West Nile virus circulation in south-eastern Romania, 2011 to 2013

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Lineage 2 West Nile virus (WNV), previously found only in sub-Saharan Africa and Madagascar, was identified in Hungary in 2004 and has rapidly expanded in Europe in the past decade. Following a significant outbreak of West Nile fever with neurological cases caused by lineage 1 WNV in Romania in 1996, scattered cases have been recorded in the south-east of the country in each transmission season. Another outbreak, affecting a larger area and caused by lineage 2 WNV, was recorded in 2010. We analysed human sera from neuroinvasive West Nile fever cases and mosquitoes, sampled in south-eastern Romania between 2011 and 2013, for the presence of WNV genome, and obtained partial NS5 and envelope glycoprotein sequences. Human- and mosquito-derived WNV sequences were highly similar (99%) to Volgograd 2007 lineage 2 WNV and differed from isolates previously detected in central and southern Europe. WNV was detected in one pool of *Culex pipiens* s.l. males, documenting vertical transmission. Lineage 4 WNV, of unknown pathogenicity to mammals, was found in the amphibian-feeding mosquito *Uranotaenia unguiculata* from the Danube Delta. Our results present molecular evidence for the maintenance of the same isolates of Volgograd 2007-like lineage 2 WNV in south-eastern Romania between 2011 and 2013.

Introduction

WNV is by far the most widely distributed arbovirus. It belongs to the Japanese encephalitis antigenic complex of the family *Flaviviridae*, transmitted in an avian cycle by ornithophilic mosquitoes, mainly of the genus *Culex*. Mammals can also be infected, but are considered dead-end hosts because viraemia is generally too low to infect mosquitoes. Eighty per cent of human infections are asymptomatic, and less than 1% of clinical cases lead to neuroinvasive disease [1,2].

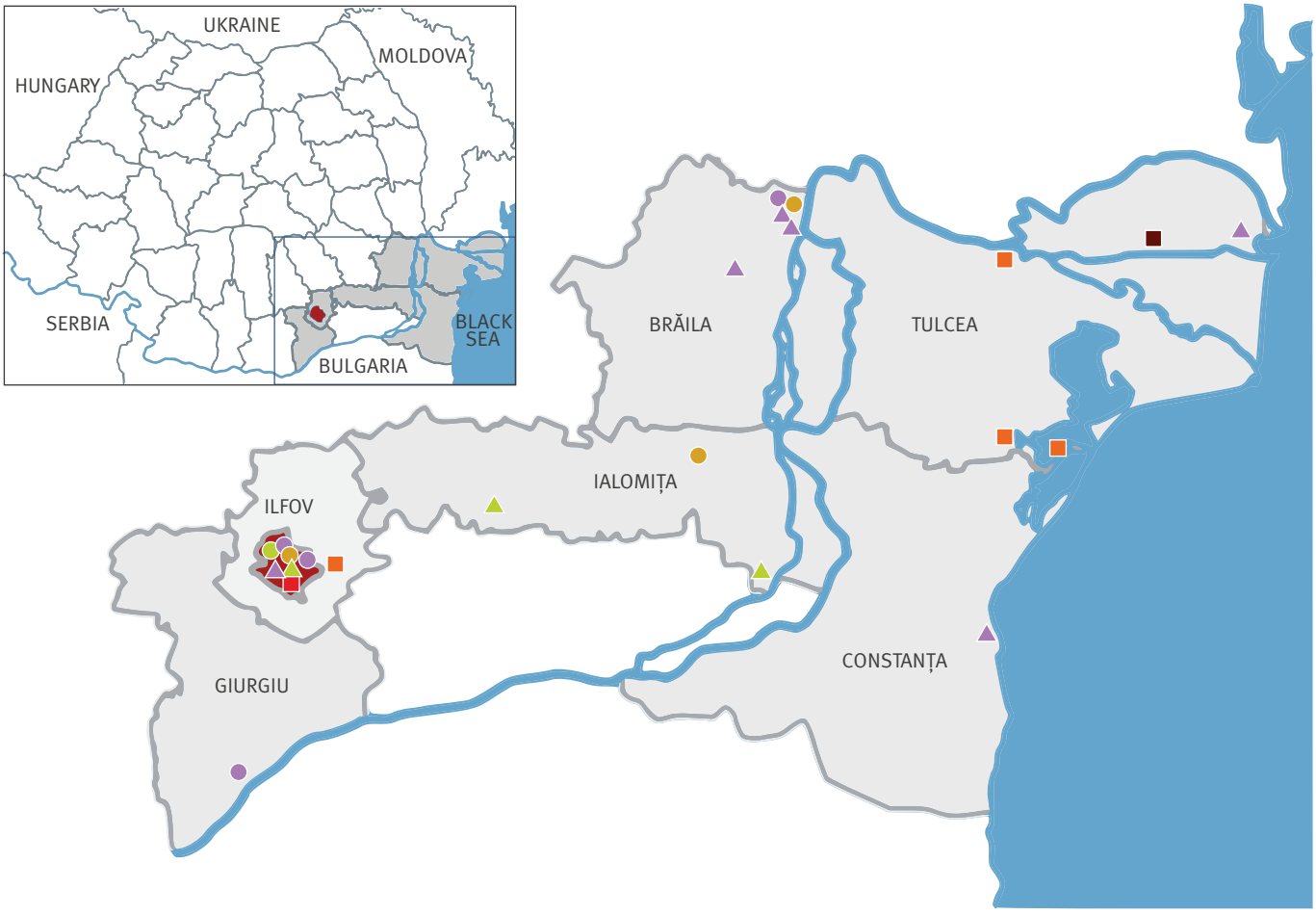
Evidence of WNV circulation in Europe dates back to the early 1960s [3], lineage 1 isolates being responsible for the major outbreaks. A recent retrospective study indicates the circulation of lineage 2 WNV in birds (*Sylvia nisoria*) in Cyprus as early as 1968 [4]. Starting in 2004, lineage 2 WNV was identified in Hungary in birds of prey and was subsequently found again in Hungary in 2005 and in Austria in 2008 and 2009 [5-7]. Erroneously considered to be non-pathogenic for humans and with a distribution restricted only to sub-Saharan Africa and Madagascar [8], lineage 2 isolates caused outbreaks of WNV infection in Russia (2007), Greece (2010–2013), Romania (2010), Italy (2011, 2012), Serbia (2012, 2013) and Croatia (2012, 2013) [9-21].

Following the unprecedented epidemic of West Nile fever in south-eastern Romania in 1996 [22], caused by a lineage 1 strain [23], scattered human cases were recorded every year until a second significant outbreak occurred in 2010 [14]. In the latter, the affected area also included counties in the north-east of the country as well as in Transylvania, beyond the Carpathian mountains. The WNV detected in one human serum was lineage 2, closely similar to an isolate from a patient during the outbreak in Volgograd, Russia in 2007 [14].

According to the data provided by the National Institute of Public Health, laboratory-based surveillance of neuroinvasive West Nile infection was carried out in 2012 as previously described [14], and detected one probable and 14 confirmed cases among 128 suspected cases. The majority of West Nile fever cases were recorded in south-eastern Romania: seven in Bucharest

Figure 1

Distribution of confirmed human cases of West Nile fever selected for molecular investigation and mosquito collection sites by county, Romania, 2011–2013



WNV: West Nile virus.
Counties shown at NUTS 3 level. Bucharest city is highlighted in russet.

city, two (one probable and one confirmed case) in the adjacent Ilfov county and in Ialomița (one case), Brăila (two cases), and Giurgiu (two cases) counties. A single case was also recorded in Iaşi, north-eastern Romania. Dates of onset were from 31 July to 11 September 2012. One death was recorded. In 2013, 22 confirmed and two probable cases were recorded among 142 suspected cases tested. Dates of onset were from 17 July to 19 September 2013. No deaths were reported (source: National Centre for Surveillance and Control of Communicable Diseases, National Institute of Public Health). The majority of West Nile fever cases in that year also occurred in south-eastern Romania: Ialomița (four confirmed cases), Brăila (one probable and four confirmed cases), Tulcea (one probable case and two confirmed cases), Constanța (two confirmed cases), Bucharest city (one confirmed case) and the adjacent Ilfov county (one confirmed case). Another five cases were recorded in the north-eastern part of the country: Iași (two confirmed cases), Galați (two confirmed cases) and Bacău (one confirmed case). Bacău county is a new affected area. Three cases were recorded in

Table 1

Mosquito pools tested for West Nile virus genome, Romania, 2011–2013

Collection area	2011 pools	2012 pools	2013 pools
	Tested/real-time RT-PCR-positive/sequenced		
Tulcea county	95/3/2	388/70/10	508/109/16
Bucharest and surroundings	9/0/0	87/5/1	98/2/1
Total number of mosquito pools/total number of mosquitoes	**104/3,291**	**475/12,159**	**606/15,405**

the central part of the country (Mureş and Sibiu counties). Sera from these three cases were also tested for the presence of tick-borne encephalitis virus-specific antibodies because this flavivirus had previously been found to be circulating in this area.

Here we present the molecular characterisation of WNV circulating between 2011 and 2013 in humans and mosquitoes in south-eastern Romania, an area of endemic WNV circulation as shown by previous human and animal host surveillance studies [24,25].

Methods

Human cases

Sera collected in 2012 and 2013 from patients with confirmed WNV neuroinvasive infection living in south-eastern Romania were included in this study (Figure 1). Only samples collected in the first seven days post onset were selected for molecular investigation. Viral RNA was extracted from sera using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany).

Mosquito collection and processing

Adult mosquitoes were collected from two main areas: Tulcea county and Ilfov county including Bucharest. Four sites were investigated in Tulcea county: Mila 26 (in the core of the Danube Delta), Sălcioara and Grindul Lupilor (on the Razim lagoon shore) and Tulcea city (Figure 1). In Tulcea county, mosquitoes were collected by overnight capture in cylindrical traps baited with birds (chickens) and small rodents (guinea pigs) or collected from vegetation with a backpack aspirator. In the Danube Delta and the lagoon shore, collections were performed for periods of five to eight days in August and September 2011 and from May to October in 2012 and 2013. In Bucharest and the periurban area of the city, mosquito captures were performed between July and September (2011–13) using CDC Gravid Traps [26] and BG Sentinel Traps (Biogents AG, Germany). Mosquitoes were also collected from resting sites such as hallways of buildings using hand aspirator.

For RNA extraction (QIAamp Viral RNA Mini Kit, Qiagen, Hilden, Germany), the mosquitoes were processed as follows: they were identified using an entomological key [27], and pooled by species, sex and physiological age in pools never exceeding 50 individuals.

Molecular analysis

All samples (mosquito pools and human sera) were screened for the presence of WNV genome by one-step real-time RT-PCR using a commercial kit (West Nile Virus Real-TM, Sacace Biotechnologies). Positive samples were further tested by RT-PCR using primers VD8, FU2, cFD3 and WNV9368f, targeting the NS5 genomic region [5,28,29]. When possible, a fragment spanning the envelope glycoprotein region (E) was amplified using primers WNVII 870f and WNVII 1630r [5]. All amplicons were sequenced (3130 Genetic Analyzer, Applied Biosystems) and the resulting sequences were aligned with ClustalW, BioEdit version 7.0.5.3 [30]. Maximum-likelihood phylogenetic analysis was conducted with Mega 6 software [31], which was also used for choosing the fittest nucleotide substitution model. The reliability of the phylogenetic trees was tested with 1,000 bootstrap replicates.

Results

Human samples

In 2012, serum samples from eight patients with serologically confirmed WNV infection, all in the acute phase of the disease, were tested by real-time RT-PCR.

Table 2

Mosquito species found to be positive for West Nile virus by sequencing and real-time RT-PCR, Romania, 2011–2013

Mosquito species	2011 pools	2012 pools	2013 pools
	Sequenced/real-time RT-PCR-positive/pools tested		
Culex pipiens s.l.	2[a]/2/68	5[a]/47[b]/343	13[a]/51/369
Culex modestus	0/1/21	3[a]/15/56	0/46/131
Coquillettidia richiardii	NA	0/5/30	2[a]/8/54
Anopheles hyrcanus	NA	1[a]/4/23	0/3/21
Uranotaenia unguiculata	NA	2[c]/3/4	2[c]/2/4
Ochlerotatus caspius	NA	0/1/10	NA
Anopheles maculipennis complex	NA	NA	0/1/18

NA: not applicable.

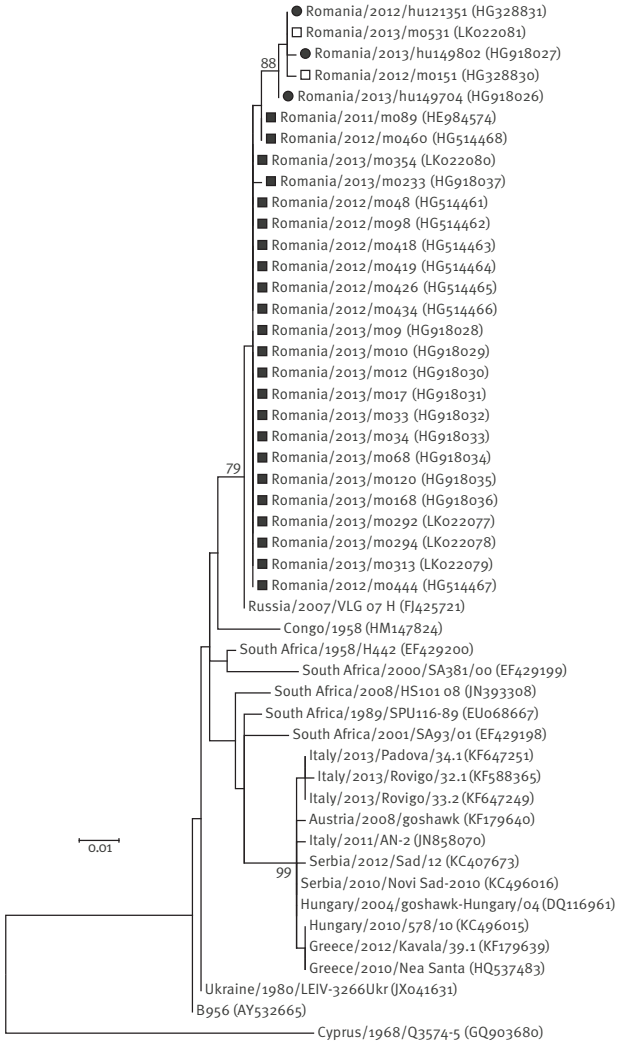
[a] West Nile virus lineage 2.

[b] 46 pools of females and one pool of males.

[c] West Nile virus lineage 4

Figure 2

Phylogenetic tree of lineage 2 West Nile viruses based on NS5 partial sequences, Romania, 2011–2013



Black squares: sequences obtained in this study from mosquitoes collected in Danube Delta (Mila 26); white squares: sequences obtained in this study from mosquitoes collected in Bucharest city; black circles: sequences obtained in this study from human sera. Numbers at nodes represent the bootstrap percentages (values<70% are not shown).

The analysis was conducted on a 466 nt sequence (nt positions 9,463–9,928 in isolate Reb_VLG_07_H, GenBank acc. no. FJ425721) using maximum-likelihood method, Kimura 2-parameter model, 1,000 bootstrap replicates. A sequence obtained from strain Q3574–5 (Cyprus, 1968; GenBank acc. no. GQ903680) was used as an outgroup.

All patients lived in south-eastern Romania: Bucharest city (four cases) and the counties of Brăila (two cases), Giurgiu (one case) and Ialomița (one case). WNV genome was detected by real-time RT-PCR (Ct values: 28.1–34.4) in four of these samples: Bucharest city (two cases), Brăila (one case) and Ialomița (one case) (Figure 1).

In 2013, nine serum samples fulfilled the inclusion criteria: Bucharest city (two cases) and Brăila (three cases), Constanța (one case), Ialomița (two cases), Tulcea (one case) counties. WNV genome was found in the samples from one patient living in Bucharest city and two from Ialomița county (Ct values: 29.85–33.65) (Figure 1).

In summary, during the two years of investigation, we detected seven positive serum samples in the PCR screening assay. Only one sample from 2012 and two from 2013 yielded an NS5 amplicon suitable for obtaining a DNA sequence. None of the above samples yielded an E amplicon suitable for sequencing.

Mosquito samples

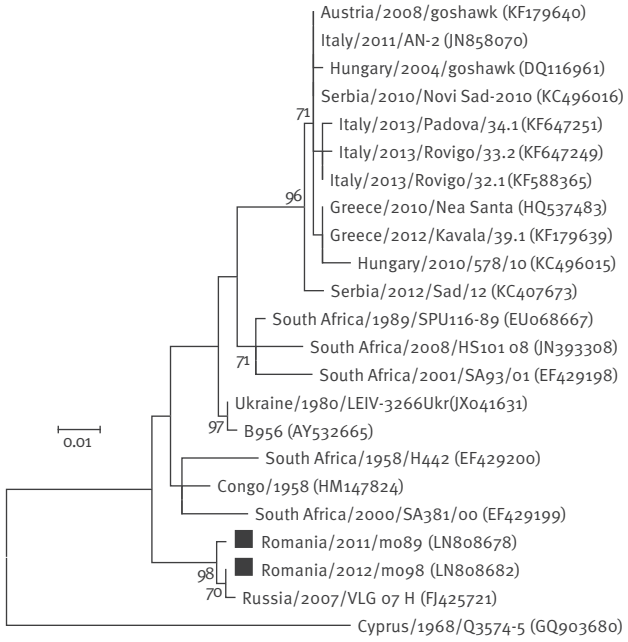
In 2011 to 2013, we collected and analysed 30,855 mosquitoes. About 75% of these insects were captured in the Danube Delta, and 189 of the 1,185 mosquito pools tested were real-time RT-PCR-positive for WNV genome (Table 1). As expected, the majority of these pools consisted of *Culex pipiens* s.l., followed by *Cx. modestus*. Other mosquito species were also found real-time RT-PCR-positive for WNV genome (Table 2). Of interest was the detection of WNV genome in a pool of *Cx. pipiens* s.l. males collected in 2012 in Bucharest. Unfortunately, no amplicon for sequencing could be obtained from this sample. During the three years of the study, we obtained 30 DNA sequences for WNV NS5 derived from mosquito pools containing *Cx. pipiens* s.l., *Cx. modestus*, *Anopheles hyrcanus*, *Coquillettidia richiardii* and *Uranotaenia unguiculata* species (Table 2). Two partial E sequences were also obtained from *Cx. pipiens* s.l. mosquitoes collected in the Danube Delta in 2011 and 2012.

In 2012 and 2013, we identified the WNV lineage 2 genome in sera collected from three patients with meningoencephalitis living in south-eastern Romania. The first detection was in August 2012 in the acute phase serum of a resident of Bucharest. In September 2013, similar WNV isolates were found in a patient living in Bucharest and in a resident of Ialomița county.

Our phylogenetic analysis based on NS5 partial sequences (Figure 2) indicated that the viruses in circulation in Romania between 2011 and 2013 were very similar to a lineage 2 WNV isolated during the outbreak in Volgograd, Russia, in 2007; all our mosquito and human-derived sequences were more than 99% similar to that isolate. The same strain of lineage 2 WNV, 99% identical to Volgograd 2007, has been circulating in Bucharest since 2010 [14]. Although the investigated genomic region is strongly conserved, a bootstrap value of 79% from the node relating the Romanian sequences to the Volgograd 2007 isolate indicated the robustness of the analysis. It is worth mentioning that the sequences obtained from human specimens clustered with two sequences derived from two pools of *Cx. pipiens* s.l. females collected in 2012 and 2013 in Bucharest area. All sequences derived from mosquitoes collected in the same ecosystem (Danube Delta, Mila 26 collecting site), regardless of the year of collection, clustered in a single subclade comprised of three

Figure 3

Phylogenetic tree of lineage 2 West Nile viruses based on envelope glycoprotein (E) sequences, Romania, 2011–2012



Black squares: sequences obtained in this study from mosquitoes collected in Danube Delta (Mila 26). Numbers at nodes represent the bootstrap percentages (values<70% are not shown).

The analysis was conducted on a 460 nt sequence (positions 934–1,393 in isolate Reb_VLG_07_H, GenBank acc. no. FJ425721) using maximum-likelihood method, Tamura-Nei model, 1,000 bootstrap replicates. A sequence obtained from strain Q3574–5 (Cyprus, 1968; GenBank acc. no. GQ903680) was used as an outgroup.

major groups that differed by a small number of synonymous and non-synonymous nucleotide substitutions.

Furthermore, the phylogenetic analysis based on E partial sequences confirmed the topology of the NS5 tree, placing the Romanian sequences in the same clade with the isolate obtained in 2007 in Volgograd, Russia (Figure 3).

An interesting finding was the detection of lineage 4 WNV in four pools of *Ur. unguiculata* mosquito collected in 2012 and 2013 (Figure 4). Similar strains have already been reported by Russian authors from this mosquito species, known to feed on amphibians, and from frogs collected in Volga Delta, as well as from *Dermacentor marginatus* ticks collected in the Caucasus [32].

NS5 and E partial sequences described in this study are available in GenBank under the following accession numbers: HE984574, HE984575, HG328830, HG328831, HG514461-HG514468,HG918026-HG918037, LK022077-LK022085, LN808678 and LN808682.

Discussion

As shown by sequencing, Volgograd 2007-like lineage 2 WNV isolates were found both in patients with neurological WNV infections (2012 and 2013) and in mosquito vectors (2011–13) in south-eastern Romania.

In 2011, Volgograd 2007-like isolates were detected in two pools of *Cx. pipiens* s.l. mosquitoes collected from the Danube Delta. In 2012, in this area, the same virus was found in nine mosquito pools consisting of specimens belonging to three species: *Cx. pipiens* s.l. (five pools) and *Cx. modestus* (three pools) mosquitoes, already known as WNV principal vectors in Europe [23,33], but also in *An. hyrcanus* (one pool). The results for 2013 in the Danube Delta indicated the presence of the same lineage 2 WNV in 15 mosquito pools belonging to two species: *Cx. pipiens* s.l. (13 pools) and *Cq. richiardii* (two pools). Also, in 2012 and 2013, we detected Volgograd 2007-like isolates in two pools of *Cx. pipiens* s.l. mosquitoes collected in Bucharest city. In Europe, WNV has been previously detected in *Cq. richiardii* and *An. hyrcanus* in the Volga Delta [32].

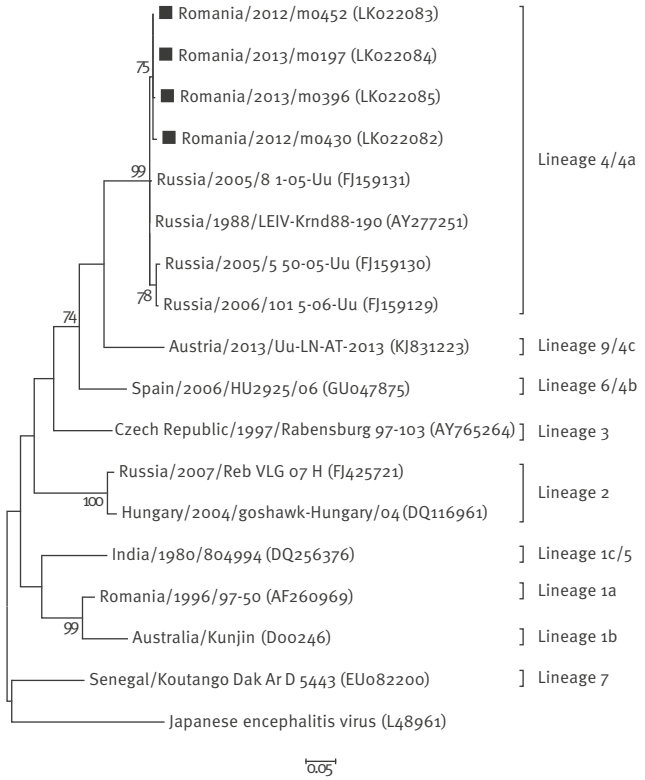
WNV genome was also detected in one pool of *Cx. pipiens* s.l. males collected in 2012 in Bucharest city. To our knowledge, this is the first direct field evidence of vertical transmission of WNV in Europe. WNV has previously been detected in *Culex* spp. males in Kenya [34] and in North America [35,36]. Vertical transmission has also been documented in overwintering *Culex* spp. females [37] and is thought to represent a mechanism for WNV maintenance [38]. The persistence of WNV in a temperate climate may be achieved by overwintering of infected arthropod vectors and by long–term infection in birds [39], and may explain endemic WNV circulation. Indeed, maintenance of this WNV lineage 2 Volgograd-like strain in the same area for three years has been documented by us.

Initially considered to be non-pathogenic for humans [40], lineage 2 WNV was detected in Europe in 2004, in a goshawk (*Accipiter gentilis*) in south-eastern Hungary. It then became established and caused sporadic cases of infection in wild birds, sheep, horses and humans [5,41]. In the following years, similar isolates were detected in birds of prey in eastern Austria [7] and in *Culex* sp. mosquitoes and collared doves (*Streptopelia decaocto*) in Italy [42]. During the period from 2010 to 2013, lineage 2 WNV isolates similar to those in central Europe caused major outbreaks in Greece [10-13]. The Greek isolates from 2010 and 2012 had unique amino acid substitutions (V119I in NS2B; H249P in NS3; S14G, T49A and V113M in NS4) compared with the isolates from Hungary and Austria, which might explain their high pathogenicity [43].

Although there is a high degree of similarity (96%) between our sequences and those obtained from other isolates circulating in central and southern Europe in recent years, our sequences clearly clustered with the Volgograd 2007 isolate. This distribution of a distinct strain of lineage 2 WNV in eastern Europe may be related to the Mediterranean/Black Sea flyway of northward migrating birds, as it is documented that

Figure 4

Phylogenetic tree of lineage 4 West Nile viruses based on NS5 partial sequences, Romania, 2012–2013



Black squares: sequences obtained in this study from mosquitoes collected in Danube Delta (Mila 26). Numbers at nodes represent the bootstrap percentages (values<70% are not shown).

Analysis was conducted on a 365 nt sequence (positions 9,479–9,843, in isolate LEIV-Krnd88–190, GenBank acc. no. AY277251) using maximum-likelihood method, Kimura 2-parameter model, 1,000 bootstrap replicates. A sequence obtained from a Japanese encephalitis virus isolate (GenBank acc. no. L48961) was used as an outgroup.

Lineages were defined as previously proposed [48].

birds play a crucial role in the spread of the virus [44]. The Volga and Danube deltas are also connected by autumn migration, and there is evidence that WNV may be introduced as birds travel to their owerwintering sites in Africa [45]. Our findings, together with other published data suggest that at least two independent introduction events of two different lineage 2 WNV strains occurred in Europe, followed by their subsequent endemisation.

The presence of WNV lineage 4 identified in two consecutive years (2012–13) in four pools of *Ur. unguiculata* mosquitoes captured in the Danube Delta is worth mentioning. Studies between 2002 and 2006 in the Volga Delta, an ecosystem similar to that of the Danube Delta, demonstrated the presence of WNV lineage 4 in *Ur. unguiculata* and in the lake frog *Rana ridibunda* [46]. Lineage 4 (isolate LEIV-Krnd88–190) was first identified in 1988 in *Dermacentor marginatus* ticks collected in the north-west Caucasus [32]. Attempts to propagate the virus in suckling mice or in mammalian or mosquito cell lines have failed. The strains of this lineage seem to be associated with arthropods and amphibians and their pathogenicity for vertebrates is not characterised [46]. Recent studies conducted in Spain and Austria [47,48] reported two WNV strains of unknown pathogenicity which can be assigned to new lineages closely related to lineage 4. The Spanish WNV was found in *Cx. pipiens* mosquitoes, while the Austrian virus was detected in *Ur. unguiculata* mosquitoes. It has been proposed based on NS5 partial sequences that WNV isolates previously found in *Ur. unguiculata* mosquitoes and *Dermacentor marginatus* ticks from Russia should be grouped in a clade designated lineage 4 or 4a, while the sequence from Spain should be classified in lineage 4b or 6, and the WNV sequence identified in mosquitoes from Austria should be comprised in lineage 4c or 9 [48]. As shown in Figure 4, WNV sequences derived from *Ur. unguiculata* mosquitoes collected in Romania clearly cluster in the proposed lineage 4/4a, along with the Russian isolates. It has been speculated that less or non-pathogenic WNV strains may infect birds, conferring them immunity, thus limiting the spread of pathogenic strains [47].

Conclusion

The neurovirulent strain Volgograd 2007-like of lineage 2 WNV has been circulating in Romania in mosquito populations and causing disease in humans since at least 2010, as shown by previous [14] and present findings. The distribution of this strain may be linked to the flyways connecting Africa to eastern Europe and the Danube and Volga deltas, followed by virus maintenance and endemic circulation in the region.

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Conflict of interest

None declared.

Authors' contributions

SD, IRPG and GO set up and performed molecular diagnostic, sequencing and phylogenetic analysis. IRPG, DB and CSC performed serological diagnosis of human cases. AIC and IRPG performed molecular detection tests in mosquitoes pools. AS managed West Nile fever surveillance program. FLP and EF performed field collection of mosquitoes, their identification and processing for molecular analyses. SD and CSC wrote the paper. PR coordinated the entomological work and provided a critical review of the manuscript.

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