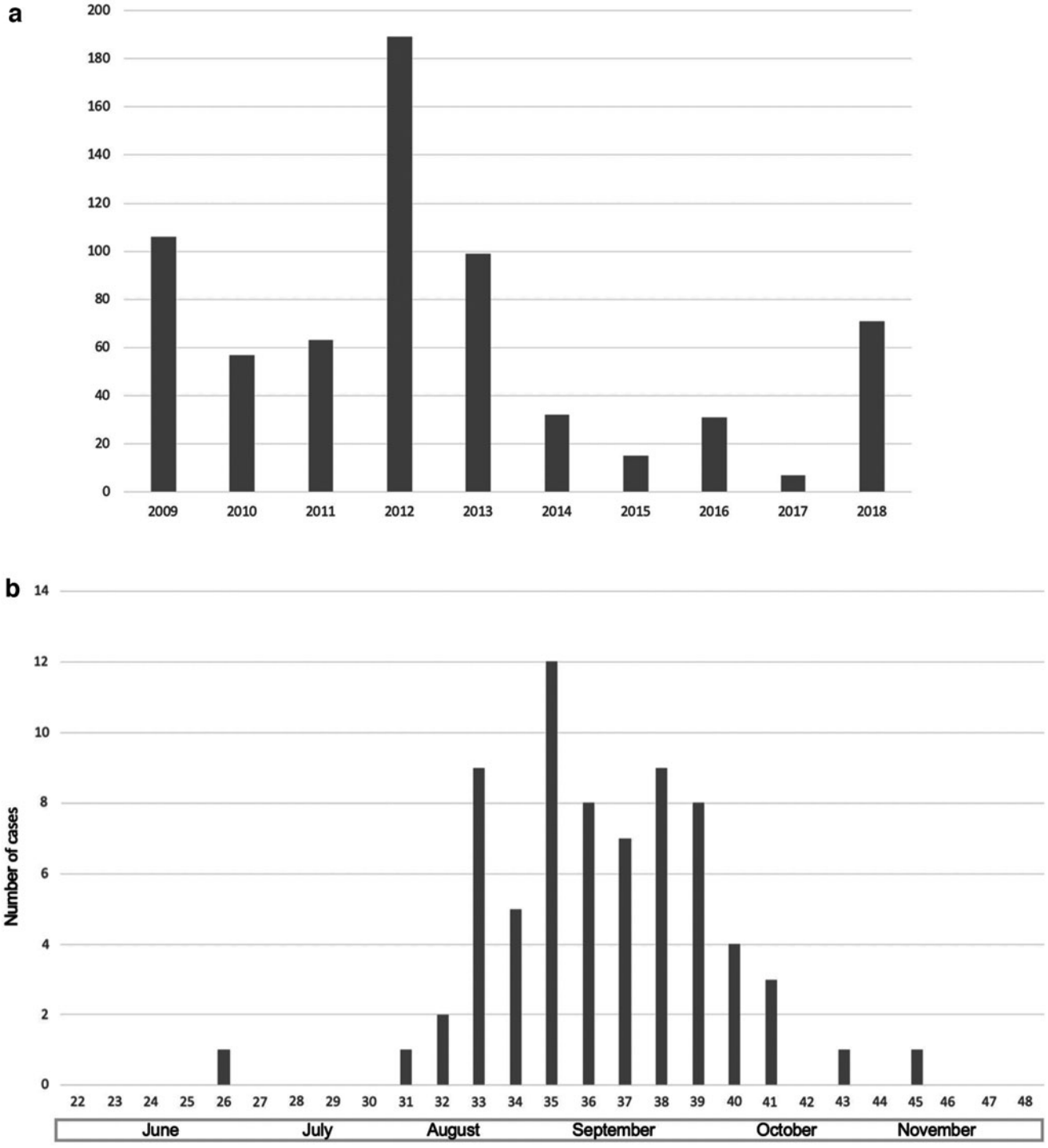

FIG. 2. Number of SINV diagnoses in 2009–2018 **(a)** and epidemic curve of SINV 2018 in Finland **(b)** (National Infectious Disease Registry maintained by the Finnish Institute for Health and Welfare 2019).

RNA Mini Kit (Qiagen) or QIAamp 96 Virus QIAcube HT Kit (Qiagen) according to manufacturer's instructions. Samples were studied using SINV real-time RT-PCR according to primers, probe, and protocol of Sane et al. (2012a) with the exception of using TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) reagents. Real-time RT-PCR was repeated for positive samples, except one human serum in which we had no volume enough left. BHK-21 (baby hamster kidney) and Vero E6 (green monkey kidney) cells grown in 6-well plates were infected with 100 μL of mosquito homogenate or human serum and observed daily for cytopathic effects for duration of 2 weeks.

For molecular identification purposes mitochondrial cytochrome c oxidase subunit 1 (COI) gene region PCR (Folmer et al. 1994) was performed for the nucleic acids extracted from virus positive mosquito pool using DreamTaq DNA polymerase

(Thermo Fisher Scientific), and the product was subjected to Sanger (University of Helsinki, Institute of Biotechnology, DNA sequencing and genomics) and Next-Generation sequencing (in-house Illumina MiSeq) sequencing.

SINV genomes were sequenced from the viral isolates using Illumina MiSeq. RNA was extracted from isolation culture supernatants with TRIzol Reagent (Thermo Fisher Scientific), and libraries were prepared using NEBNext Ultra RNA Library Prep Kit (New England Biolabs) according to manufacturer's instructions. Low quality (quality score <30) sequences were removed using Trimmomatic followed by *de novo* assembly using Megahit and reassembly against the *de novo* assembled consensus sequences using BWA-MEM algorithm (Li 2013, Bolger et al. 2014, Li et al. 2015).

Phylogenetic tree was constructed using complete coding sequences of SINV. The sequences that form northern European subgroup of SINV genotype I (Ling et al. 2019) were downloaded from the GenBank and aligned using ClustalW algorithm implemented in MEGA7 (Kumar et al. 2016). The alignment was manually edited to include only the concatenated open reading frames of SINV genome. The best fit substitution model was estimated using jModeltest2 (Kumar et al. 2016). Phylogenetic tree was constructed using Bayesian Monte Carlo Markov Chain method and GTR + G substitution model implemented in MrBayes (Ronquist and Huelsenbeck 2003). The posterior probabilities are shown for each node. Viral sequences obtained in this study are deposited to GenBank with acc. nos. MN389434 (*Ochlerotatus* sp) and MN389435, MT270144, and MT270145 (human serum isolates).

Results

One out of 52 mosquito pools and eight out of 223 human sera were positive in SINV real-time RT-PCR. Out of the eight positive human sera, two were samples from patients with serodiagnosis and six were from patients without diagnosis. Six of these samples were repeatedly positive. With one sample, the PCR could not be repeated because of lack of the volume of RNA and one sample was negative when repeated. Virus isolation confirmed the RNA positivity in the mosquito pool (Sample FIN_2018_M_P1) and in three patient serum samples (FIN_2018_H_37; 25 and 05). The isolates formed cytopathic effect (CPE) in 2–4 days in both of the tested cell lines.

The studied mosquito pools represented mostly *Ochlerotatus* genus ($n = 35$), but also *Culex* ($n = 2$), *Aedes* ($n = 13$), and *Coquillettidia* ($n = 2$) genera were identified and pooled for screening. The SINV positive mosquito pool consisted of 13 individual female *Ochlerotatus* mosquitoes collected on 13th of August 2018 using Prokopack aspirator during evening from the yard of a rural settlement next to Mekrijärvi (Fig. 1) and stored in Virocult medium. Nucleotide Basic Local Alignment Search Tool (BLAST) results of the Sanger sequenced COI PCR product of 530 bp (MN422093), and the sequences obtained from NGS sequencing of the same PCR product (data not shown) were in line with the morphological identification of *Ochlerotatus* genus, but failed to provide reliable identification of the exact species comprising the pool. The sequence from Sanger sequencing showed highest nucleotide identity to *Ochlerotatus communis* (<95%), whereas the NGS sequencing results shared highest percentage identities to *Ochlerotatus annulipes* from Sweden (96.79%) and *Ochlerotatus excrucians* from the United States (96.04%) and furthermore demonstrated numerous polymorphic sites that may result from *multiple species* existing in the pool.

In the phylogenetic analysis of the obtained SINV complete genome data, the virus isolate from mosquito pool FIN_2018_M_P1 clustered with a virus strain isolated from mosquitoes from the same area in Ilomantsi, Finland, 2005. Within the same clade, also one of the human isolates Strain FIN_2018_H05 was located (Fig. 3). The patient serum isolate FIN_2018_H_37 was clustered with strains isolated from mosquitoes and woodpigeon in Southwest Germany 2009–2016 (Jost et al. 2010, Scheuch et al. 2018). The human serum isolate FIN_2018_H_25 was clustered with previous Finnish human blood isolate from 2002 Ilomantsi and Swedish Lovanger strain from *Culiseta morsitans*, 2013. These subclusters formed a larger group together with strains from Finland, Russia, Germany, and Sweden. The SINV serum isolate FIN_2018_H_37 was obtained from the acute-phase sample of a SINV diagnosed 75-year-old male who had no recent travel history and, thus, likely obtained the infection in his residence commune of Laukaa, Central Finland (Fig. 1). The isolate FIN_2018_H_25 was from a serum sample of a 48-year-old female who lived in Tampere, and FIN_2018_H_05 was from a 36-year-old female from Kuopio. These two female patients did not have serological diagnosis, as only a single sample with no antibodies detected was received by the laboratory. Before this study, the isolates from patients in Finland originated from whole blood ($n = 1$) and skin lesions ($n = 3$) from Ilomantsi and Kiihtelysvaara (Kurkela et al. 2004).

Discussion

In this study we detected SINV RNA initially in serum samples of eight patients but when repeated, one of the samples remained negative. This sample had shown positivity only at late cycles in the initial screening. Two of the SINV RNA positive patients were from the group of patients with serological diagnosis based on paired samples, and the RNA positivity was confirmed by virus isolation in one of them. We detected SINV RNA repeatedly also in additional five patients, who had no serodiagnosis and isolated the virus in two of them. This was surprising as, before this work, SINV has been rarely isolated from human sera, and the SINV RNA detection has been considered not suitable for diagnostic use (Sane et al. 2012a). These undiagnosed SINV RNA positive patients were suspected for SINV infection, but were left undiagnosed likely due to the early sampling time point and the lack of a consequent sample. These results encourage further studies on SINV patient serum samples and their wider research use in SINV RNA and virus detection.

The sequence analysis of the three virus isolates obtained in this study from patients originating from different geographical locations during the epidemic season of 2018 suggested the strains to be divergent from another raising the question of possible strain characteristics in relation to pathogenic properties, viremia levels, and its duration. Unfortunately, for the patients lacking the serodiagnosis no clinical data were available, including the timing of disease onset in regard to sampling time point.

In Finland, the mosquito isolate obtained in this study, and in the previous work involving the same area (Sane et al. 2012b), are all most likely originating from *Ochlerotatus*

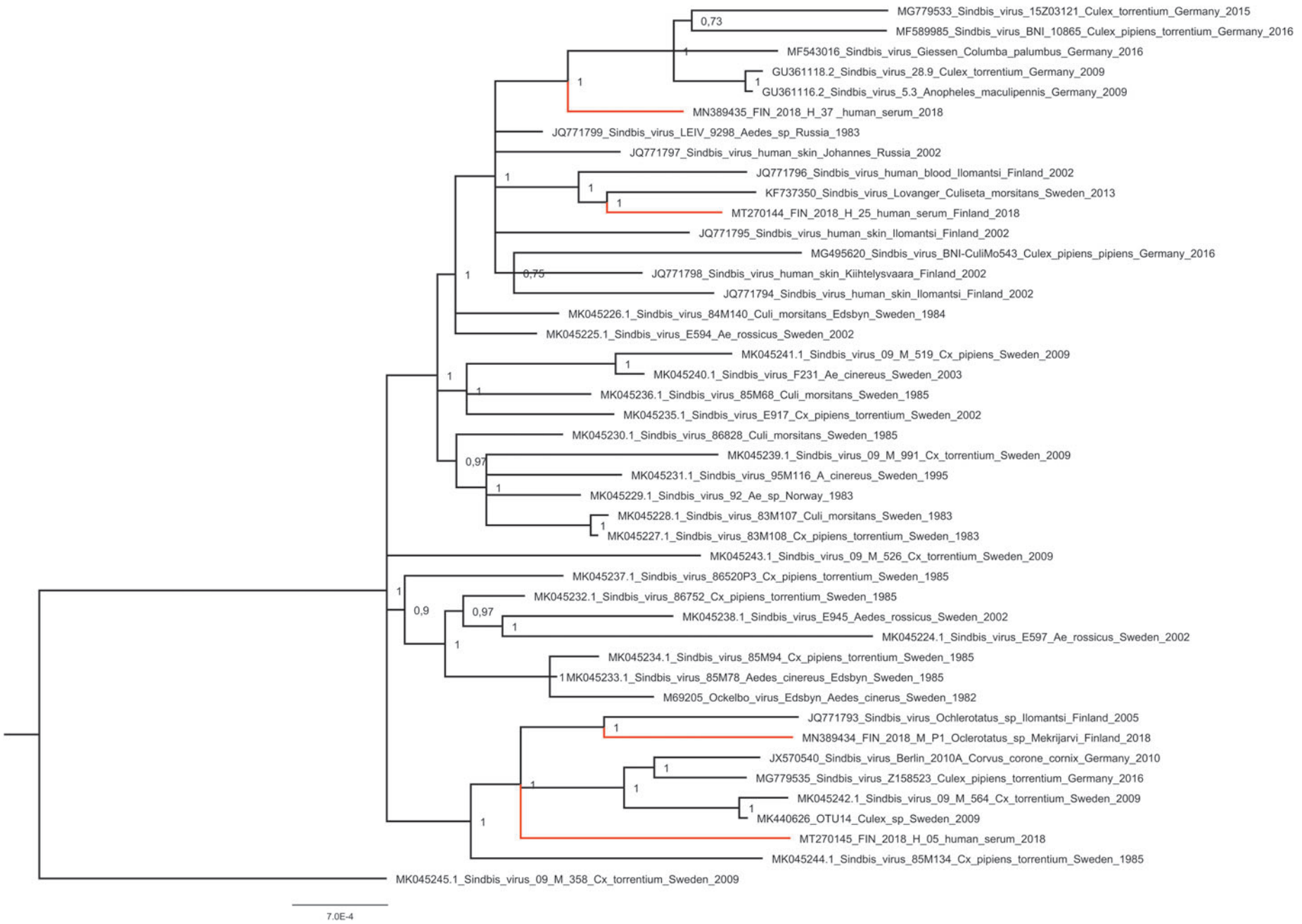

FIG. 3. Phylogenetic tree based on complete coding sequences of SINV. SINV sequences obtained in this study are deposited to GenBank with acc. nos. MN389434 (*Ochlerotatus* sp), MN389435, MT270144, and MT270145 (human serum isolates). Color images are available online.

species. Yet, in the previous study by Sane et al. (2012b) this was not confirmed through morphological species identification of mosquitoes tested positive for SINV. The ornithophilic *Culex* and *Culiseta* mosquitoes are globally considered the main vector genera for SINV, with virus detection and isolation in several countries, including Kenya, Saudi Arabia, Israel, Germany, and Sweden (Lundstrom et al. 1990, Hesson et al. 2015, Ling et al. 2019). However, SINV has also been detected from other mosquito genera, such as *Aedes* (Lvov et al. 1984, Ling et al. 2019) and *Ochlerotatus* (Tingstrom et al. 2016), but their relevance for SINV circulation in nature or role in transmission to humans is not well characterized.

In this study, the morphological characteristics identified the virus positive mosquitoes as members of *Ochlerotatus* genus, but the reliable molecular identification of the species was restricted due to the sample pooling and the lack of high identity sequences in the databases. Unfortunately, the morphological identification to species level was not definitive as the voucher specimens had lost some of the features necessary for species level identification, and there are several species of *Ochlerotatus* mosquitoes in Finland. Interestingly, *Ochlerotatus* spp. have been reported to be frequent man biters in Finland in contrast to *Culex* spp considered to be more ornithophilic (Utrio 1979). Thus *Ochlerotatus* spp. could be involved in transmission of SINV to humans. Further work on morphological and genetic characterization of the local vector species in Finland would be needed to unravel the possible vector effects to the special epidemiological characteristics of SINV in Finland.

Interestingly, strains clustering phylogenetically represent regions with different epidemiological characteristics. Besides Finland, the epidemic SINV with human disease is known to occur in Russia (Laine et al. 2004) and Sweden (Lundstrom et al. 1991, Ahlm et al. 2014) and very rarely in Germany (Jost et al. 2011). In Finland SINV patients are diagnosed yearly with occasional outbreaks. The known risk factors for contracting SINV infection in Finland include exposure to mosquito bites during outdoor activities and living in rural areas (Jalava et al. 2013). In contrast in Germany, where no human outbreaks have been documented, SINV has been detected from urban environments, including ornithophilic *Culex* spp. mosquitoes and urban bird species (Jost et al. 2010, Eiden et al. 2014, Scheuch et al. 2018). In Finland SINV has been associated with migratory and resident birds and especially with grouse that inhabit rural forests. The resident grouse and passerines have been shown to have antibodies against SINV, and the fluctuation of the grouse population density has been found to correlate with human epidemics in Finland (Kurkela et al. 2008, Jalava et al. 2013). Whether the changing climate or different SINV strains would enable urban SINV circulation also outside Germany, and in Finland, remains to be studied.

Conclusion

We conclude that SINV in Finland is genetically more divergent in Finland than previously known, and the results from patient sera demonstrate its potential in SINV detection and strain characterization for research use. The phylogenetic analysis of the new strains provides evidence of SINV strain transfer and circulation within Europe. SINV strains from Finland grouped closely with strains from Sweden and Germany that in contrast to Finland represent areas with rare or nonexisting SINV disease. The possible effects of local vector species selection involved in SINV transmission and the role of *Ochlerotatus* mosquitoes as possible bridge vectors of SINV to humans in Finland and elsewhere in Northern Europe remains to be studied.

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Author Disclosure Statement

No conflicting financial interests exist.

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