UNIVERSITY OF PÉCS

Doctoral School of Biology and Sportbiology

Exploring the genetic diversity of mosquito-derived viruses in Central Europe with *in vitro* and molecular biological methods

PhD Thesis

Brigitta Zana

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Supervisor Head of the doctoral school

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Abbreviations

WNV	West Nile virus
CHIKV	Chikungunya virus
ZIKV	Zika virus
YFV	Yellow fever virus
ssRNA	single-stranded RNA
(+)ssRNA	positive-sense single-stranded RNA
(-)ssRNA	negative-sense single-stranded RNA
ORF	Open reading frame
ER	Endoplasmic reticulum
IFN	Interferon
JEV	Japanese encephalitis virus
TBEV	Tick-borne encephalitis virus
USUV	Usutu virus
ISFVs	Insect-specific flaviviruses
dISFVs	dual-host affiliated insect specific flaviviruses
cISFV	classical insect specific flaviviruses
NKV	no-known-vector flaviviruses
CFAV	Cell-Fusing Agent virus
KRV	Kamiti River virus
CHAOV	Chaoyang virus
DONV	Dongang virus
MMV	Marisma mosquito virus
MBV	Mosquito-borne flaviviruses
CPE	Cytopathogenic effect
RdRp	RNA-dependent RNA polymerase
dsRNA	double-stranded RNA
PLV	Parry's Lagoon virus
KHV	Koyama Hill virus
NDiV	Nam Dinh virus

CavV	Cavally virus
MénoV	Méno virus
ExoN	Exoribonuclease
mRNA	messenger RNA
CDS	Complete coding sequence
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription PCR
SISPA	Sequence-Independent Single-Primer Amplification
NCBI NR	National Center for Biotechnology Information non-redundant protein
MUSCLE	Multiple Sequence Alignment online tool
MCMC	Markov chain Monte Carlo
aa	amino acid
nt	nucleotide
Pro/P	Proline
Asn/N	Asparagine
His/H	Histidine
Ser/S	Serine
Thr/T	Threonine
Gly/G	Glycine
Ala/A	Alanine
Glu/E	Glutamic acid
Met/M	Methionine
Val/V	Valine
WNND	West Nile neuroinvasive disease
ECDC	European Centre for Disease Prevention and Control
Ct	cycle threshold
ADNS	Animal Disease Notification System
UTR	untranslated region
AnFV	Anopheles flavivirus
FTA	Flinders Technology Associate

1. General introduction

Pathogenic viruses are major causative agents of morbidity and mortality among animals and humans. The efficient transmission of viruses between susceptible hosts is the main driving force of these agents to persist in nature and to cause disease. Arthropod-borne viruses (arboviruses) are a group of viral pathogens that require hematophagous arthropods, mainly mosquitoes and ticks, to transmit viruses between vertebrates via blood feeding (**Figure 1.**) (Mueller and Cao-Lormeau, 2018). In arbovirus transmission, mosquitoes are counted as the most important vectors, although many are transmitted by ticks, phlebotomine sand flies, and other arthropods (Rückert and Ebel, 2018). Mosquito-borne diseases cause millions of infections annually. For example, dengue cases have risen 8-fold during the last two decades from 505,430 cases in 2000 to over 5,2 million in 2019 and 3,8 million in 2022 (WHO; ECDC, 2022).

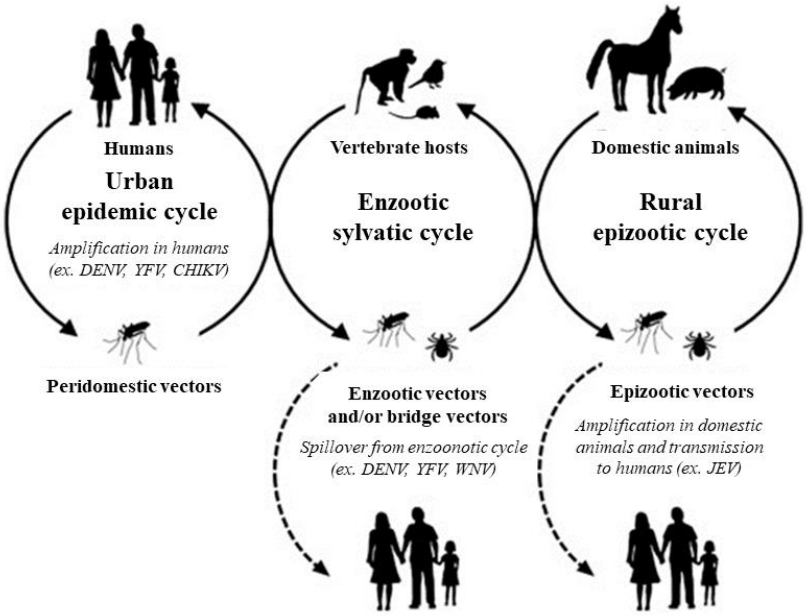


Figure 1. Arbovirus transmission cycles (Mueller and Cao-Lormeau., 2018).

Many factors collectively influence the spread of mosquito-derived arboviruses. The vector competence of a mosquito is a crucial factor in transmitting a given virus since different mosquito species vary in their blood-feeding behaviour and host preference. Furthermore, arboviruses must cross specific barriers within the mosquito host for successful transmission, and they require active virus replication in both the arthropod vector and the

vertebrate host (Rückert and Ebel, 2018). Of the major barriers that viruses must cross, the first is the midgut infection barrier, which means viruses must successfully infect and replicate within the midgut epithelial cells (midgut infection barrier). After that, viruses must cross the basal lamina enclosing the midgut epithelium (midgut escape barrier). Before the viruses enter the next barrier (salivary gland infection barrier) and infect the salivary gland, they replicate in different mosquito tissues such as the fat body, haemocytes, nerve and muscle tissues. Upon salivary gland infection, mosquitoes can inoculate the virus into the host during a blood meal (salivary gland escape barrier) (Rückert and Ebel, 2018; Franz et al., 2015).

The increasing global trade and travel, urbanization, and climate change significantly contribute to the exchange of pathogens and susceptible vectors across continents (Conway et al., 2014; Rückert and Ebel, 2018). The latter factors allow the expansion of previously non-endemic viruses and provide novel opportunities for establishing new host-virus relationships. For example, the emergence of West Nile virus (WNV), Chikungunya virus (CHIKV), and Zika virus (ZIKV) in new territories during the last decades. Since WNV had been introduced into Europe by migratory birds from Sub-Saharan Africa in the 1900s, the virus emerged through human activity in the United States in 1999 and rapidly spread throughout the country and in the Americas via migratory birds (Gould et al.,2017; Rückert and Ebel, 2018). The maintenance of WNV in nature is linked to an enzootic transmission cycle between Culex spp. and birds. In general, Culex spp. obtain their blood meal from birds; however, humans and horses can serve as incidental hosts, which makes Culex spp. efficient vectors to transmit WNV and other zoonotic arboviruses (Rückert and Ebel, 2018). The Chikungunya virus was originally endemic in Sub-Saharan Africa. In the middle 2000s, the virus spread globally from Africa into Asia, islands of the Indian Ocean, and temperate areas in Europe. The virus has now become endemic in the Americas (Rückert and Ebel, 2018; Mayer et al., 2017). In the case of CHIKV, *Aedes aegyipti* mosquito species were counted as the primary vector. However, due to a mutation in the CHIKV genome, *Aedes albopictus* had become capable of successfully transmitting the virus, which largely contributed to the dispersion of the virus into urban regions where *Ae. aegypti* is not abundant. Furthermore, the domestic form of *Ae. aegypti* had been accidentally shipped across the Atlantic and/or Pacific Ocean (Gould et al., 2017; Mayer et al., 2017). Zika virus has emerged from Africa and has become a global pathogen through travel and the introduction of the infected mosquitoes into naïve places where the presence of competent

vectors (*Ae. albopictus* and *Ae. aegypti*) further facilitated the virus emergence (Mayer et al., 2017).

Unfortunately, there are any or just limited specific treatments and prophylaxis for diseases caused by arboviruses. The prevention of contact between the vector and host using repellents, pesticides, physical barriers, and traps against vector species is the most effective way to prohibit infection and arbovirus emergence (**Figure 2.**) (Rückert and Ebel, 2018; Conway et al., 2014).

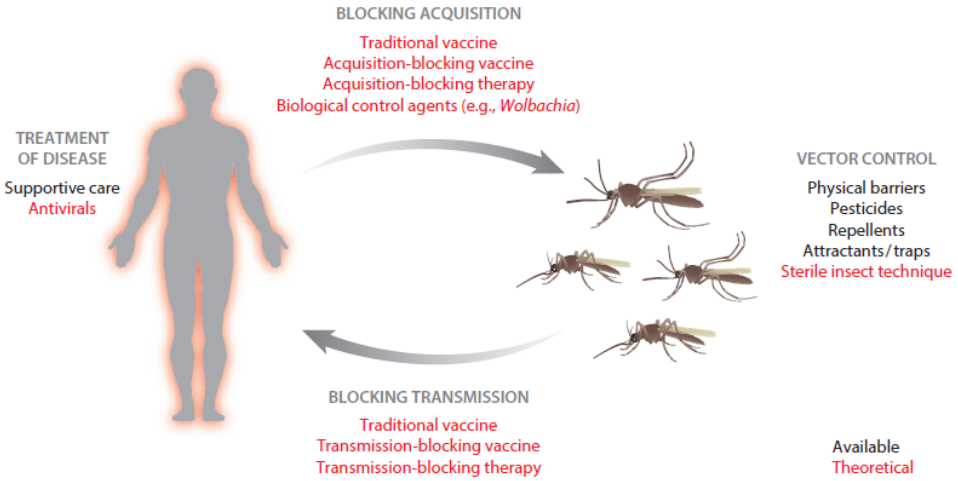


Figure 2. Available (*black*) and theoretical (*red*) interventions against arboviral diseases (Conway et al., 2014).

2. Introduction

2.1 Flaviviridae, Flaviviruses

Yellow fever virus (YFV), a prototype member of the *Flaviviridae* family, was the first human virus discovered more than a century ago (Knipe et al., 2007). Since then, this family has been extended and currently consists of four genera: *Flaviviruses*, *Pestiviruses*, *Pegiviruses* and *Hepaciviruses* (ICTV). More than 70 viruses are listed in the *Flavivirus* genus (Knipe et al., 2007). Flaviviruses have a common virion structure. The mature virions bounded by the envelope are round and 50 nm in diameter. The outermost layer of the virion is a glycoprotein coat consisting of 180 repeating envelope (E) protein units combined with membrane (M) proteins. The surface of a virion shows icosahedral symmetry on which the envelope dimers form 30 groups of "rafts" (**Figure 3.**). The flaviviral genome is approximately 11,000 nucleotides long positive-sense ssRNA that contains I type CAP at the 5' end and lacks the 3' end poly(A)-tail and encodes a single long open reading frame (ORF) (Samuel MA and Diamond MS, 2006; Barrows et al., 2018) (**Figure 4.**).

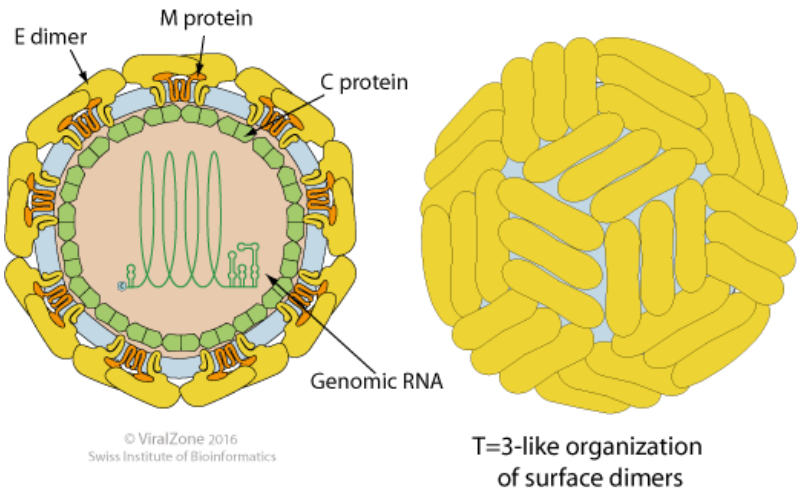


Figure 3. Virion structure of flaviviruses (ViralZone; https://viralzone.expasy.org/43) (Date of Access: 10.08.2022).

Translation of ORF produces a long polyprotein that is co- and post-translationally cleaved into three structural (capsid, premembrane and envelope) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (**Figure 4.**).

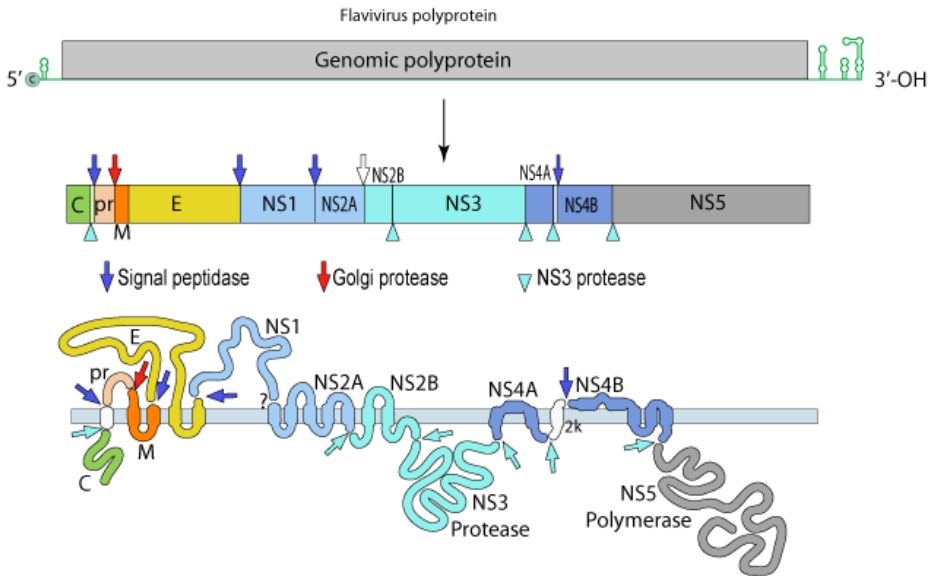


Figure 4. Genome organization of flaviviruses (ViralZone; https://viralzone.expasy.org/43) (Date of Access: 10.08.2022).

Capsid (C) protein binds viral RNA and contains a C-terminal hydrophobic domain that serves as a signal peptide for endoplasmic reticulum (ER) CF translocation of prM. Premembrane (prM) protein blocks premature viral fusion and chaperone envelope (E)-protein folding while envelope protein mediates viral attachment, membrane fusion and viral assembly. NS1 is secreted from infected cells and has cofactor activity for the viral replicase. Mutation of the N-linked glycosylation sites in NS1 can lead to RNA replication and virus production failures. NS2a may participate in virus assembly and inhibits interferon (IFN) responses, while NS3 has protease, NTPase, and helicase activities. NS2b is a cofactor involved in NS3 proteolytic activity. NS4a and NS4b have a role in the modulation of IFN signalling, while NS5 encodes the RNA-dependent RNA polymerase and methyltransferase enzymes (Samuel MA and Diamond MS, 2006; Knipe et al., 2007).

During virion entry, which is initiated by the engagement of the E protein with the unknown cellular receptor (or receptors), receptor-mediated endocytosis of the virus occurs. Viral fusion with the endosomal membrane is triggered by the low-pH environment within the endosomal vesicle, leading to virion uncoating and release of the viral positive-sense single- stranded RNA ((+) ssRNA) genome into the cytoplasm. Then it is translated by host cell machinery into a single polyprotein at the ER and cleaved co- and post-translationally by viral and cellular proteases into ten mature proteins. The structural proteins (capsid, precursor membrane and envelope) become incorporated into the virion, while the non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) form the replication complex on modified ER membranes for the synthesis of full-length negative-sense ssRNA

((−)ssRNA) intermediates which will serve as templates for the synthesis of full-length (+)ssRNAs. After virion assembly and encapsidation of viral genome occurring on rough ER membranes, immature virions are transported through the Golgi apparatus resulting in glycosylation of the E protein followed by the furin-mediated cleavage of prM protein to mature membrane protein, M. Finally, mature virions are transported to the plasma membrane and released by exocytosis.

The majority of flaviviruses are classified as arthropod-borne viruses transmitted horizontally between vertebrate hosts and arthropod vectors (mosquito or tick). At the same time, other groups of viruses within this genus are also maintained in nature by horizontal transmission and infect vertebrate hosts (rodent and bat), but their vector is still unknown (King et al., 2015; Blitvich et al., 2015; Hoshino et al., 2009). During the last decade, a group of flaviviruses was recognized that appears to replicate only in mosquitoes but cannot replicate in vertebrate hosts (King et al., 2015; Blitvich et al., 2015; Calzolari et al., 2016).

Flaviviruses cause infection with various outcomes ranging from asymptomatic or mild illness such as fever, rash, and joint pain to severe or fatal haemorrhagic fever or neurological disease. Mammals and birds represent the vertebrate hosts of flaviviruses, and these viruses affect hundreds of millions of individuals every year. Protective vaccines are available only against YFV, Japanese encephalitis virus (JEV) and Tick-borne encephalitis virus (TBEV). Unfortunately, there are no available vaccines or therapies against the more pathogenic flaviviruses where the prevention of insect bites represents the major defence (ICTV, Apte-Sengupta 2014).

2.1.1 West Nile virus

West Nile virus (WNV) was discovered in Uganda in 1937 (Smithburn et al., 1940). Since then, the disease (West Nile fever) caused by the virus has become a major public health concern worldwide (Gray et al., 2014). Up to now, nine genetic variants of the virus have been identified. However, the infectivity and neuroinvasive tendencies of lineages have been largely different (Kemenesi et al., 2014; Pachler et al., 2014; Papa et al., 2011). Of the genetic variants, lineages 1 and 2 possess the largest human health risk worldwide (Gray et al., 2014). A higher degree of infectivity and neuroinvasive potential was attributed to lineage 1 WNV until the appearance of a large number of lineage 2 WNV strains in South Africa with high infectivity and neuroinvasive properties (Venter and Swanepoel, 2010). Until 2004, only the presence of lineages 1 and 3 WNV strains had been demonstrated in

Europe (Papa et al., 2011). Following the first emergence of lineage 2 WNV in Hungary in 2004 (Bakonyi et al., 2006), this genetic variant was characterized by an explosive spread and genetic diversification in the region (Bakonyi et al., 2013). It was responsible for several epidemics across Europe (Italy, Greece, Serbia, Austria, etc.) in human and animal populations. For example, compared to the previous seven years (n = 1832), in 2018 sharp increase in cumulative autochthonous WNV infections (n = 2 083) was reported in Europe (ECDC, 2018).

West Nile virus is transmitted by mosquitoes of the *Culex* genus. However, there is a growing concern about the role of recently expanding invasive Aedes mosquitoes throughout Europe, establishing novel transmission scenarios (Napp et al., 2018). The virus is maintained in nature in an enzootic cycle between mosquitoes and birds where birds serve as amplifying hosts while mosquitos as vectors. Survival of the pathogen in nature is primarily provided by birds enabling the virus to be further distributed by mosquitoes. Humans and horses serve as incidental or dead-end hosts that may be infected through mosquito bites or, in rare cases, transfusion of transplanted blood or organ transplantation (Ciota et al., 2013; Paphitou et al., 2017). In most human cases, the infection does not have severe consequences, and the infection remains asymptomatic. However, in 30% of the cases, milder flu-like symptoms or encephalitic diseases develop (Zannoli and Sambri, 2019). The first signs of viral illness occur within 2 to 14 days of infection. Viral infections can lead to 1% of severe neuroinvasive diseases (encephalitis and meningitis), especially in the elderly, children, and immunosuppressed people. Within these groups, mortality rates may reach up to 20% (Ulbert, 2011). Those who have undergone neurological illness may suffer from lasting symptoms for months or years, such as memory loss, muscle weakness, headache, tiredness, dizziness, depression, and hearing loss (Anastasiadou et al., 2013). The disease caused by West Nile fever leaves transient and lifelong protection (Ulbert, 2011). Currently, a vaccine for human health is not available against the West Nile fever virus (Poore et al., 2017; Rossi et al., 2010).

2.1.2 Usutu virus

The virus was first identified in a mosquito sample in South Africa in 1959 (Woodall, 1964). The first known European appearance of the virus was retrospectively confirmed in Italy in 1996 (Weissenböck et al., 2013). Since then, the virus has been detected in many European countries, including Austria, Hungary, Spain, Switzerland, Belgium, the Czech Republic, and Germany. Hitherto, six genetic variants of the virus can be distinguished (Engel et al., 2016). The persistence of the Usutu virus (USUV) in nature is linked to a mosquito-bird-mosquito enzootic cycle, in which mosquitoes act like vectors, and birds participate as amplifying hosts in the system (Zannoli and Sambri, 2019). Usutu virus is a neglected mosquito-borne flavivirus causing high mortality among captive bird species. Sporadic human cases can occur, putting the virus under consideration as a potential public health problem (Engel et al., 2016). Clinical manifestation of USUV is similar to WNV infection, while compared to WNV, USUV seems more pathogenic for some bird species and responsible for less human disease (Zannoli and Sambri, 2019).

2.2 Flaviviridae, Mosquito-specific flaviviruses

During the last decade, a group of flaviviruses has been recognized that appears to replicate only in mosquitoes and cannot replicate in vertebrate hosts. These flaviviruses show global distribution and have been categorized as insect-specific flaviviruses (ISFVs) (King et al., 2012; Blitvich and Firth, 2015; Calzolari et al., 2016). This group can be divided into two separate phylogenetic groups. The first group (classical ISFVs) consists of flaviviruses that are phylogenetically distinct from all other known flaviviruses, such as the Cell-Fusing Agent virus (CFAV) and Kamiti River virus (KRV). While the second group (dual host affiliated ISFVs) consists of viruses that are phylogenetically linked to the arthropod-borne and no-known vector flaviviruses, such as Chaoyang virus (CHAOV) and Donggang virus (DONV) (Blitvich and Firth, 2015). Interestingly, integrated flavivirus-like sequences have been found incorporated into the genome of different mosquito species. These sequences can be highly fragmented and may contain internal stop codons, but several encode intact ORFs. Moreover, transcription of some integrated sequences can occur, leading to the detection of flavivirus-like RNA in an organism, which may mistakenly refer to active flavivirus infection (Blitvich and Firth, 2015). Although their incidental role in nature is barely understood, many recent studies focusing on determining the presumable role of ISFVs show

that some ISFVs tend to enhance or suppress the replication of flaviviruses associated with human diseases (Calzolari et al., 2016; Blitvich and Firth, 2015; Goenaga et al., 2015; Carvalho et al., 2021).

2.2.1 Marisma mosquito virus

Marisma mosquito virus (MMV) was first isolated from an *Ochlerotatus (Aedes) caspius* mosquito pool in Spain in 2012 (Vázquez et al., 2012). Since the first detection, the presence of this virus has been confirmed in Italy from *Ochlerotatus (Aedes) caspius* mosquito pools as well by two consecutive studies published in 2013 (Pautasso et al., 2013) and 2014 (Rizzo et al., 2014). According to phylogenetic analysis, MMV segregate into a separate clade along with the Lammi virus and Chaoyang virus within the mosquito-borne flaviviruses (MBV) group indicating these three viruses may represent a separate putative antigenic group within MBV. *In vitro* experiments after the first passage on monolayers of C6/36, Vero and BHK-21 have revealed cytopathogenic effect (CPE) (cell detachment) after 5-7 days post-infection with MMV. Interestingly, after the first passage, neither CPE nor positive PCR amplification was detected on supernatants suggesting MMV does not replicate in vertebrate cells (Vázquez et al., 2012).

2.3 Insect-specific viruses

Technological advances in the field of sequencing and bioinformatic analyses led to the discovery of a diverse group of viruses, namely insect-specific viruses (ISVs). A hallmark characteristic of ISVs is that they exclusively infect mosquitoes and phlebotomine sandflies and cannot replicate in vertebrate hosts and their cell lines. ISVs belong to several viral families and taxon, such as *Flaviviridae* (detailed in chapter 1.2), *Togaviridae*, *Peribunyaviridae*, *Phenuiviridae*, *Rhabdoviridae*, *Mesoniviridae*, *Tymoviridae*, *Birnaviridae*, *Nodaviridae*, *Reoviridae*, *Parvoviridae*, *Iridoviridae*, *Permutotetraviridae*, *Iflaviridae*, *Orthomyxoviridae*, *Totiviridae* and the proposed taxon *Negevirus* (Bolling et al. 2015, Carvalho et al., 2021). According to available data, vertical transmission is the primary route used by ISVs for their maintenance in nature; however, some studies highlighted the possibility that veneral and horizontal transmission also may occur. Several studies focused on revealing the possible role of ISVs in nature. Based on the currently available data, ISVs may have a function in the suppression of arbovirus replication, providing a biological tool for arbovirus control. Furthermore, ISVs can be reliable agents for vaccine and diagnostic platform developments as well (Carvalho et al., 2021).

2.3.1 Taxon: Negevirus

Negeviruses are newly identified taxa consisting of a diverse group of viruses isolated from mosquitoes and phlebotomine sandflies (Nunes et al., 2017). Negeviruses have ~10 kb long non-segmented ssRNA genome with positive-sense encoding three open reading frames (ORFs), of which ORF1 encodes the methyltransferase, FTsJ-like helicase, viral RNA helicase, and the RNA-dependent RNA polymerase (RdRp), while ORF2 encodes viral glycoproteins, and ORF3 encodes virion membrane proteins (Nunes et al., 2017; Suvanto et al., 2020). Viral particles have a spherical structure with a size of 45-55 nm in diameter.

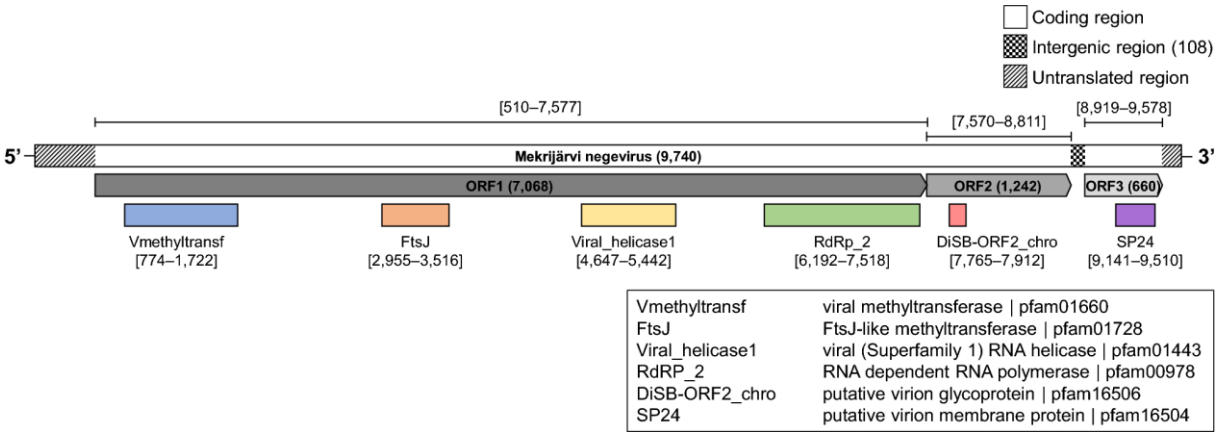


Figure 5. Genome organization of the Mekrijärvi negevirus. (Suvanto et al., 2020)

Negeviruses display extensive geographical distribution, broad host range and relatively high infection rates in some mosquito species. Based on phylogenetic analysis, Negeviruses form two distinct phylogenetic groups, the *Nelorpivirus* and *Sandewavirus*, within the taxon. They are most closely related to plant-infecting viruses belonging to *Cilevirus*, *Higrevirus* and *Blunervirus* genera. Negeviruses replicate to high titres in different invertebrate cell lines; however, they cannot infect laboratory animals and vertebrate cell lines. The maintenance of these viruses in nature is unknown. The genetic similarity of Negeviruses with some plant viruses suggests horizontal transmission through floral and extrafloral nectaries since mosquitoes and sandflies gain essential carbohydrates from plants. It has been recently suggested that transovarial (vertical) transmission also can occur since negevirus had been found in larva of the Aedes mosquito (Nunes et al., 2017).

2.3.2 Mesoniviridae, genus: Alphamesonivirus

Mesoniviridae belongs to the *Nidovirales* order along with three other genetically distinct families - *Coronaviridae*, *Arteriviridae* and *Roniviridae*. Viruses of *Nidoviralaes* order possess enveloped positive-sense single-stranded RNA (+ssRNA) genomes ranging in lengths from ~12-31 kilobases (Newton et al., 2020).

Since the independent discovery of the members of type species, Alphamesonivirus 1, including Nam Dinh virus (NDiV) (Nga et al., 2011) and Cavally virus (CavV) (Zirkel et al., 2011), eight additional mesonivirus species have been classified (Alphamesonivirus 2 – Alphamesonivirus 9) (ICTV, 2020).

Due to the restriction of their replication to only insects and still no available evidence to infect or cause any disease in vertebrates, *Mesoniviridae* may represent a unique order within the Nidovirus family. However, they are of great interest since their structural and genetic similarities to other members of the *Nidovirales* order, and their genome may provide an evolutionary link between small (arteriviruses) and large (coronaviruses and roniviruses) nidoviruses (Newton et al., 2020; Vasilakis et al., 2014).

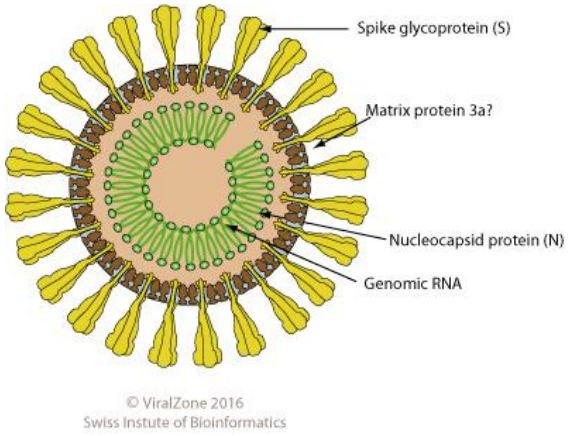


Figure 6. Virion structure of *Mesoniviridae* (ViralZone, https://viralzone.expasy.org/4776) (Date of Access: 10.08.2022).

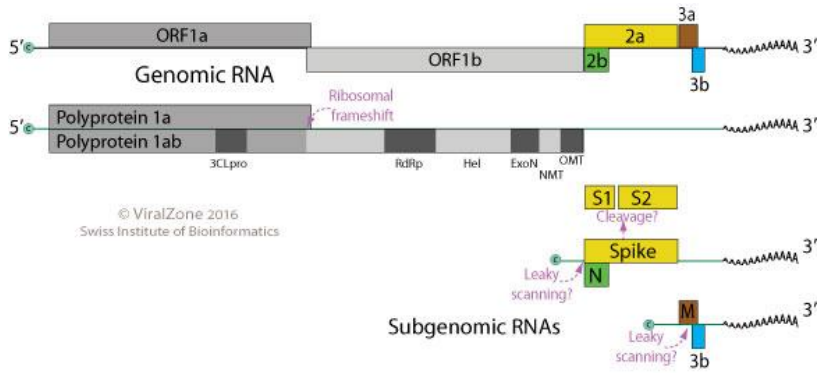


Figure 7. Genome organization of *Mesoniviridae* (ViralZone, https://viralzone.expasy.org/4776) (Date of Access: 10.08.2022)

Mesoniviridae RNA coding for seven major open reading frames (ORFs), excluding Méno virus (MénoV), only encodes six. Between the two largest ORFs, ORF1a and ORF1b, a few nucleotides long overlapping region takes place, which functions as a ribosomal frameshift signal allowing the two respectively encoded proteins, pp1a and pp1ab, to be expressed. Key replicative enzymes, such as RNA-dependent RNA polymerase (RdRp) and 3'-to 5' exoribonuclease (ExoN), regulating numerous products modulating viral expression and replication are encoded by ORF1a and ORF1b. The RNA processing enzyme, ExoN, is exclusively encoded by nidoviruses, presumably controlling replication efficiency, and may have opened the way to the emergence of viruses with ssRNA genomes larger than ~20 kb. The remaining ORFs are translated from subgenomic mRNAs expressing structural proteins and other proteins regulating virus-host interactions, such as spike glycoprotein (S protein, S1 and S2 subunit), nucleocapsid protein (N) and membrane-spanning proteins (M) (Newton et al., 2020, Nga et al., 2011).

Based on the current scientific literature, Mesoniviruses represent a common element in mosquito virome with extensive geographic distribution and broad host range (Vasilakis et al., 2014; Newton et al., 2020).

2.3.3 Unclassified ssRNA viruses, Daeseongdong virus

Daeseongdong virus was discovered from *Culex pipiens* mosquitoes collected in the Republic of Korea in 2012. The genome sequences of these two novel viruses designated Daeseongdong virus 1 and Daeseongdong virus 2 are 9,632 bases containing three complete coding sequences (CDSs) and 4,742 bases with two complete CDSs, respectively. Based on the available sequence data, Daeseongdong virus 1 and Daeseongdong virus 2 are distantly related to *Negevirus* and Drosophila A virus, respectively (Hang et al., 2016).

2.4 Reoviridae, genus Orbivirus

The genome of orbiviruses is composed of 10 linear double-stranded RNA (dsRNA) segments encoding 10 proteins, of which 7 are structural (VP1-VP7) and 3 are non-structural. Each viral particle contains one copy from each dsRNA segment (ICTV).

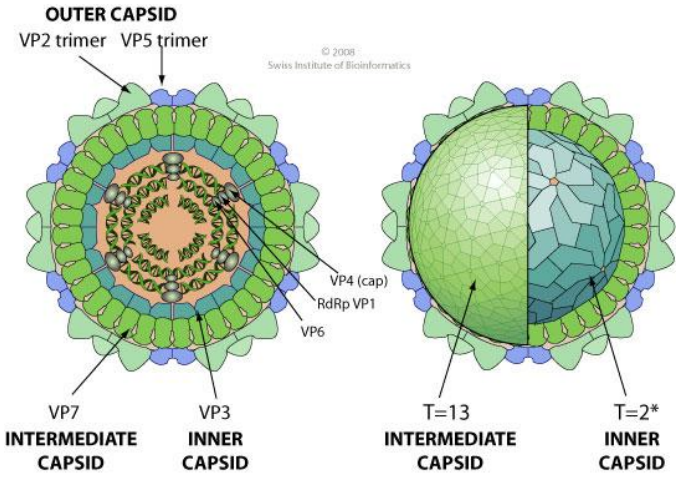


Figure 8. Virion structure of Orbiviruses (ViralZone, https://viralzone.expasy.org/106) (Date of Access: 22.08.2022)

The transmission of orbiviruses is related to different arthropod vectors, such as mosquitoes, midgets and ticks. Wilde range of vertebrate hosts can be affected by orbivirus infection. Several orbiviruses have economic importance due to severe diseases affecting livestock (Ejiri et al., 2014; Harrison et al., 2016). Not all orbiviruses are arthropod-borne viruses since Parry's Lagoon virus (PLV), an insect-specific orbivirus, displays host restricted replication strategy (Colmant et al., 2017; Harrison et al., 2016). Koyama Hill virus (KHV) is a member of the species *Umatilla virus*, which was isolated from the ornithophilic

Culex sasai mosquito species in Japan. Results of *in vitro* analyses revealed that both invertebrate (c6/36) and vertebrate (CCL-141) cell lines support the replication of the virus, suggesting the virus is maintained in nature through bird-mosquito transmission cycle; however, evidence for infection among birds (wild or poultry) caused by KHV have not been found yet (Ejiri et al., 2014).

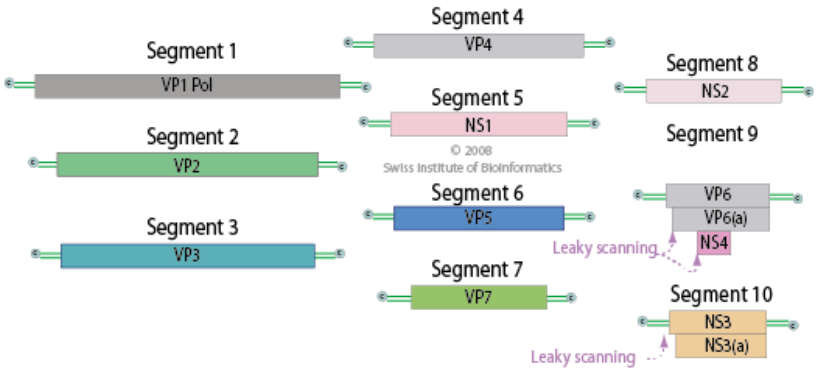


Figure 9. Genome organization of Orbiviruses (ViralZone, https://viralzone.expasy.org/106) (Date of Access: 22.08.2022)

3. Aims of the study

- Understanding the genetic diversity of mosquito-derived viruses on the vector-pathogen interaction level. It can ultimately lead to novel solutions and a better understanding of arbovirus interactions and emergence worldwide.
- The main goal of this thesis is the establishment a European virus isolate collection with *in vitro* virus isolation experiments and to describe this collection with the extensive evolutionary and genomic characterization of these mosquito-derived viruses.
- To evaluate a proof-of-concept study, where we combine traditional virological methods with modern genomic sequencing and establish a local virus isolate bank to aid future competence, interaction, and transmission studies.

4. Materials and methods

4.1 Sample origins

Mosquitoes were collected with EVS CO2 Mosquito Trap baited with dry ice and white light from mosquito breeding areas near Pécs and Debrecen in Hungary from May to September 2013. Large-scale mosquito surveillance was conducted in the Vojvodina province of Serbia in 2013 and 2014. Mosquitoes were trapped with CDC light traps baited with dry ice in multiple sampling events during the breeding season from May to September. Each specimen was determined by species according to their taxonomic keys (Becker et al., 2003). Samples were grouped by species, collection site and date and pools were created consisting of a maximum of 50 individuals per pool. All collected mosquitoes were transported to the laboratory on dry ice and kept frozen at −80 °C until further processing.

Entomological surveillance of larvae and adult stages of mosquitoes was further performed in Hungary, examining the adjacent area of horse WNV cases in Dunaföldvár in 2018. We conducted a follow-up sampling for the wintering sites of mosquitoes in the ~1 km area of these horse cases to assess the overwintering WNV infection capacity regarding local mosquitoes. Larvae collection was performed using the standard dipping method, while adult stages were collected via CDC light traps (BioQuip Products, CA, USA) and hand aspirators. Larvae samples and adult mosquitoes were pooled, including a maximum of 20 specimens per tube, and subjected to molecular analysis.

Further WNV-positive samples and sample data were retrieved in a country-wide collaborative effort from multiple institutions. Human sample data was generously provided by the National Public Health Center, horse serologic data were obtained from the University of Veterinary Medicine Budapest, and animal-derived WNV sequences were provided by the University of Veterinary Medicine, the National Food Chain Safety Office, and PROPHYL Ltd. Mohács, Hungary. Passive surveillance regarding wild bird mortality cases was performed at the Veterinary Diagnostic Directorate of the National Food Chain Safety Office. Bird carcasses examined in the avian influenza monitoring scheme were also tested for flavivirus infection, as previously described (Weidinger et al., 2019). Pathology, histology, and polymerase chain reaction (PCR) were performed, and partial WNV sequences were obtained from positive cases.

4.2 Nucleic acid preparation

Mosquito samples were homogenized with Minilys® personal homogenizer (Bertin Corp., USA) by adding two pieces of 2,5-2,8 mm diameter glass beads (Kisker Biotech GmbH & CO., Germany) and 500 µl EMEM (Lonza, Switzerland) to each tube and homogenized for 30 sec at maximum speed. After homogenization, samples were centrifuged at 15,000 *g* for 10 min. For nucleic acid extraction, 200 µl of supernatants were used following the manufacturer's protocol of Geneiad Viral Nucleic Acid Extraction Kit II (Geneaid). The extracted nucleic acid was eluted in 50 µl of nuclease-free water and stored at -80 °C until further laboratory processes.

Total RNA was extracted using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) in the case of virus isolates, northern goshawk, and horse brain tissue samples.

4.3 Polymerase Chain Reactions (PCRs)

In general, mosquito pools, bird and horse samples were tested with a TaqMan real-time RT-PCR targeting the NS3 region of the WNV genome, using reagents of the OneStep RT-PCR Kit (Qiagen) with the following cycling conditions: reverse transcription at 50 °C for 30 min and 95 °C for 15 min, then 40 cycles of denaturation at 95 °C for 20 s, annealing at 51 °C for 20 s, and elongation at 72 °C for 20 s. After that, WNV-positive mosquito, northern goshawk, and horse samples were subjected to a nested reverse transcription-PCR (RT-PCR) system targeting the NS5 gene, as described in (Vázquez et al., 2012) and a reverse transcription-PCR (RT-PCR) system targeting the NS3 gene (WNV_NS3_F 5'-AGCMGGAAARACRCGCAAGA-3' and WNV_NS3_R 5'-GGTCTTTCCAACATACTCAG-3') to amplify a longer genomic region regarding sequencing. To detect additional flaviviruses, mosquito pools were subjected to nested reverse transcription - polymerase chain reaction (RT-PCR) using degenerated primers targeting the conserved NS5 gene of flaviviruses (Kuno et al., 1998; Scaramozzino et al., 2001). For the first round PCRs, OneStep RT-PCR Kit (Qiagen) was used as suggested by the manufacturer, while the second round PCRs were done with GoTaq® G2 Flexi DNA Polymerase Kit (Promega, USA) according to the manufacturer's instructions. To obtain the complete genome of WNV and USUV, long-range PCRs were conducted by Superscript™ III Reverse Transcriptase (Invitrogen) resulting four large fragments. In the case of USUV,

large amplicons were subjected to nested PCRs using Phusion U Hot Start PCR Master Mix (Thermo Scientific) resulting in overlapping fragments of the genome.

Oligonucleotides used for the detection of WNV and additional flaviviruses in our samples are summarized in Table 1.

4.4 Sanger sequencing

Amplicons in the case of USUV and flaviviruses amplified by nested PCR system were purified with Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, Taiwan). PCR products were subjected to bidirectional sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on the ABI Prism 310 genetic analyzer platform (Applied Biosystems).

Table 1. Oligonucleotides used for detection of WNV and additional flaviviruses

Type of PCR	Target	Primer name	Primer sequence	Location	Final amplicon size	Ref
Quantitative RT PCR	West Nile virus	WNV 5009f	5'-GAACGTCAGGTTCCCCCATT-3'	NS3	93 bp	Bakonyi et al., 2013
		WNV 5103r	5'-GGCGCTTATGTATGAACCATTAGG-3'			
		WNV 5050p	5'-ATTGGATTGTATGGGAACGGCGTCATC-3'			
Semi nested PCR	Flaviviruses	cFD2 R	5'-GTGTCCCAGCCGGCGGTGTCATCAGC-3'	NS5	250 bp	Kuno et al., 1998
		FS778 F	5'-AARGGHAGYMCDGCHATHTGGT-3'			Scaramozzino et al., 2001
		MAMD F	5'-AACATGATGGGRAARAGRGARAA-3'			
Nested PCR	West Nile virus	1NS5F	5'-GCATCTAYAWCAYNATGGG-3'	NS5	1019 bp	Vázquez et al., 2012
		1NS5Re	5'-CCANACNYNRTTCCANAC-3'			
		2NS5F	5'-GCNATNTGGTWYATGTGG-3'			
		2NS5Re	5'-CATRTCTTCNGTNGTCATCC-3'			
Quantitative RT PCR	Usutu virus	UsuFP	5'-CAAAGCTGGACAGACATCCCTTAC-3'	NS5	103 bp	Nikolay et al., 2014
		UsuRP	5'-CGTAGATGTTTTCAGCCCACGT-5'			
		UsuP	5'-AAGACATATGGTGTGGAAGC-3'			

4.5 Next-Generation Sequencing

4.5.1 Ion Torrent PGM

Long-range PCR amplicons, obtained in the case of WNV, were subjected to library preparation for Ion Torrent using the NEBNext® Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs, USA) with KAPA Adapter Kit (KAPA Biosystems, UK). Emulsion PCR to obtain clonally amplified fragments was carried out using the Ion OneTouch™ 200 Template Kit (Life Technologies, USA) on OneTouch version 2 equipment (Life Technologies, USA) as recommended by the manufacturer. Templated beads were enriched using an Ion OneTouch™ ES pipetting robot (Life Technologies, USA). The 200 bp sequencing protocol was performed on a 316 chip (Life Technologies, USA) using the Ion Torrent PGM (Life Technologies, USA) semiconductor sequencing equipment. Trimmed sequence reads were used for *de novo* assembly utilizing the MIRA (version 3.9.17) (Chevreux et al., 1999). Additional bioinformatic analyses and validation with remapping were performed using the CLC Genomics Workbench (version 6.5.1; http://www.clcbio.com) and the DNAStar (version 12; http://www.dnastar.com).

4.5.2 Oxford Nanopore sequencing

Mosquito virus isolates from *in vitro* virus propagation experiments were subjected to Oxford Nanopore sequencing. Before Nanopore sequencing, virus isolates were exposed to enrichment protocol. Samples were enzymatically treated for 2 hours at 37 °C with a mixture of 1 µl micrococcal nuclease (NEB, USA) and 2 µl of benzonase (Merck Millipore, USA) and 7 µl of homemade buffer (1M Tris, 100 mM $CaCl^2$ and 30 mM $MgCl^2$, pH 8) as detailed previously Conceição-Neto, N. *et al*. 2015. Thereafter, nucleic acid was extracted from samples using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). Samples were then subjected to the Sequence Independent Single Primer Amplification (SISPA) approach (Song et al., 2017). cDNA amplification was performed by an AMV Reverse Transcriptase (Promega) according to the provided manual by the manufacturers using the FR26RV-N (5′-GCCGGAGCTCTGCAGATATCNNNNNN-3′) primer. After that, ds cDNA was amplified by a DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the supplied protocol using the FR20RV (5′-GCCGGAGCTCTGCAGATATC-3′) primer. Amplified cDNA was purified by NucleoSpin® Gel and a PCR Clean-up kit (Macherey-Nagel, Düren, Germany), and quantified using a Qubit dsDNA BR Assay kit (Thermo Fisher

Scientific). Libraries were prepared using the PCR Barcoding Kit (SQK-PBK004) and protocol by Oxford Nanopore Technologies. R9.4.1 flow cell and MinKNOW v4.5 software were used for sequencing. Base-calling of raw data was done by guppy (ONT guppy v4.4.2.) high accuracy base-calling algorithm (dna_r9.4.1_450bps_hac config file). As the first step of sequence data analysis, demultiplexing and trimming of barcodes were performed with guppy as well, using default parameters of the 'guppy_barcoder' runcode. For long-read metagenomic classification, Centrifuge (Kim et al., 2016) and Kraken 2 (Wood and Salzberg, 2014) metagenomic classification algorithms were applied using the full NCBI NR (National Center for Biotechnology Information non-redundant viral protein and viral genomic reference databases) (https://ftp.ncbi.nlm.nih.gov/refseq/release/viral/) (Accessed on 08.11.2021). Further sequence manipulations were done by Geneious Prime program. Mapping of data packages of each sample to reference sequences was run by using the Minimap2 plugin (Li, 2018) within the Geneious Prime 2022.2.2 (https://www.geneious.com) program. Based on metagenomic results, we selected the most fitted reference sequence for polishing and to create a consensus sequence with the medaka tool. The generated consensus sequences from medaka analyses and from mapping by the Minimap2 were aligned using the MAFFT plugin (Katoh, 2013), and the base-calling errors were manually corrected in Geneious Prime 2022.2.2.

4.6 Phylogenetic analyses

Basic sequence manipulation and verification were performed using GeneDoc version 2.7 software (Nicholas et al., 1997). Nucleotide sequences were aligned by ClustalX version 2.0 software (Larkin et al., 2007) and MUSCLE Multiple Sequence Alignment online tool (Edgar, 2004), respectively. Phylogenetic trees were constructed with MEGA version 7.0 software (Kumar et al., 2016).

Model selection in reference to accurate phylogenetic analysis was performed using the PhyML 3.0 online tool Substitution model selection section. Phylogenetic tree reconstruction was implemented using the PhyML 3.0 online tool, with the TN93+G+I substitution model performing nonparametric bootstrap analysis with 1000 replicates (Guindon et al., 2010). Trees were edited using the iTol online tool (Letunic and Bork, 2019).

Bayesian coalescent analyses and time-calibrated phylogeny were used to reconstruct the evolutionary dynamics of WNV. The phylogenetic tree was calibrated by attributing the sampling dates to the tips of the tree and using an uncorrelated relaxed clock with the lognormal distribution. Subsequently, the analyses were performed using Beast v. 1.10 software with

TN93+F+G4 substitution model. The Markov chain Monte Carlo (MCMC) analysis was run for 30 million generations and sampled every 30,000 steps. The convergence assessment based on the Effective Sample Size (ESS > 200) was performed in Tracer v1.6.0. Tree Annotator program was used to summarize the trees in a maximum clade credibility (MCC) tree with a 10% burn-in. The resultant tree was visualized in FigTree v1.4.4 program.

4.7 In vitro virus propagation

C6/36 (*Aedes albopictus*) cells (ATCC® CRL-1660™) were maintained in EMEM (Lonza, Switzerland) supplemented with 10 % Fetal Bovine Serum (Biosera, France) and 1 % Penicillin-Streptomycin (Lonza, Switzerland) at 28 °C until 70 % confluency in a 24-well plate. Before inoculation, 200 µl of supernatant from each mosquito homogenate was treated with an antibiotic cocktail at 37 °C for 1 h containing 1 µl Ampicillin ((stock cc. 100 mg/ml) Duchefa Biochemie, The Nederlands) 1 µl Gentamicin ((stock cc. 50 mg/ml) Lonza, Switzerland)) and 5 µl Penicillin-Streptomycin ((stock cc. 10.000 U/ml) Lonza, Switzerland)). After spent media was discarded from C6/36 cell monolayers, cells were incubated with the treated supernatants from mosquito homogenates for 1 h at 37 °C. Thereafter, cells were supplemented with 1 ml of extra fresh medium and were monitored for cytopathogenic effect for 7 days post-infection. After 7 days, cells were frozen at -80 °C and thawed to lyse the cells, and 200 µl of the inoculum was used for each of three additional passages from the previous plates.

5. Results and discussion

5.1 West Nile virus

As a result of the large-scale mosquito surveillance in Vojvodina province of Serbia in 2013, several novel WNV strains were detected, mainly in *Culex pipiens* species (Zana et al., 2016). Ten thousand nine hundred forty-eight nucleotides of the viral genomes were determined from all six WNV strains (GenBank accession numbers: KT757318 – KT757323), which covered the complete coding region (3434 aa). The highest nucleotide identity (99%) of the Serbian strains was identified with the Nea Santa-Greece-2010 strain (GenBank: HQ537483). Comparing our WNV strains to the Nea Santa-Greece-2010 strain, six amino acid changes were found in different positions of the polypeptide coding region (**Table 3.**). Several previously identified amino acid substitutions associated with altered pathogenicity and virulence of WNV were also described, such as Proline (Pro, P) at position $NS1_{250}P$, Asparagine (Asn, N) changes at the positions $NS1_{130}N$, $NS1_{175}N$, $NS1_{207}N$ and $E_{153/154}N$ of the *N*-linked glycosylation sites (Whiteman et al., 2011; Samuel and Diamond, 2006; Moudy et al., 2011). More interestingly, Pro amino acid substitution at position 249 of the NS3 region ($NS3_{249}P$) has been also detected in all six strains (**Table 2.**). This mutation was previously described as a potential marker of increased neuropathogenicity (Papa et al., 2011). Furthermore, we revealed parallel mutations between the helicase domain of the NS3 protein and the NS4B TM protein. Although mutations in the NSB4 TM protein were previously noted by Papa et al. (2011), the parallelism and potential functional association with $NS3_{249}P/H$ loci were not mentioned before. Strains with $NS3_{249}H$ (Histidine, His) substitution have parallel $NS4B_{14}S$ (Ser, S) and $NS4B_{49}T$ (Threonine, Thr) mutations, while those viruses with $NS3_{249}P$ amino acid variant have $NS4B_{14}G$ (Glycine, Gly) and $NS4B_{49}A$ (Alanine, Ala). Amino acid substitutions compared to other WNV lineage 2 strains are summarized in Tables 2 and 3.

Phylogenetic and sequence analyses of the complete coding sequence revealed that the 2013 Serbian strains belonged to the group of WNV genetic lineage 2. As the phylogenetic tree clearly shows, the Serbian WNV strains compose a single monophyletic group which unambiguously clusters with other Greek strains from 2010 and 2013 containing $NS3_{249}P$ mutation (**Figure 10a**). Due to the lack of WNV sequences with $NS3_{249}P$ mutation from Italy, only a single partial NS3 sequence was available, which serves as evidence for the presence of $NS3_{249}P$ strains in the country (**Table 2**; **Figure 10b**). This observation, along with phylogenetic- and molecular characterization suggests multiple introductions of different WNV strains from other European territories. Based on the complete coding sequence and partial NS3

phylogenetic analyses, a notable aggregation of $NS3_{249}P$ possessor strains in a separate clade from other European strains can be observed.

Mosquito surveillance, vector competence studies, human or animal case reports and serosurveillance activities are all essential pillars for monitoring the risk of WNV infections in Europe and predicting future outbreaks. In addition, examining locally circulating WNV strains for the early detection of those emerging mutations or amino acid alternations that may affect neurovirulence or pathogenicity is essential for evaluating the possible impact of forthcoming outbreaks and understanding viral evolution.

Here we examined the entire coding region of six WNV strains for amino acid alternations, described in previous studies as putative virulence factors. Asparagine changes in all examined lineage 2 sequences at the 3 *N*-linked glycosylation sites of NS1 ($NS1_{130}N$, $NS1_{175}N$, $NS1_{207}N$) and E ($E_{153/154}N$) proteins were identified. Amino acid changes might be important due to the special functions of these proteins. NS1 has a well-known cofactor activity of the viral replicase, while (Whiteman et al., 2011; Samuel and Diamond, 2006) E protein is responsible for the attachment to the cell surface, membrane fusion and viral assembly (Moudy et al., 2011). In the case of the WNV strain, which was originally introduced to New York in 1999 (HQ596519), E protein also proved to be a molecular determinant ($E_{153/154}N$) of neuroinvasiveness in a previous study (Shirato et al., 2004). At the NS3 genomic region, we detected the recently described $NS3_{249}P$ substitution in all Serbian strains analysed in this study (Papa et al., 2011).

The NS3 protein of most WNV lineage 2 strains contain His at the amino acid position 249, which is also characteristic of most neuroinvasive lineage 1 strains (Papa et al., 2011). In previous experimental studies, $NS3_{249}P$ substitution of NS3 protein was associated with increased viraemia and mortality among American crows (*Corvus brachyrhynchos*), which might have had a positive influence on the increased virus transmission rates (Brault et al., 2007; Papa et al., 2011; Samuel and Diamond, 2006). Furthermore, $NS3_{249}P$ mutants with increased viraemia and elevated thermotolerance may show greater pathogenicity (Brault et al., 2007). This important locus was only detectable in a single lineage 2 Italian sequence, in several Greek strains and the present Serbian lineage 2 viruses (**Table 2.**). The introduction of the new WNV strains possessing $NS3_{249}P$ substitution in Serbia could serve as an explanation for the increased number of WNND cases in 2012-2013 in the country (Popovic et al., 2013).

Additionally, we revealed parallel mutations between the NS3 protein helicase domain and NS4B TM protein ($NS3_{249}H$ with $NSB_{14}S$ and $NS3_{249}P$ with $NS4B_{14}G$), which might influence the putative protein-protein interaction between the two viral proteins.

Table 2. Previously identified amino acid substitutions for pathogenicity and virulence of WNV compared in different WNV lineage 2 strains. Parallel mutations between $NS3_{249}$H/P and $NS4B_{14}$ and $NS4B_{49}$ observed in the current study are emphasized in bold. Abbreviations: Asn, asparagine; Pro, proline; His, histidine; Gly, glycine; Ser, serine; Thr, threonine; Ala, alanine (Zana et al., 2016).

				Protein, amino acid position									
				E	**NS1**				**NS3**	**NS4B**			
Strain	**Country**	**GB number**	**Year**	154	130	175	207	250	**249**	**14**	23	32	**49**
Nea Santa-Greece-2010	Greece	HQ537483	2010	Asn	Asn	Asn	Asn	Pro	**Pro**	**Gly**	Thr	Asn	**Ala**
Greece/2013/Xanthi_3	Greece	KJ883345	2013	Asn	Asn	Asn	Asn	Pro	**Pro**	**Gly**	Thr	Asn	**Ala**
Greece/2013/Thessaloniki_4	Greece	KJ883346	2013	Asn	Asn	Asn	Asn	Pro	**Pro**	**Gly**	Thr	Asn	**Ala**
Italy-2012	Italy	JX878386	2012	-	-	-	-	-	**Pro**	-	-	-	-
Serbia/2013/Vojvodina-1	Serbia	KT7573718	2013	Asn	Asn	Asn	Asn	Pro	**Pro**	**Gly**	Thr	Asn	**Ala**
Serbia/2013/Vojvodina-2	Serbia	KT7573719	2013	Asn	Asn	Asn	Asn	Pro	**Pro**	**Gly**	Thr	Asn	**Ala**
Serbia/2013/Vojvodina-3	Serbia	KT7573720	2013	Asn	Asn	Asn	Asn	Pro	**Pro**	**Gly**	Thr	Ser	**Ala**
Serbia/2013/Vojvodina-4	Serbia	KT7573721	2013	Asn	Asn	Asn	Asn	Pro	**Pro**	**Gly**	Thr	Asn	**Ala**
Serbia/2013/Vojvodina-5	Serbia	KT7573722	2013	Asn	Asn	Asn	Asn	Pro	**Pro**	**Gly**	Thr	Asn	**Ala**
Serbia/2013/Vojvodina-6	Serbia	KT7573723	2013	Asn	Asn	Asn	Asn	Pro	**Pro**	**Gly**	Thr	Asn	**Ala**
-	DRC	HM147824	1958	Asn	Asn	Asn	Asn	Pro	**His**	**Ser**	Ala	Ser	**Thr**
SA93/01	South Africa	EF429198	2001	Asn	Asn	Asn	Asn	Pro	**His**	**Ser**	Ala	Ser	**Thr**
Goshawk-Hungary/04	Hungary	DQ116961	2004	Asn	Asn	Asn	Asn	Pro	**His**	**Ser**	Thr	Asn	**Thr**
Austria/2008_gh	Austria	KF179640	2008	Asn	Asn	Asn	Asn	Pro	**His**	**Ser**	Thr	Asn	**Thr**
Novi Sad-2010	Serbia	KC496016	2010	Asn	Asn	Asn	Asn	Pro	**His**	**Ser**	Thr	Asn	**Thr**
Cz 13-502	Czech Republic	KM203863	2013	Asn	Asn	Asn	Asn	Pro	**His**	**Ser**	Thr	Asn	**Thr**
Cz 13-329	Czech Republic	KM203861	2013	Asn	Asn	Asn	Asn	Pro	**His**	**Ser**	Thr	Asn	**Thr**
Italy/2013/Rovigo/32.1	Italy	KF588365	2013	Asn	Asn	Asn	Asn	Pro	**His**	**Ser**	Thr	Asn	**Thr**

Table 3. Unique amino acid substitution changes in the Serbian lineage 2 WNV strains compared to Neo Santa-Greece-2010 virus. Changes are emphasized in bold. Abbreviations: Thr, threonine; Ala, alanine; Glu, glutamic acid; Gly, glycine; Met, methionine; Val, valine; Ser, serine (Zana et al., 2016).

Strain	**GenBank No.**	**Protein, amino acid position**					
		NS1			NS2A	NS4A	NS5
		77	109	166	155	61	650
Serbia/2013/Vojvodina-1	KT7573718	Thr	Glu	Met	**Val → Ala**	Ser	Thr
Serbia/2013/Vojvodina-2	KT7573719	Thr	Glu	Met	**Val → Ala**	Ser	Thr
Serbia/2013/Vojvodina-3	KT7573720	**Thr → Ala**	**Glu → Gly**	Met	Val	**Ser → Gly**	**Thr → Ala**
Serbia/2013/Vojvodina-4	KT7573721	**Thr → Ala**	**Glu → Gly**	**Met → Val**	Val	Ser	Thr
Serbia/2013/Vojvodina-5	KT7573722	**Thr → Ala**	**Glu → Gly**	Met	Val	Ser	Thr
Serbia/2013/Vojvodina-6	KT7573723	**Thr → Ala**	**Glu → Gly**	Met	Val	Ser	Thr

Phylogenetic relationships with other European strains suggest multiple introduction events to Serbia, as mentioned before (Petrović et al., 2013; Kemenesi et al., 2014). Serbian WNV strains from mosquito samples of 2013 were clustered with Greek strains from 2010 and 2013. This suggests the possible origin of these strains from Greece, which explains the presence of $NS3_{249}P$ substitution in the Serbian samples. Based on the phylogenetic analyses, a possible WNV introduction from Greece to Italy in 2012 may also be assumable based on a partial NS3 sequence from Italy (JX878386), which also contains the $NS3_{249}P$ alternation as the Greek and Serbian samples. Previously detected WNV strains from Serbia (2010 and 2012) unambiguously clustered with other European WNV strains with the absence of the $NS3_{249}P$ alternation. The presence of $NS3_{249}P$ mutant WNV strains in Serbia may explain the increased number of West Nile neuroinvasive disease (WNND) cases in the outbreak of 2013 and warn of possible more severe outbreaks in the affected areas in the future.

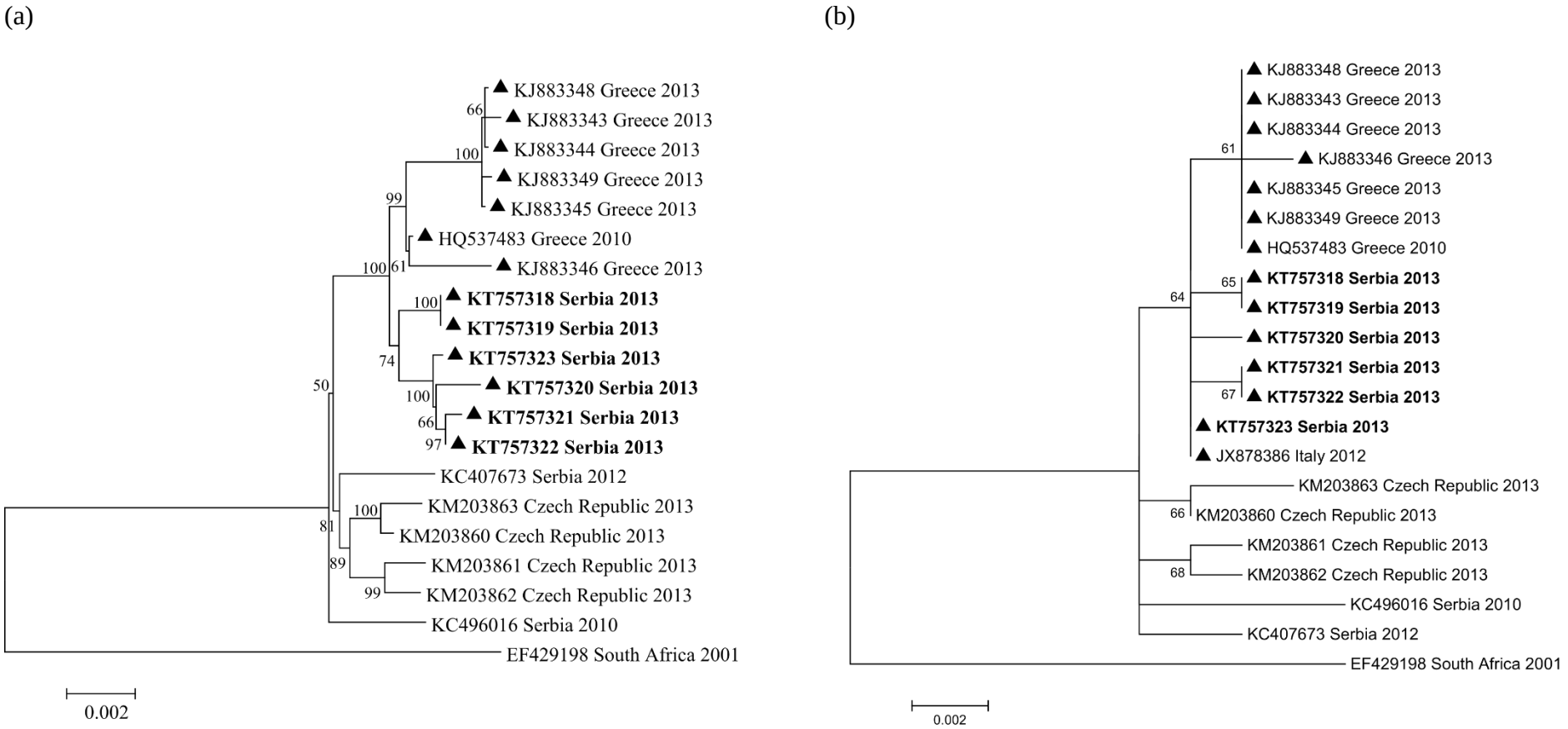


Figure 10. Phylogenetic analyses of West Nile virus lineage 2 strains detected in Serbia. Phylogenetic trees were constructed based on a complete coding region of the virus (a) and a partial region (743 nt) of the NS3 (b) gene. Strains that possessed the NS3249P mutation are marked with black triangular. The phylogenetic trees were constructed using the Maximum-Likelihood method, based on the General Time Reversible model with Gamma distribution (GTR+G). The number of bootstraps for simulations was 1000 (Zana et al., 2016).

The year 2018 was characterized by an extraordinary increase in human and animal cases in the Central-Eastern European region, including Hungary. In a collaborative effort, we shared, summarized and analysed the genetic and serologic data of WNV infections from multiple professional sources and institutions in Hungary. We compared human and veterinary serologic data, along with NS5 and NS3 gene sequence data, through 2018 (Zana et al., 2020). Except for two samples (one horse and one goshawk), all sequence data were generated and kindly shared with us for further analyses by colleagues of the National Public Health Center, Dr Károly Erdélyi and colleagues of The National Food Chain Safety Office.

During the transmission seasons between 2014 and 2017, n = 80 human patients of the n = 103 reported cases were examined using molecular diagnostic methods. Altogether, n = 32 (40.0%) were positive by both diagnostic real-time and confirmatory nested RT-PCR assays. In contrast, during the transmission season of 2018, n = 225 autochthonous and imported WNV cases were reported to the European Centre for Disease Prevention and Control (ECDC, 2018). In total, n = 53 patients were found to be positive for WNV RNA by diagnostic PCR screening, while only those samples from n = 46 patients (27.7%) were eventually confirmed by WNV nested RT-PCR and sequencing. Comprehensively, the results suggest a 1.4-fold increase in the number of PCR-positive cases within a single transmission period compared to the total number of cases from the previous four years (**Figure 11.** and **Figure 12.**). These results are consistent with serological data, which showed a nine-fold increase in the cumulative number of human WNV cases (Nagy et al., 2019).

The geographic distribution of infected birds coincided with the zones regarding PCR-positive human cases (**Figure 11.**, **Figure 12.** and **Figure 13.**). From the 150 wild and exotic bird carcasses examined from January to November 2018, we identified 14 cases of WNV-related mortality. The first WNV case was detected on 2 July in a hooded crow (*Corvus cornix*) carcass from Debrecen, while the last two positive bird cases were submitted on 4 September, including a white stork (*Ciconia ciconia*) and a peregrine falcon (*Falco peregrinus*) from Tiszaalpár and Pilisvörösvár, respectively. The wide range of affected species comprised the hooded crow (3), black-headed gull (*Chroicocephalus ridibundus*) (1), greenfinch (*Chloris chloris*) (1), mute swan (*Cygnus olor*) (1), monk parakeet (*Myiopsitta monachus*) (2), great tit (*Parus major*) (1), goshawk (*Accipiter gentilis*) (1), African penguin (*Spheniscus demersus*) (1), kea (*Nestor notabilis*) (1), peregrine falcon (1), and the white stork (1). As expected from our earlier experience (Bakonyi et al., 2013; Erdélyi et al., 2007) and regional studies (Hubálek et al., 2018), birds of prey were found affected, and sporadic mortality cases of hooded crows were identified at two locations. Although active monitoring of European corvid species was

suggested by several studies (Rizzoli et al., 2015; Napp et al., 2019), we only detected 6 specimens (1 magpie, 2 Eurasian jays, and 3 hooded crows) from the 130 examined corvid carcasses during the past 14 years of passive bird mortality monitoring. Nevertheless, starting active surveillance of selected corvid species in the future may prove beneficial in the early detection of seasonal WNV circulation.

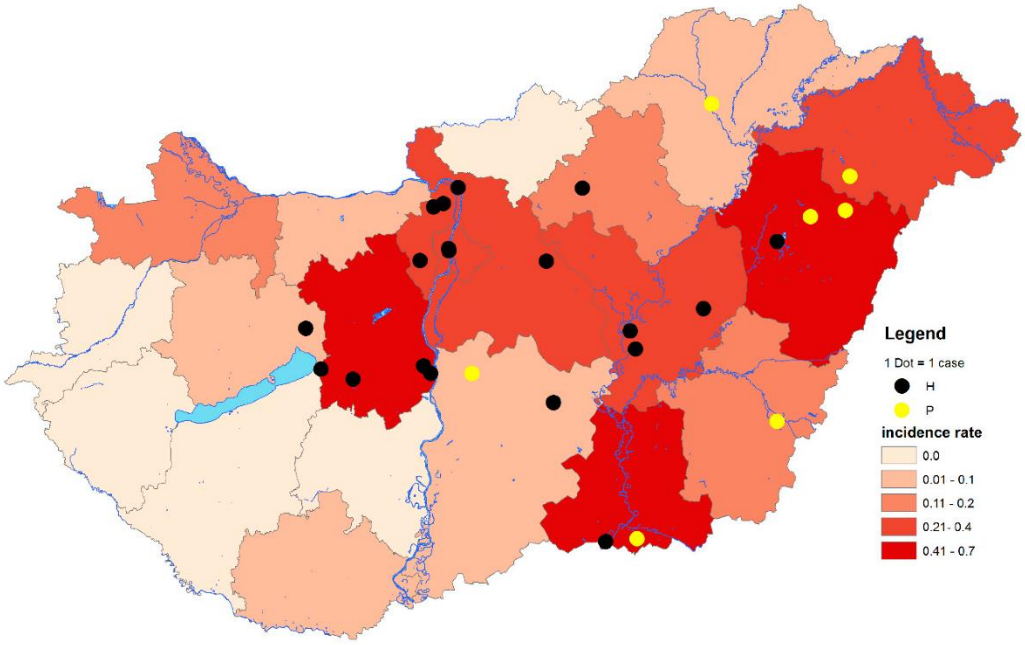


Figure 11. Geographical distribution of West Nile virus (WNV) polymerase chain reaction (PCR)-positive cases at the LAU 2 level and cumulative incidence rates of autochthonous and imported human WNV infections at NUTS 3 level of Hungary, from 2014 through 2017. The total number of human WNV cases between 2014 and 2017: n = 103. Black dots indicate PCR-positive samples containing histidine at amino acid position NS3249. Yellow dots indicate PCR-positive samples containing proline at amino acid position NS3249. LAU 2: Local Administrative Units level 2. NUTS 3: Nomenclature of territorial units for statistics level 3. IR: Incidence rates (number of WNV human infections/100,000 inhabitants) (Zana et al., 2020).

During the entomological surveillance regarding infected horse cases in Dunaföldvár, we revealed the presence of abundant larval breeding sites within close proximity to the horses (swimming pool with a neglected water body and rainfall pools in artificial containers). *Culex pipiens* larvae in the swimming pool were found to be PCR-positive for WNV RNA. However, we measured an extremely low viral load, represented with PCR Ct (cycle threshold) values of 40–45, which prevented any successful in vitro isolation or sequencing attempts. No other mosquito species were trapped or found around the site. The total set of 36 pools from overwintering mosquitoes (*Culex pipiens*, *Anopheles maculipennis*, *Culiseta annulata*) were found to be negative for WNV RNA, which supports the previous observation regarding low-level infection rates being sufficient for the persistence of WNV in overwintering mosquito

populations (Rudolf et al., 2017). In addition to the role of mosquitoes, persistent bird infections should also be considered as a possible way of virus persistence and overwintering, as previously reported (Bakonyi et al., 2016). However, a more detailed and extensive sampling strategy likely will verify this observation in future studies.

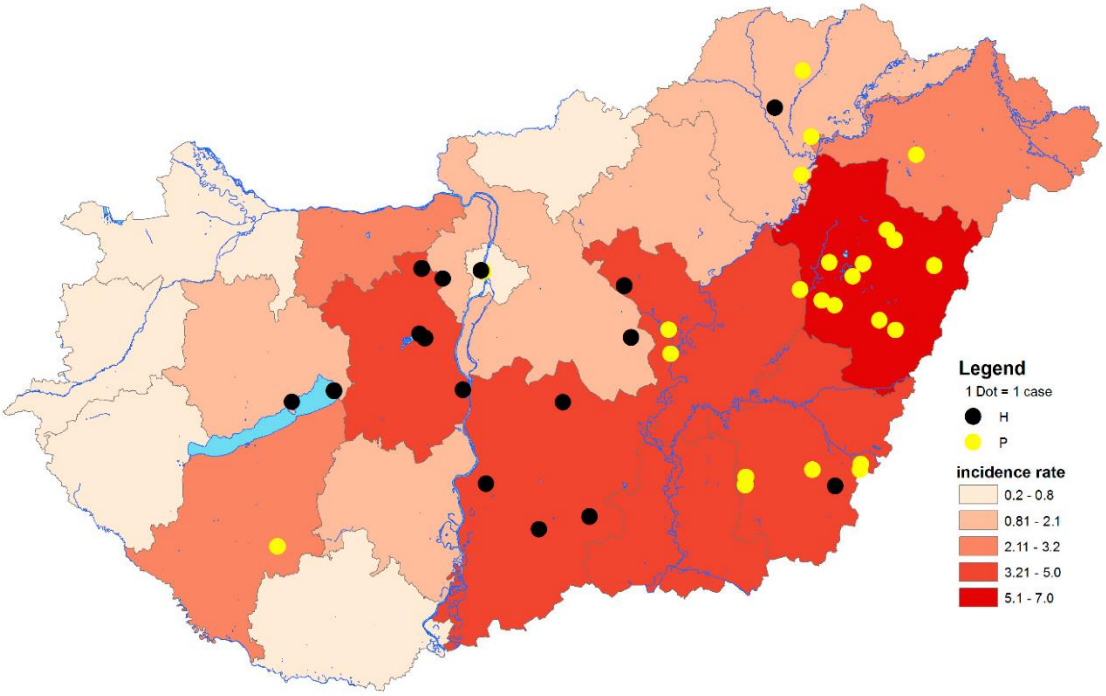


Figure 12. Geographical distribution of West Nile virus (WNV) PCR-positive cases at LAU 2 level and cumulative incidence rates of autochthonous human WNV infections at NUTS 3 level of Hungary, 2018. The total number of autochthonous human WNV cases in 2018: n = 215. Black dots indicate PCR-positive samples containing histidine at amino acid position NS3249. Yellow dots indicate PCR-positive samples containing proline at amino acid position NS3249. LAU 2: Local Administrative Units level 2. NUTS 3: Nomenclature of territorial units for statistics level 3. IR: Incidence rates (number of WNV human infections/100,000 inhabitants) (Zana et al., 2020).

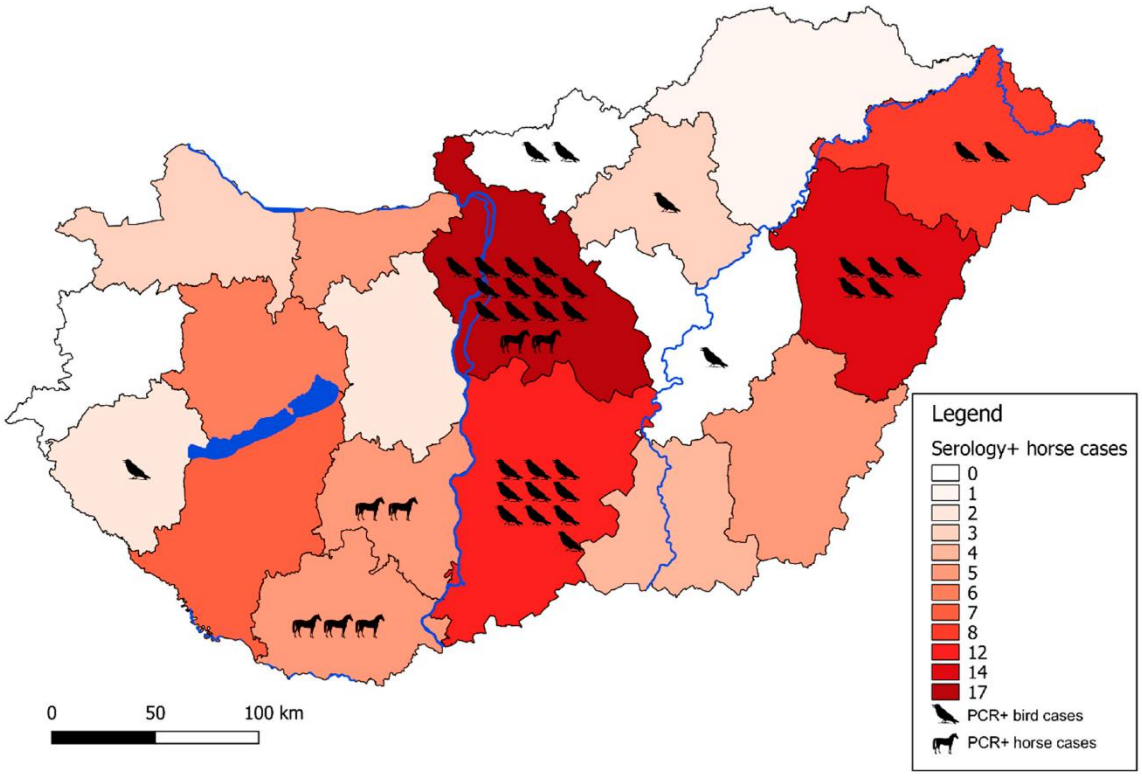


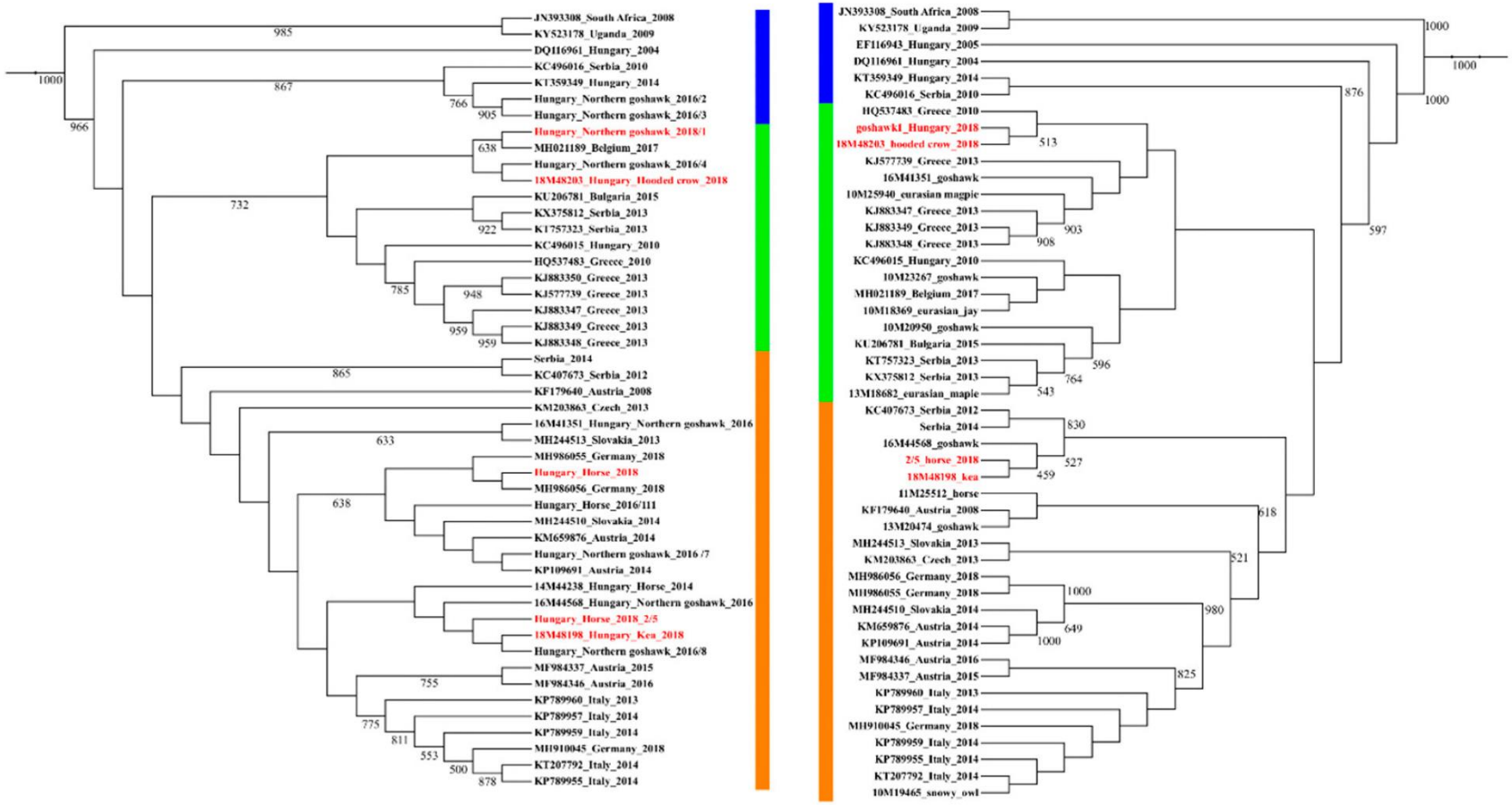
Figure 13. Geographical distribution of enzootic WNV cases. The colour background of each county represents the number of IgM seropositive horses in that specific region from 2018, as annotated on the sidebar. Individual PCR-positive animal cases are indicated with respective pictograms for each case in that specific region (Zana et al., 2020).

Equine cases related to WNV are reported through the Animal Disease Notification System (ADNS) of the European Commission. During the transmission season of 2017, three cases were reported that originated from Hungary (Gray and Webb, 2014). In contrast to the previous year, in 2018, 91 equine cases were detected by serologic survey and virus neutralization tests. As a result, the number of cases implies an approximately 30-fold increase in WNV cases among equines.

Attempts to isolate the virus on C6/36 and VeroE6 cell lines proved to be successful in the case of one *Culex pipiens* mosquito pool (n = 50) homogenate from the Serbian/Hungarian border and one northern goshawk (*Accipiter gentilis*) brain tissue homogenate. Unfortunately, repeated attempts to isolate the virus from horse brain tissue homogenates failed regarding both cell lines. Following the third blind passage, we could not detect WNV RNA in the supernatants of lysed cells by the previously mentioned real-time RT-PCR system, likely due to the loss of viability of virus particles as a consequence of the condition of carcasses at the time of collection, handling, and storage methods of the samples.

The maximum likelihood phylogenetic analysis regarding WNV strains resulted in two bush-like groups, representing the two major subclades of WNV strains circulating simultaneously in Central Europe as previously described by others (Chaintoutis et al., 2019). Our phylogenetic analysis revealed that the circulating WNV strains of 2018 were segregated into two different subgroups, indicating the simultaneous circulation of members from these two clades of the virus in Hungary in 2018. Partial NS3 and NS5 sequences both supported this observation. One of these phylogenetic clusters was related to members of the Balkans subclade, while the other contained WNV strains mainly from Southern and Western European countries. One Western European sequence, which was imported to Belgium from Hungary, was grouped into the Balkanian subclade. This offers a more precise evolutionary origin regarding this imported case than previously published (Wollants et al., 2018) (**Figure 14.**). The progenitor strains were segregated outside the two groups, advisably indicating the order of WNV strains' emergence in time and route of introduction to Hungary. In contrast to Italy, where mostly endemic clades persist (Veo et al., 2019), we reported two WNV strains simultaneously co-circulating in Hungary.

Figure 14. Phylogenetic representation of partial NS3 (left side) and NS5 (right side) gene sequences of Hungarian horse and wild bird samples compared to cognate sequences from the region, collected between 2010 and 2018. Progenitor African, Hungarian, and one Serbian WNV lineage 2 strains are highlighted with a blue outline, while the two major subclades are indicated with green (Balkanian subclade) and orange (Central/South-West European subgroup) outlines. Samples from 2018, Hungary, are highlighted in red colour. Note MH021189 as an imported case from Hungary to Belgium (Zana et al., 2020).



In our study, human-derived sequences permitted detailed phylogenetic analysis, mainly due to the short length of sequences, which resulted in a highly polytomic tree structure. Therefore, we indicated the presence of the two major genetic subclades on a heat map structure (**Figure 15.**), which substantially supports the findings of the phylogenetic analysis regarding animal-derived sequences. We hypothesize the dominance of these two subclades in the affected countries in our region during the 2018 season, and quite possibly prior. However, retrospective studies may likely verify this assumption in other countries of the region as well.

Our results were further supported by Bayesian time-scale phylogeny (**Figure 16.**). Strains of the 2018 outbreak most likely originated from a diversification event in Hungary between 2010 and 2013, and they are represented by both subclades. Although this analysis is highly theoretical, it provides additional evidence for the absence of novel emerging strains. Our data is also confirmed by the more detailed work about the evolutionary dynamics of lineage-2 WNV (Chaintoutis et al., 2019).

Figure 15. Human-derived, partial WNV NS3 sequences from the 2018 season. The figure represents similarity scores of all available human derived WNV partial NS3 sequences from 2018. The two dominant genetic subclades are highlighted on the dendrogram with green and peach colour (Zana et al., 2020).

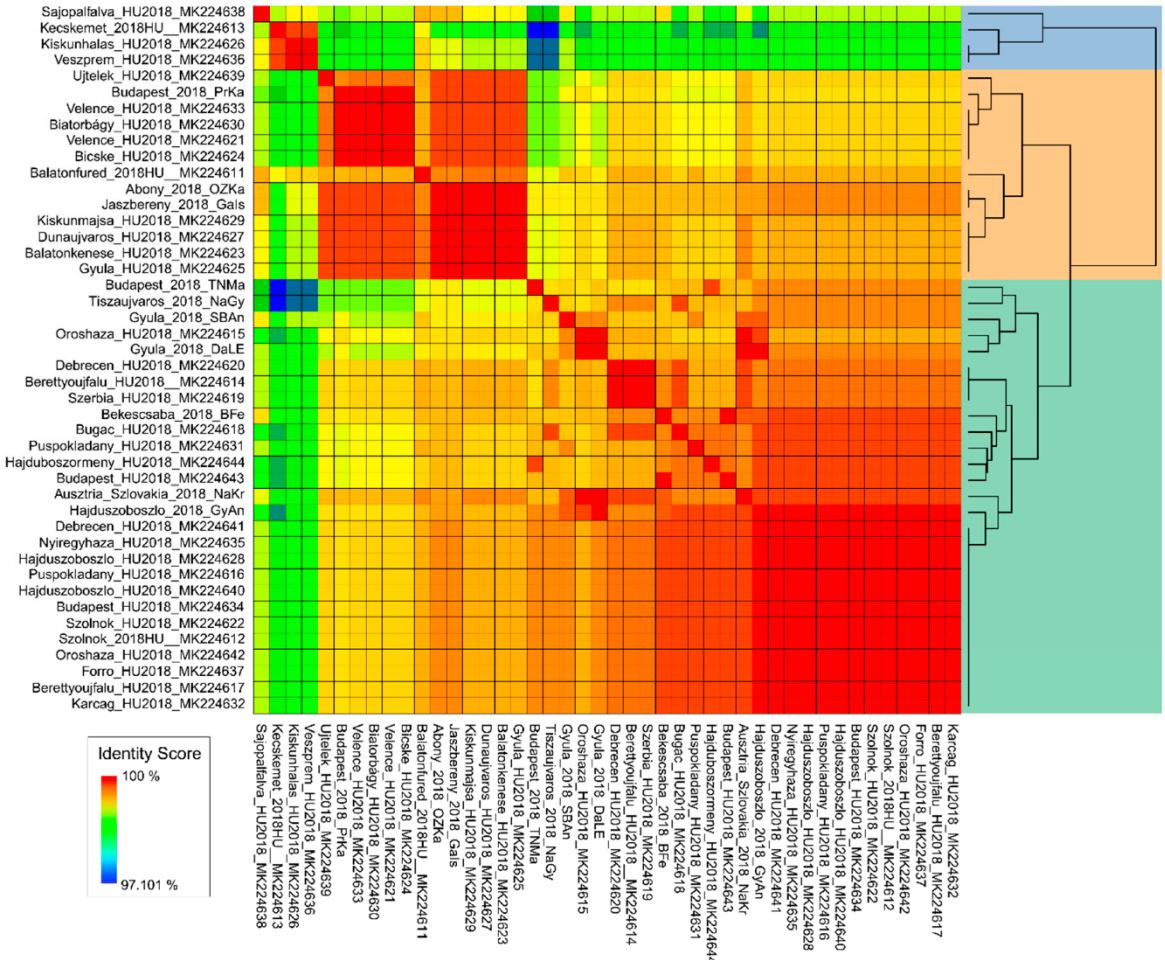
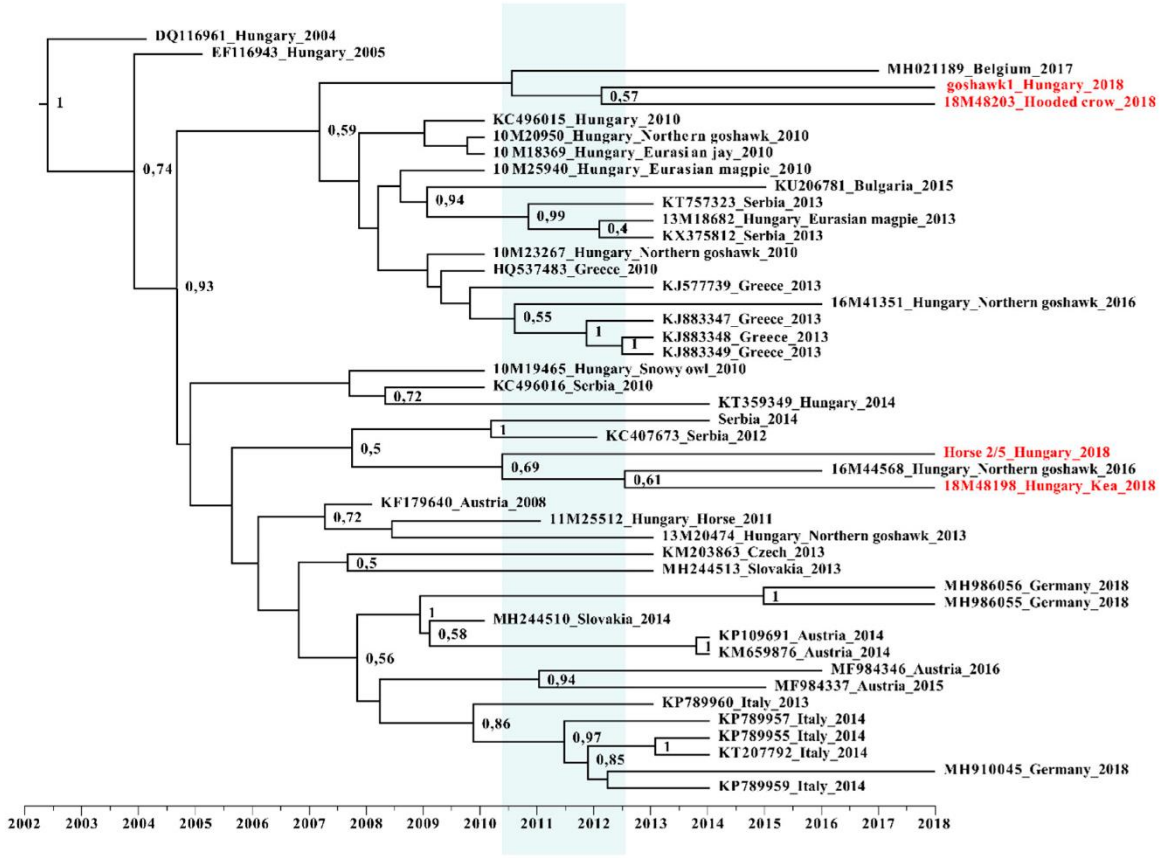


Figure 16. Time-calibrated Bayesian maximum clade credibility phylogenetic reconstruction of the evolution of Hungarian wild bird and horse samples. Partial NS5 gene sequences of this study, along with cognate sequences, were included in this dataset. Samples from 2018 are highlighted in red, while the timeframe of origin is indicated with a blue background (Zana et al., 2020).



In summary, we analysed the complete coding region of six WNV strains from mosquito samples during the outbreak of 2013, in Serbia. The $NS3_{249}P$ amino acid substitution has been described in all strains, which was previously described as a potential marker of increased neuropathogenicity. Additionally, parallel mutations between NS3 protein helicase domain and NS4B TM protein were described, which might influence the protein-protein interaction, and therefore the RNA binding of NS3, which may facilitate viral replication. These results may support future surveillance activities by shedding light on the importance of examining mutations possibly affecting neuropathogenicity. This may lead to more precise predictions of the possible outcome of future outbreaks.

During the transmission season of 2018, Hungary was widely affected by the WNV epidemic. To date, the exact scenario and main triggering factors are not fully understood. In a country-wide, multi-approach investigation, we aimed to understand the underlying factors lurking behind the situation of 2018 in Hungary. For this purpose, we analysed human and veterinary sequences along with country-wide human and veterinary serologic data.

Based on our sequence and phylogenetic data, the situation of 2018 was more likely caused by endemic strains rather than recently introduced novel WNV strains. We identified the presence of multiple phylogenetic subclades (the Balkans subclade and the Central/South-West European subgroup) in Hungary before the epidemic in 2018. Therefore, we hypothesize that the main trigger factors behind the outstanding case numbers during 2018 were likely the result of favourable environmental conditions for mosquito vectors and the increased contact of these mosquitoes with native animal and human populations. However, we do not have an integrated surveillance system in the country providing a long-term comprehensive dataset to support this theory. Therefore, at this level, it is just a logical hypothesis. Since previous studies pointed out Hungary as an important ecological niche for virus diversification and dissemination in our geographic area (Chaintoutis et al., 2019), a One Health approach should be implemented to fully understand the ecological background factors driving annual human and veterinary cases. Our work also reflects the urgent need for a country-wide, organized surveillance system, based on rapid and extensive data sharing regarding the West Nile virus.

5.2 Usutu virus

For virus surveillance, female mosquitoes were collected as part of national mosquito control activities in Vojvodina province. Altogether, 23,753 adult female mosquitoes were collected from urbanized, human-inhabited areas and typical mosquito breeding sites within cities and small villages from May to October 2014. Mosquitoes were trapped with CDC light traps baited with dry ice at 59 sampling sites belonging to 9 municipalities. Specimens were grouped by a maximum of 50 individuals per sampling event, species and collection site into pools and were subjected to USUV-specific nested reverse transcription-polymerase chain reaction (Kemenesi et al., 2018).

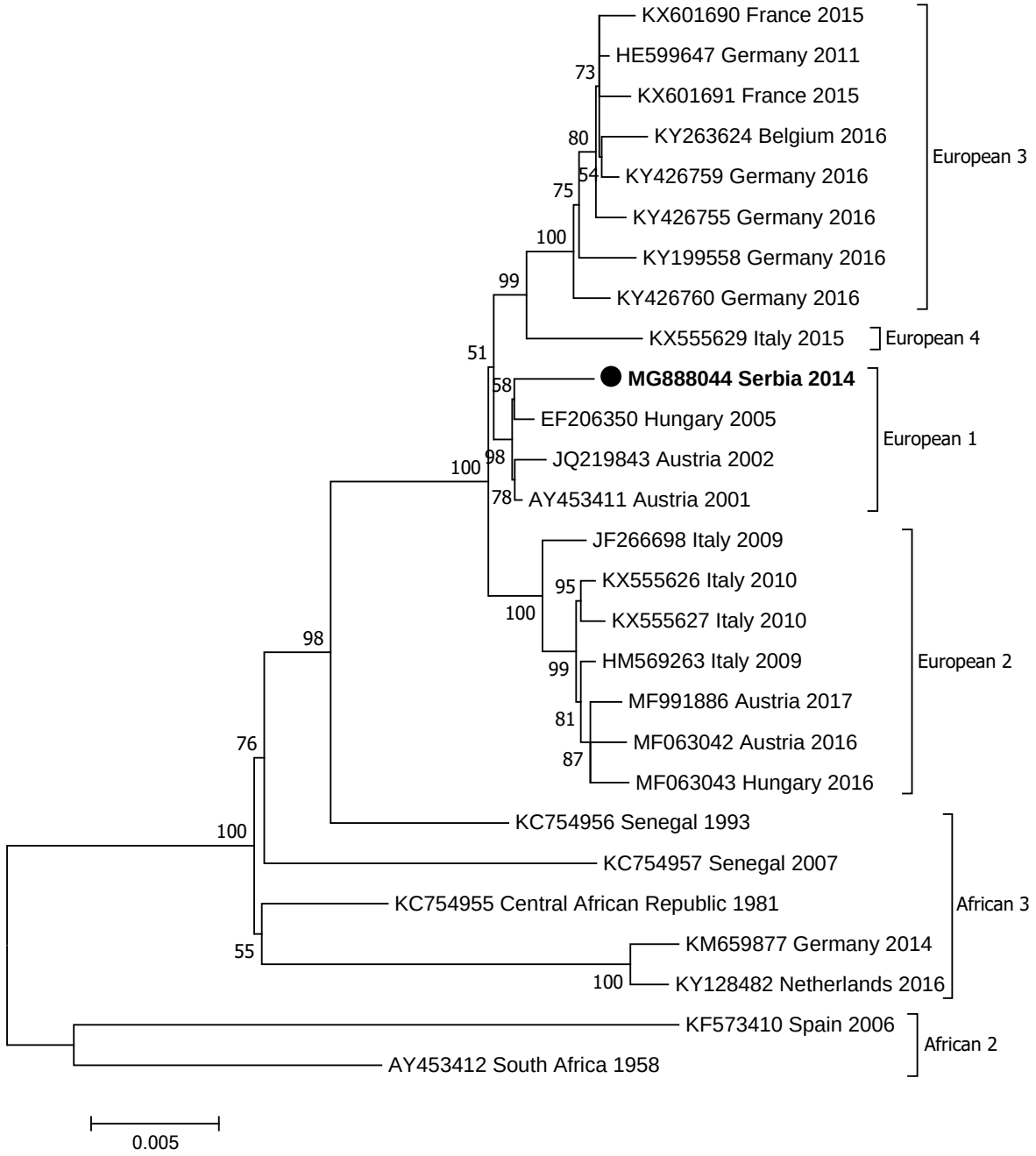
The most abundant sampled species were *Culex pipiens* (n=11,099, 47% of all mosquito specimens), followed by *Aedes vexans* (n=10,005, 42% of all mosquito specimens). Dominant species of the investigated area were *Ochlerotatus caspius* (n=1574, 7%), *Ochlerotatus sticticus* (n=559, 2.4%) and *Coquillettidia richiardii* (n=332, 1.4%) as well. All other species were represented with a considerably lower number (n < 100, <0.5% of all mosquito specimens). A total of 1,437 female mosquitoes (68 pools) were collected in May, n=4252 (158 pools) in June, n=1512 (52 pools) in July, n=13,683 (403 pools) in August, n=54 (2 pools) in September and n=2815 (70 pools) in October.

Out of the 753 pools sampled, the presence of USUV RNA was confirmed in 3 pools of *Culex pipiens* mosquitoes, collected in August from Titel (45°12′N, 20°18′E, 1 positive out of 38 pools) and Zrejanin (45°22′N, 20°23′E, 2 positives out of the 330 pools).

Cx. pipiens was frequently described as a primary vector of the virus across Europe (Ashraf et al., 2015; Fros et al., 2015; Nikolay, 2015; Cadar et al., 2017a). However, *Cx. pipiens* mosquitoes are originally considered with ornithophilic feeding behaviour (Brugman et al., 2017), and the two distinct biotypes (*Cx. p. pipiens* and *Cx. p. molestus*) show remarkable physiological and behavioural differences. While the *Culex p.* biotype *pipiens* rarely bite humans and seems to be strictly ornithophilic (i.e., bird-biting host preference), females of the biotype molestus are mainly anthropophilic (Becker et al., 2012). At the same time, human host preference was identified in the case of pipiens and molestus forms and their hybrids as well (Martínez-de la Puente et al., 2016). The adaptation of *Cx. pipiens* mosquitoes to human-altered environments led to their global distribution through dispersal via humans and combined with their mixed feeding patterns on birds and mammals (including humans), predestine them as bridge vectors for pathogens transmitted between mammals and birds (Ashraf et al., 2015; Fros et al., 2015; Nikolay, 2015; Cadar et al., 2017a). However, we did not identify members of the

Cx. pipiens complex to biotype level, mosquitoes of the molestus form are more frequently found in urban than in natural areas (Martínez-de la Puente et al., 2016), and biotype molestus was identified already in Novi Sad, Serbia (Becker et al., 2012).

Figure 17. The evolutionary history was inferred based on 10,752 nucleotide genome fragments by using the Maximum Likelihood method based on the Tamura-Nei model (+G). The best-fit nucleotide substitution model was selected based on the Bayesian information criterion as implemented in the MEGA software. The scale bar indicates evolutionary distance (Kemenesi et al., 2018).



Regarding the close geographic location, same vector species and the 100% identity of partial NS5 sequences, we subjected one positive pool for longer genomic fragment amplification and used it as a reference strain from the region. The entire coding region of the virus was determined along with a partial 3′ UTR fragment (Accession number: MG888044) with a putative polyprotein encoding open reading frame of 3,434 amino acids.

Representative members of known putative genetic lineages were selected (Africa 2 and 3; Europe 1–4) to infer presumed evolutionary relationships to known genetic variants. Africa 1 genetic variant (KC754958) was excluded from the analysis for a better resolution of the tree. Based on phylogeny results, the Serbian USUV strain from 2014 was unambiguously clustered to the European genetic lineage 1 clade originally emerged in Austria, in 2001 (**Figure 17.**). The presence of this lineage in Serbia indicates a geographic expansion to southward territories in the Balkanian peninsula and urges the establishment of surveillance systems in neighbouring countries as well, especially where serologic evidence of the virus was already published (e.g., Croatia) (Barbic et al., 2013; Vilibic-Cavlek et al., 2014; Santini et al., 2015). It is unclear if the serologic evidence of USUV in the Southern part of the Balkans in Greece indicates the circulation of the same genetic variant or if it is evidence of the presence of another genetic lineage of USUV (Chaintoutis et al., 2014). Intensified surveillance efforts are needed to clarify this. The implementation of such a program could serve to clarify if this genetic lineage reached a wide distribution on Balkan Peninsula or if other genetic variants are co-circulating. Based on the evidence of human infection with USUV (Bakonyi et al., 2017b; Percivalle et al., 2017; Grottola et al., 2017; Cadar et al., 2017b), it is necessary to expand the surveillance efforts to human samples and note the presence of the virus in the region for public health authorities and blood transfusion services.

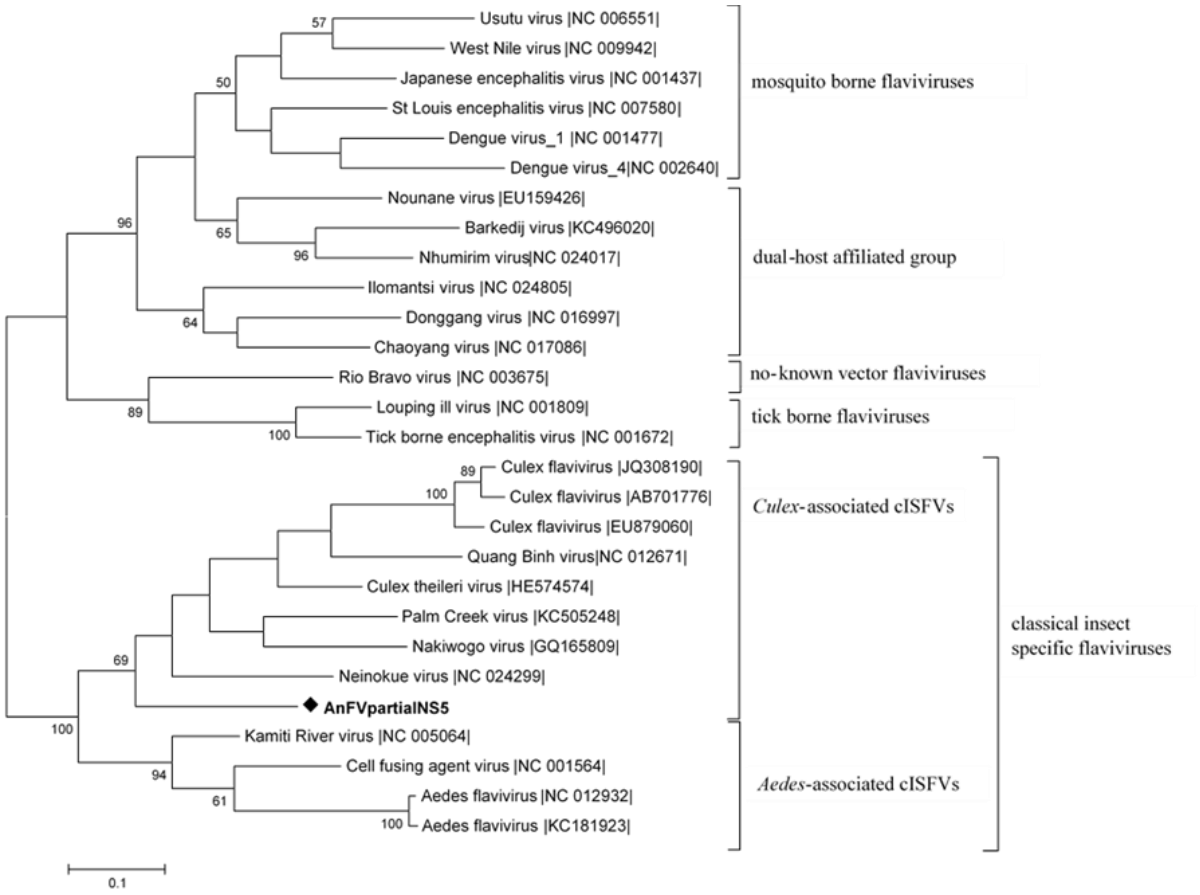
This work represents the first evidence for the geographic expansion of European lineage 1 to southern territories in the Balkanian peninsula and provides the first genetic data of USUV in the region. However, it is necessary to further investigate locally circulating strains to clarify any possible strain exchange, as published recently from Hungary and Austria (Bakonyi et al., 2017a).

5.3 *Anopheles flavivirus*

Anopheles hyrcanus mosquito samples were collected in multiple locations from Hungary and were tested for the presence of flavivirus-related sequences (Zana et al., 2017). Altogether 283 female *An. hyrcanus* mosquitoes were collected and combined in 8 pools. Flavivirus-related sequences were detected in 1 and 2 pools collected in Pécs and Kisvárda, respectively. Nucleotide BLAST homology revealed a putative novel ISFV, which showed the highest nucleotide identity of 76% to the Nakiwogo virus (Cook et al., 2009). To evaluate the taxonomic status of our sequence, we aligned the nucleotide sequences of the most frequent representatives of arthropod-borne (tick and mosquito) flaviviruses, nearly all representatives of insect-specific flaviviruses from both classical (cISFVs) and dual-host affiliated ISFVs and one representative from not-known vector (NKV) flaviviruses. Examined sequences showed the formation of the same main phylogenetic clusters described previously. Classical ISFVs generated distinct phylogenetic groups from all other known flaviviruses and separated into two main clades composed of cISFVs which are usually associated with Aedes spp. mosquitoes or Culex spp. mosquitoes (**Figure 18.**) (Blitvich and Firth, 2015; Cook et al., 2009; Kenney et al., 2014). Our putative novel ISFV sequence branched within the cluster of cISFVs. Besides, it showed homology with Culex-associated insect-specific flaviviruses and formed a distinct branch within this cluster, indicating that anopheles flavivirus (AnFV) is a possible new member of the Culex-associated insect-specific flaviviruses (**Figure 18.**). Anopheles hyrcanus is known for its potential role as Plasmodium sp. and Dirofilaria sp. vector (Seidel et al., 2013; Kemenesi et al., 2015). Furthermore, the presence of the Tahyna virus was also described in this mosquito species (Hubalek et al., 2014). However, its possible capability for hosting flaviviruses is described in this study for the first time. Other species within the Anopheles genus were described previously in association with flaviviruses, precisely with Quang Binh and Culex flaviviruses, which were reported in Anopheles sinensis mosquitoes (Calzolari et al., 2016).

Although we described a potential novel member within the group of ISFVs, a relatively short fragment analysed is not sufficient to make long-term or even final conclusions. Further experiments and field screening of *An. hyrcanus* are needed to clarify the presence and exact position of this tentatively novel member within the Flavivirus genus.

Figure 18. Phylogenetic tree of the novel AnFVpartialNS5 strain and other members of the genus Flavivirus for the partial NS5 and complete genome nucleotide dataset. The phylogenetic tree was constructed from the nucleic acid sequence alignments using the maximum likelihood method based on the General Time Reversible model (GTR+G+I) of the program MEGA v6.0 software. The number of bootstrap replications was 1000 (Zana et al., 2017).



5.4 *In vitro* virus propagation

For this study, we used the remaining mosquito samples collected in Serbia, 2014 originally utilized for surveillance studies of the Usutu virus and West Nile virus (Kemenesi et al., 2018; Zana et al.,2020). During the *in vitro* surveillance altogether, we screened 283 mosquito pools for viral cytopathic effect on C6/36 (*Aedes albopictus*) cell line. From the inoculated samples, 72 showed cytopathic effects after the second passage. Metagenomic analyses of 72 virus isolates proved the presence of diverse mosquito-specific viruses in 68 samples belonging to several mosquito species from different collection sites in Serbia (**Table 5**.).

Table 4. Pivot table of the result of *in vitro* surveillance

mosq_species	Daeseongdong virus	Negevirus	Alphamesonivirus	Orbivirus	Flavivirus
Ae. vexans	5	15	10		
An. maculipennis		1			
Cu. annulata		1			
Cx. pipiens	3	35	25	1	1
Oc. caspius		5	1		1
Oc. sticticus		2	1		
Sum	**8**	**59**	**37**	**1**	**2**

After metagenomic analyses of virus isolates, Negeviruses and Alphamesoniviruses occurred in the highest proportion in the sequenced samples, 59 and 37 respectively, then the next abundant virus proved to be daeseongdong virus in 8 samples, then flavivirus in 2 and orbivirus in one sample. Most virus isolates were derived from *Aedes vexans* and *Culex pipiens* mosquito samples, of which Negeviruses and Alphamesoniviruses occurred in the highest proportion (**Table 4.**). Our results support and broaden the wide geographical distribution of these viruses stated by previous studies (Warrilow et al., 2014; Birnberg et al., 2020; Nunes et al., 2017) since so far, there has been no evidence of the presence of these viruses in Serbia.

Daeseongdong virus was isolated from *Culex pipiens* and *Aedes vexans* mosquito samples collected in distinct locations of the country. Daeseongdong virus is a poorly investigated virus, however, its presence in mosquito samples (*Culex pipiens*) collected in South Korea and honey-baited Flinders Technology Associates (FTA) cards samples in Spain suggests wild geographic distribution (Hang et al., 2016; Birnberg et al., 2020). The result of

our *in vitro* surveillance attempts further strengthen the worldwide distribution of the Daeseongdong virus and prove its broader host preference.

Based on preliminary BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analyses, the segments of the new orbivirus isolate are closely related to the Umatilla virus, Stretch Lagoon orbivirus and Koyama Hill virus, respectively, members of Umatilla virus species which also represents a separate serogroup within orbivirus genus. Based on this and comparing our results to previous studies this orbivirus belongs to the Umatilla virus species although phylogenetic analyses and sequence comparison of conserved T2 and VP7 (T13) protein-coding sequences and serological cross-reaction tests related to VP7 (T13) protein are sufficient to differentiate within virus species and specify the virus serogroup (Belaganahalli et al., 2011; Ejiri et al., 2014). Our results represent the first evidence of the presence of the Umatilla virus species in Serbian mosquitoes.

Marisma mosquito virus is a dual host affiliated insect-specific virus (dISFs) (Guzman et al., 2018) which presence in *Ochlerotatus caspius* mosquitoes was confirmed by previous studies in Spain and Italy in 2012 and 2014, respectively (Vázquez et al., 2012; Rizzo et al., 2014). Our results provide the first evidence of the presence of the virus in Serbia, also isolated from *Oc. caspius* mosquito species. As suggested by multiple studies, dISFs show a close phylogenetic relationship and antigenic potential to cISFs such as pathogenic flaviviruses such as WNV or ZikV. Furthermore, they might have an impact on arbovirus infection and transmission in a co-infected mosquito host and hide the potential to develop antiviral agents or vaccines (Guzman et al., 2018).

However, most of the phylogenetic and genomic information originates from metagenomic investigations, and most ISVs have not been isolated yet (Atoni et al., 2019; Guzman et al., 2018). To understand the transmission mechanisms of ISVs in mosquito populations, their relationships with mosquitoes, their modulation activity on arbovirus replication in vectors and their impact on vector competence, virus isolation is a crucial step. Furthermore, as it is reviewed, most *in vitro* co-infection studies provide contradictory results due to the lack of antiviral RNA interference response of mainly used C6/36 cell lines (Bolling et al., 2015; Guzman et al., 2018; Carvalho et al., 2021). Besides, quite a few results of *in vivo* experiments are available and seem inconclusive as well. To determine the actual modulation activity and their possible role in arbovirus control as it is suggested in the case of Wolbachia, more efficient functional *in vivo* studies are needed focusing on locally collected mosquitoes to investigate locally circulating viruses in mosquitoes and their effect on each other (Carvalho et al., 2021; Guzman et al., 2018; Bolling et al., 2015).

Table 5. Samples and results of *in vitro* virus propagation study

Sample ID	Locality	Municipality	Collection date	mosq_species	number/pool	Identified virus			
132	Novi Sad - Novi Naselje	Novi Sad	27.08.2014	*Ae. vexans*	50		Negevirus		
142	Futog I	Novi Sad	17.07.2014	*Cx. pipiens*	50				Orbivirus
147	Subotica V (Vet Stanica)	Subotica	02.08.2014	*Ae. vexans*	6	Daeseongdong virus	Negevirus		
148	Subotica V (Vet Stanica)	Subotica	02.08.2014	*Cx. pipiens*	14	Daeseongdong virus	Negevirus	Alphamesonivirus	
149	Novi Sad IX (Centar)	Novi Sad	25.07.2014	*Cx. pipiens*	26		Negevirus		
151	Kelebija	Subotica	02.08.2014	*Cx. pipiens*	18		Negevirus	Alphamesonivirus	
154	Novi Sad (Kamenjar)	Novi Sad	27.08.2014	*Ae. vexans*	50	Daeseongdong virus	Negevirus		
157	Novi Sad IV (Kamenjar)	Novi Sad	27.08.2014	*Ae. vexans*	50		Negevirus		
158	Novi Sad IV (Kamenjar)	Novi Sad	27.08.2014	*Ae. vexans*	4		Negevirus	Alphamesonivirus	
159	Čurug	Žabalj	08.07.2014	*Cx. pipiens.*	50		Negevirus		
162	Čurug	Žabalj	08.07.2014	*Cx. pipiens*	50	Daeseongdong virus	Negevirus		
165	Kisač	Novi Sad	27.08.2014	*Oc. caspius*	7		Negevirus		
182	Elemir	Zrenjanin	29.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus	
191	Begeč II (shoreline)	Novi Sad	27.08.2014	*An. maculipennis*	3		Negevirus		
196	Begeč II (shoreline)	Novi Sad	27.08.2014	*Ae. vexans*	50	Daeseongdong virus	Negevirus		
197	Begeč II (shoreline)	Novi Sad	27.08.2014	*Ae. vexans*	50			Alphamesonivirus	
200	Begeč II (shoreline)	Novi Sad	27.08.2014	*Ae. vexans*	50			Alphamesonivirus	
201	Hajdukovo	Subotica	02.08.2014	*Cx. pipiens*	17		Negevirus		
202	Subotica I (pull)	Subotica	28.06.2014	*Cu. annulata*	1		Negevirus		
204	Subotica I (pull)	Subotica	28.06.2014	*Cx. pipiens*	50		Negevirus		
208	Subotica I (pull)	Subotica	28.06.2014	*Cx. pipiens*	5		Negevirus	Alphamesonivirus	
214	Subotica III (Makova 7)	Subotica	02.08.2014	*Cx. pipiens*	29		Negevirus	Alphamesonivirus	
216	Subotica IV (Palic)	Subotica	02.08.2014	*Cx. pipiens*	50		Negevirus		
217	Subotica IV (Palic)	Subotica	02.08.2014	*Ae. vexans*	4		Negevirus	Alphamesonivirus	

Sample ID	Locality	Municipality	Collection date	mosq_species	number/pool	Identified virus		
220	Subotica IV (Palic)	Subotica	02.08.2014	*Cx. pipiens*	19		Negevirus	
221	Novi Sad XI (Klisa)	Novi Sad	10.07.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus
222	Novi Sad XI (Klisa)	Novi Sad	10.07.2014	*Cx. pipiens*	24		Negevirus	Alphamesonivirus
223	Novi Sad XI (Klisa)	Novi Sad	10.07.2014	*Ae. vexans*	1		Negevirus	Alphamesonivirus
226	Farkaždin	Zrenjanin	29.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus
227	Farkaždin	Zrenjanin	29.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus
228	Farkaždin	Zrenjanin	28.08.2014	*Oc. caspius*	2		Negevirus	
229	Farkaždin	Zrenjanin	29.08.2014	*Cx. pipiens*	7		Negevirus	
232	Novi Sad VIII (Avijatičarsko Naselje)	Novi Sad	25.06.2014	*Ae. vexans*	10		Negevirus	Alphamesonivirus
233	Tomaševac	Zrenjanin	12.08.2014	*Oc. sticticus*	2		Negevirus	Alphamesonivirus
234	Tomaševac	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus
239	Tomaševac	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus	
254	Knićanin	Zrenjanin	12.08.2014	*Ae. vexans*	50	Daeseongdong virus	Negevirus	
264	Begeč I	Novi Sad	08.10.2014	*Ae. vexans*	50			Alphamesonivirus
267	Begeč I	Novi Sad	08.10.2014	*Ae. vexans*	50		Negevirus	
280	Begeč I	Novi Sad	08.10.2014	*Ae. vexans*	26		Negevirus	Alphamesonivirus
285	Aradac	Zrenjanin	29.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus
286	Aradac	Zrenjanin	29.08.2014	*Cx. pipiens*	36		Negevirus	Alphamesonivirus
292	Vrbas	Vrbas	25.07.2014	*Cx. pipiens*	50			Alphamesonivirus
293	Vrbas	Vrbas	25.07.2014	*Oc. caspius*	2		Negevirus	
305	Ečka	Zrenjanin	12.08.2014	*Oc. caspius*	17		Negevirus	Alphamesonivirus
307	Ečka	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus
311	Ečka	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus
313	Ečka	Zrenjanin	12.08.2014	*Ae. vexans*	50			Alphamesonivirus
316	Ečka	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus
343	Klek	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus

Sample ID	Locality	Municipality	Collection date	mosq_species	number/pool	Identified virus			
347	Klek	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus	
352	Klek	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus	
355	Orlovat	Zrenjanin	29.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus	
362	Orlovat	Zrenjanin	29.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus	
366	Orlovat	Zrenjanin	29.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus	
372	Farkaždin	Zrenjanin	12.08.2014	*Ae. vexans*	50		Negevirus		
374	Farkaždin	Zrenjanin	12.08.2014	*Oc. caspius*	50		Negevirus		Marisma mosquito virus
375	Farkaždin	Zrenjanin	12.08.2014	*Cx. pipiens*	50			Alphamesonivirus	
385	Farkaždin	Zrenjanin	12.08.2014	*Ae. vexans*	50	Daeseongdong virus			
389	Farkaždin	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus		
392	Farkaždin	Zrenjanin	12.08.2014	*Cx. pipiens*	6		Negevirus		
394	Apatin	Apatin	26.06.2014	*Oc. sticticus*	21		Negevirus		
395	Apatin	Apatin	26.06.2014	*Ae. vexans*	50		Negevirus		
398	Apatin	Apatin	26.06.2014	*Ae. vexans*	50		Negevirus	Alphamesonivirus	
399	Apatin	Apatin	26.06.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus	
400	Apatin	Apatin	26.06.2014	*Cx. pipiens*	50	Daeseongdong virus	Negevirus		
430	Klek	Zrenjanin	12.08.2014	*Cx. pipiens*	50				West Nile virus
444	Klek	Zrenjanin	12.08.2014	*Cx. pipiens*	50		Negevirus	Alphamesonivirus	

6. Summary

During this doctoral thesis work, we identified the $NS3_{249}P$ substitution in Serbian mosquito samples for the first time. Our results could serve as an explanation for the increased number of WNND cases in the country in 2012-2013. Furthermore, phylogenetic analyses of complete coding and partial NS3 regions revealed close relationships with different European strains suggesting multiple introduction events into the country.

Based on our sequence and phylogenetic data, the situation of 2018 was more likely caused by endemic strains rather than recently introduced novel WNV strains. Therefore, we hypothesize that the main trigger factors behind the outstanding case numbers during 2018 were likely the result of favourable environmental conditions for mosquito vectors and the increased contact of these mosquitoes with native animal and human populations.

The results of USUV surveillance represent the first evidence for the geographic expansion of European lineage 1 to southern territories in the Balkanian peninsula and provide the first genetic data of USUV in the region.

In case of Anopheles flavivirus, we described a potential novel member within the group of ISFVs. However, the relatively short fragment analysed in this work is not sufficient to make long-term or even final conclusions therefore, further experiments and field screening of *An. hyrcanus* are needed to clarify the presence and exact position of this tentatively novel member within the Flavivirus genus.

It is also important to emphasize the limitations of our studies. Many viruses could not be isolated by *in vitro* methods, so comprehensive functional and genomic studies could not be performed with them. Furthermore, Hungary lacks a comprehensive and multi-level surveillance system that would enable complex investigations and draw reliable conclusions, creating an adequate basis for preparing for future epidemic periods.

Furthermore, we demonstrated the possibility of establishing local mosquito-derived virus collections to facilitate future co-infection and interference studies. Our results provide pillars for more detailed genetic and evolutionary analyses of our insect-specific virus isolates to support further evolutionary, vector competence, vaccine-, and diagnostic platform developments in the future. Regarding the complexity of mosquito-virus interactome, such works will represent the first step for applied arbovirus surveillance studies.

Vector surveillance is the key and primary pillar to understanding and preventing the spread of vector-borne pathogens (Fourniol et al., 2021). During the past decades, besides

conventional PCR screening of mosquitoes as a gold standard methodology (Birnberg et al., 2020), metagenomic analyses, and honey-baited FTA cards came into prominence (Birnberg et al., 2020; Atoni et al., 2018; Brinkmann et al., 2016; Coffey et al., 2014; Shi et al., 2015; Sadeghi et al., 2018; Agboli et al., 2019). These new techniques provide information not only about the presence of viruses but more comprehensive and detailed information about the mosquito virome and the genome of unknown and known viruses (Guzman et al., 2018; Sadeghi et al., 2018; Atoni et al., 2019). Furthermore, FTA cards over and above *in vitro* isolation and metagenomics may provide more reliable information than PCR screening about mosquito virome since these methods eliminate the anomalies caused by integrated pathogen sequences into the mosquito genome (Blitvich and Firth, 2015). Moreover, available virus isolates, from in vitro surveillance attempts, provide endless opportunities for both detailed knowledges of genetic information through complete genome sequencing and other omic techniques and a source for further functional experiments (Atoni et al., 2019; Carvalho et al., 2021; Guzman et al., 2018). Despite the technological advances and growing discovery of ISVs our knowledge about them is still limited. Huge gaps are generated because of the lack of *in vitro* isolation of newly discovered viruses and, the surveillance efforts are still mainly driven by human epidemics, which hampers comprehensive and systematic surveillance efforts. The priority of arboviral outbreaks generates further gaps in viral taxonomy, vector competence studies, vector geography and in vector taxonomy. All these factors, as well as the lack of virus interaction and vector competence studies, cause information diversion in the case of most virus-mosquito pairs, which hinders the network-level understanding of the arbovirus community. In addition, the conflicting results of vector competence studies suggest that the origin and species of mosquitoes, as well as the origin of the tested viruses, may play an important role, highlighting the importance of including local mosquito communities and their viruses in virus interaction and vector competence studies (Carvalho et al., 2021; Chen et al., 2022; Bolling et al., 2015).

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9. List of Publications

9.1. Publications beyond thesis topic

Zana B, Erdélyi K, Nagy A, et al. Multi-Approach Investigation Regarding the West Nile Virus Situation in Hungary, 2018. Viruses. 2020;12(1):123. Published 2020 Jan 20. doi:10.3390/v12010123

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Zana B, Kemenesi G, Antal L, et al. Molecular traces of a putative novel insect flavivirus from Anopheles hyrcanus mosquito species in Hungary. Acta Virol. 2017;61(1):127-129. doi:10.4149/av_2017_01_127

9.2 Oral and poster presentations beyond thesis topic

Zana B, Kemenesi G, Buzás D, Kurucz K, Krtinic B, Kepner A, Földes F and Jakab F: First genetic characterization of Usutu virus from Culex pipiens mosquitoes Serbia, 2014. 5th International Congress on INFECTIOUS DISEASES; Berlin, Germany, March 01-02, 2018

Zana B, Buzás D, Földes F, Kemenesi G, Urbán P, Kurucz K, Jakab F. In vitro Survey Of Mosquito-related Viruses On C6/36 Aedes albpictus Cell Line. International Meeting on Emerging Diseases and Surveillance (IMED 2018), November 9-12., 2018, Bécs, Ausztria (poster)

9.3 Publications outside thesis topic

Bányai, K., Kemenesi, G., Budinski, I., Földes, F., **Zana, B**., Marton, S., ... & Jakab, F. (2017). Candidate new rotavirus species in Schreiber's bats, Serbia. Infection, Genetics and Evolution, 48, 19-26.

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