

# West Nile virus lineage 2 isolated from *Culex modestus* mosquitoes in the Czech Republic, 2013: expansion of the European WNV endemic area to the North?

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**We report the detection and isolation of four almost identical strains of West Nile virus (WNV) lineage 2 from *Culex modestus* mosquitoes collected at three fish ponds in South Moravia, Czech Republic, during August 2013. Phylogenetic analysis demonstrated that the Czech WNV strains isolated are closely related to Austrian, Italian and Serbian strains reported in 2008, 2011 and 2012, respectively. Our findings show the current northernmost range of lineage 2 WNV in Europe.**

In South Moravia in the Czech Republic, surveillance activities for mosquitoes and mosquito-borne pathogens have been carried out for several decades, but until our findings in 2013 presented here, WNV lineage 2 (WNV-2) had not been detected.

## Background

WNV is a mosquito-borne virus (genus *Flavivirus*; family *Flaviviridae*) that is widely distributed in Africa, the Middle East, Asia and southern Europe [1] and was recently introduced in the Americas [2]. WNV circulates in natural foci between birds (as amplifying hosts) and bird-feeding mosquitoes, in Europe principally *Culex pipiens* and *Cx. modestus* [3]. Humans and horses are considered accidental dead-end hosts. Most individuals infected with WNV are asymptomatic. Symptoms may develop in 20–40% of people with WNV infection, most frequently characterised as influenza-like symptoms, (West Nile fever (WNF)). Less than 1% of infected individuals develop severe neuroinvasive disease, which can be classified into three main clinical syndromes: West Nile meningitis, West Nile encephalitis and acute flaccid paralysis [4].

Several human and/or equine WNF outbreaks have occurred in the last decades in Europe, for example, in Romania (1996), Italy (1998) and Russia (1999) [1].

From 2008 onwards, an unexpected explosive spread of WNV-2, which resulted in several hundreds of human neuroinvasive cases, has been documented in Hungary, Greece and Serbia [5-7].

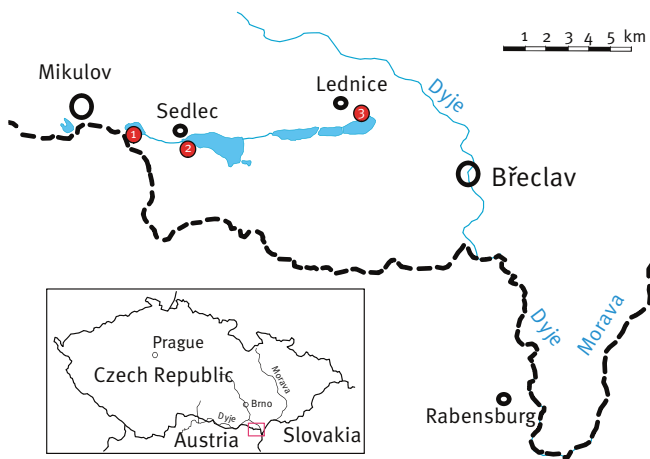
In the Czech Republic, three identical strains of WNV (proposed genomic lineage 3: Rabensburg) were isolated from *Cx. pipiens* and *Aedes rossicus* mosquitoes in 1997, 1999 and 2006 [8,9]. Although neutralising antibodies against WNV have been found rarely in humans in the Czech Republic, two confirmed cases of WNF in humans were reported after heavy floods in 1997 [10]. In addition, WNV-specific antibodies have been detected in resident wild bird species [11]. The above rare traces of WNV infections in the Czech Republic before 2008 were most likely due to WNV lineage 1. Sera collected from 163 horses, originating from 43 out of 77 administrative districts of the Czech Republic between 2008 and 2011, all proved negative for WNV antibodies [12]. Because of the rapidly changing epidemiological situation regarding WNF in Europe, we decided to perform virological surveillance of mosquitoes for WNV and related pathogenic flaviviruses (e.g. Usutu virus) to investigate the epidemiological relevance of WNF in the Czech Republic.

## Study site

In this study, mosquitoes were collected within reed belts (*Phragmites communis* alliance) of the fish ponds 'Nesyt' (48 ° 46'35"N, 16 ° 42'05"E; 176 m above sea level (a.s.l.)) and 'Nový' (48 ° 46'57"N, 16 ° 40'13"E; 177 m a.s.l.) at Mikulov, and the fish pond 'Mlýnský' at Lednice (48 ° 47'19"N, 16 ° 49'2"E; 175 m a.s.l.) during July and August 2013 (Figure 1). The climate at the ponds is relatively warm and dry: the mean annual air temperature is 9.1 °C (January -1.8 °C, July 19.2 °C); the mean annual precipitation is 571 mm

**FIGURE 1**

Locations of three study sites for *Culex modestus* trapping, South Moravia, Czech Republic, July–August 2013



Fish ponds:

- 1 Nový
- 2 Nesyť
- 3 Mlýnský

(range: 284–919 mm) (data purchased from the Czech Hydrometeorological Institute). A total of 30 species of birds have been recorded breeding in the reed belts; 51 other avian species breed in the close surroundings of the ponds and an additional 54 wild wetland and terrestrial bird species visit this habitat during their seasonal movements. Mosquitoes in South Moravia comprise 30 species of the genera *Anopheles*, *Aedes*, *Ochlerotatus*, *Culex*, *Culiseta*, *Coquillettidia* and *Uranotaenia* [13].

### Mosquito collection, molecular screening and virus isolation attempts

Mosquitoes were captured using CDC minilight-CO<sub>2</sub>-baited traps (EVS CO<sub>2</sub> Mosquito Trap, BioQuip Products, Inc., United States) placed at a height of approximately 1 m above the ground. The traps were run on two successive nights at two-week intervals. The caught insects were transported to the laboratory of the Institute of Vertebrate Biology, Brno, Czech Republic, in cooled flasks (4 to 8 °C) and stored at –65 °C until examination. They were identified under a stereomicroscope and monospecific pools consisting of 50 *Cx. modestus* females were homogenised in 1.5 ml cooled phosphate buffered saline pH 7.4 supplemented with 0.4% bovine serum albumin (Sigma) and antibiotics (PBS-BSA) and centrifuged.

Viral RNA was extracted from 140 µl mosquito homogenates using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Oligonucleotide primers targeting the NS5 region of flaviviruses were used for screening [14]. If samples were positive, a set of WNV-specific primers were used in continuous reverse transcription (RT)-PCRs for amplification of overlapping genome fragments that covered the entire genome sequences of the detected viruses [15]. Amplification products were

sequenced directly (Microsynth, Balgach, Switzerland), sequences were aligned and compiled, and identified by basic local alignment search tool (BLAST) search against the GenBank database. The WNV sequences were aligned with 25 complete or nearly complete lineage 2 WNV sequences deposited in GenBank database. Phylogenetic and molecular evolutionary analyses were conducted using neighbor-joining and maximum likelihood algorithms (MEGA version 6 [16], with 1,000 replicates for bootstrap testing) and inferred genetic relationships were shown in a phylogram.

Mosquito homogenates of WNV PCR-positive samples (20 µl) were inoculated intracerebrally into specified pathogen-free suckling ICR mice (SM). The brains of SM that succumbed to the infection were homogenised in PBS-BSA, centrifuged and passaged (intracerebrally) in a new batch of SM. Bacterial sterility of the suspensions was checked in meat-peptone and thioglycollate broths incubated at 37 °C [9].

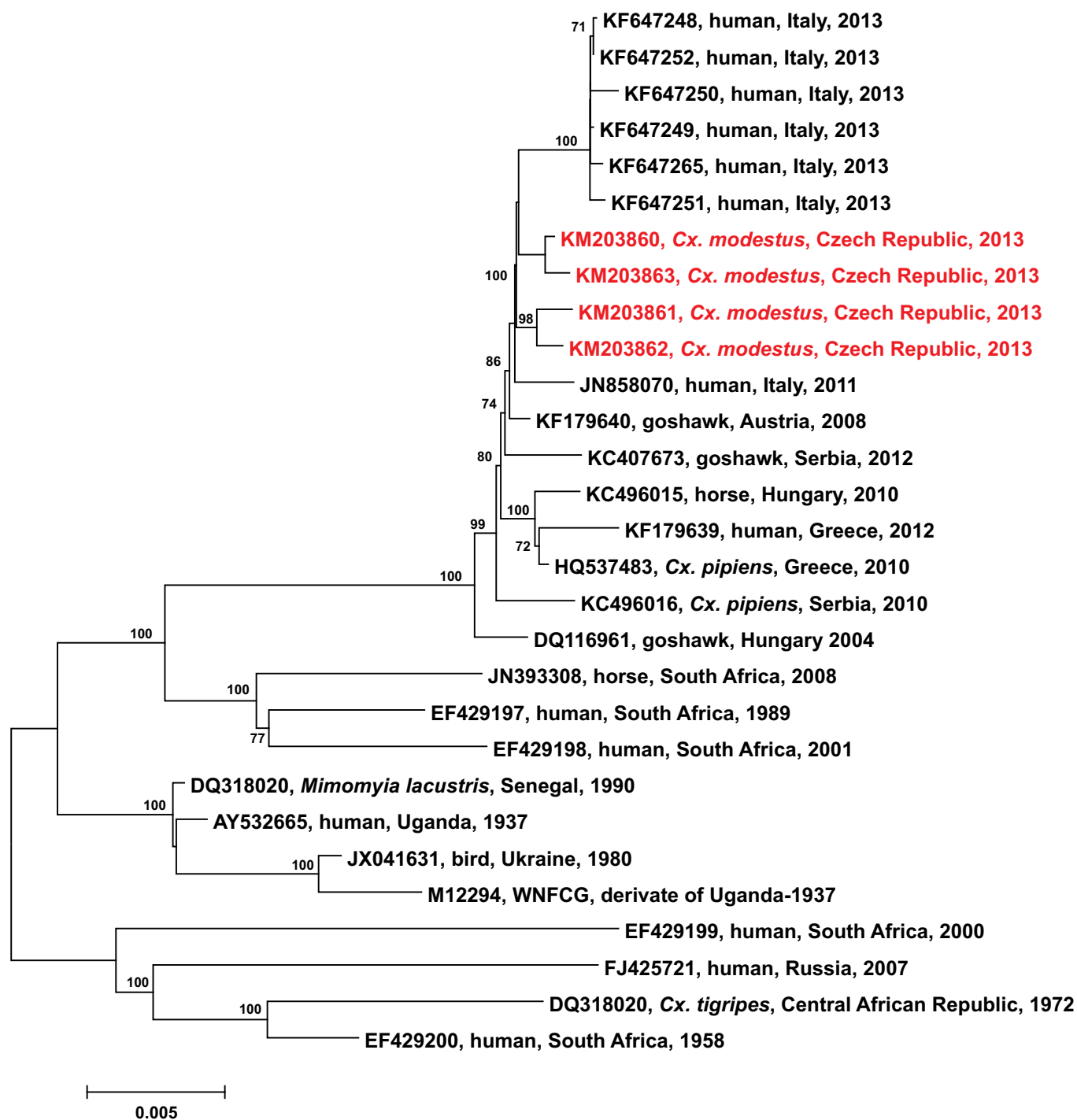
### West Nile virus prevalence in *Culex modestus* mosquitoes

A total of 32,500 female *Cx. modestus* mosquitoes in 650 pools were examined for flaviviruses by RT-PCR. RNA of lineage 2 WNV was detected in four pools of insects collected in August 2013: number 13-104 (collected at Nový fish pond), number 13-329 (collected at Nesyť fish pond), number 13-479 (collected at Mlýnský fish pond) and number 13-502 (collected at Mlýnský fish pond). The minimum prevalence rate of WNV in the examined mosquito pools was therefore 1:8,125 (0.012%). All WNV-2-positive mosquito homogenates were inoculated into SM. While number 13-329 did not kill any mice, the three others did: number 13-104 killed 6 of 11 inoculated SM within 7–8 days post inoculation (DPI) and the average survival time (AST) of SM was 7.7 days; number 13-479 killed 8 of 9 inoculated SM (6–7 DPI; AST 6.1 days); and number 13-502 killed 7 of 10 SM (6–8 DPI; AST 6.4 days). Interestingly, experimentally non-infected mothers of mice inoculated with homogenates from all three infective pools succumbed to infection seven to eight days after cannibalising their dead SM, and WNV was demonstrated by real-time RT-PCR in high concentration (10<sup>7</sup> RNA copies/ml) in the mothers' brains but not in their livers or spleens. This finding supports the hypothesis of oral infection as a (rare) alternative route of WNV transmission, for example, in raptors.

Phylogenetic analysis based on complete WNV-2 genome sequences demonstrated that the four Czech WNV strains identified form two closely related groups: number 13-104 (GenBank: KM203860) with number 13-502 (GenBank: KM203863) and number 13-329 (GenBank: KM203861) with number 13-479 (GenBank: KM203862) and that they cluster together with WNV strains from an Austrian goshawk (isolated in 2008; GenBank: KF179640), Serbian *Cx. pipiens* (in 2012; GenBank: KC407673) and Italian human (in 2011; GenBank: JN858070), while they differ partially from

**FIGURE 2**

Phylogenetic positioning of four West Nile virus strains identified in *Culex modestus* mosquitoes, South Moravia, Czech Republic, August 2013



WNV: West Nile virus.

The complete genome nucleotide sequences of the four WNV strains from the Czech Republic (marked in red) were analysed together with representative lineage 2 WNV strains by the neighbor-joining method. GenBank accession numbers, isolation sources, countries of origins and isolation years are indicated at the branches. Supporting (>70%) bootstrap values of 1,000 replicates are displayed at the nodes. The horizontal bar shows genetic distance.

other European WNV-2 strains compared. However, they are all in the same clade (i.e. central and south European WNV-2), while WNV-2 strains from Africa and Russia form distinct clades (Figure 2). Maximum likelihood analysis resulted in a similar tree topology. Although three of the four Czech isolates were found to be neuropathogenic in SM, these virus strains do not carry the putative virulence marker P249 within the NS3 region [17,18].

## Conclusions

The discovery of WNV-2 in the Czech Republic has added another country to the list of WNV risk areas in Europe. It also shows that two different lineages of WNV (lineages 2 and 3) co-circulate in the country and that *Cx. modestus* mosquito is a potential vector of WNV in reed belts of South Moravian fish ponds. This ornithophilic mosquito might play an important role in the bird–mosquito cycle of WNV in central Europe.

Our study highlights the need for epidemiological surveillance of (re-)emerging mosquito-borne viruses in central Europe. The seasonal peak activity of the adult *Cx. modestus* population in central Europe is from the beginning of July to late September [19]. Usually, the females do not enter buildings, but readily bite humans outdoors often during the day, at sun- and wind-exposed places, causing a nuisance, especially in late summer when floodwater *Aedes* and *Ochlerotatus* mosquito species have already vanished [19]. The isolation of neuroinvasive WNV strains in South Moravian fish ponds (in a popular recreational and camping area during the summer) raises the question of a possible risk of a local WNF outbreak. Given the mild climate of the 2013–14 winter, we can only speculate on the possible emergence of WNF in this year's WNV season, if favourable conditions for mass breeding of mosquitoes occur. To date, no human WNF cases have been recorded this season, which has just begun (the WNV season in central Europe starts mid-July and the majority of cases are seen in September). While infectious disease specialists in the region are aware of the WNV situation, local general practitioners should also be aware of the circulation of WNV in this area and take it into account during differential diagnosis of late-summer neuroinfections.

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## Conflict of interest

None declared.

## Authors' contributions

IR, ZH: designed, coordinated and supervised the study, performed laboratory testing, and wrote the manuscript; TB, JM:

carried out sequence analysis, processed phylogenetic data, read and revised the manuscript; LB, HB, JP, PS, KV: trapped the mosquitoes, performed molecular analyses, read and revised the manuscript; OS: trapped the mosquitoes and performed their identification, read and revised the manuscript; NN: analysed data, wrote and revised the manuscript.

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