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Mosquito-borne virus surveillance reveals co-circulation of distinct West Nile virus strains in Portugal

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Abstract

West Nile virus (WNV) is an emerging mosquito-borne pathogen in Europe, responsible for recurrent outbreaks affecting humans, horses, and wildlife. Although WNV circulation has been documented in Portugal, genomic data from mosquito populations remain scarce, limiting our understanding of viral diversity and transmission dynamics in the western Iberian Peninsula. Here, we investigated the presence and genetic diversity of WNV in mosquitoes collected from wetland ecosystems in southern Portugal in 2025.

Mosquitoes were collected and grouped into pools by collection date, location, and species. Pools were screened for WNV RNA by RT-qPCR, and positive samples were subjected to whole-genome sequencing using a hybrid-capture target enrichment method. Phylogenetic analyses were conducted to determine the evolutionary relationships of the detected viruses with previously reported WNV strains circulating worldwide.

WNV RNA was detected in several mosquito pools, predominantly in *Culex univittatus*, from which seven near-complete viral genomes were recovered. All sequences belonged to WNV lineage 1A but segregated into two distinct genetic clusters. One cluster grouped with strains previously reported from the Iberian Peninsula, whereas the second formed a divergent variant previously undetected. These findings indicate the co-circulation of genetically distinct WNV strains in Portuguese wetlands.

Our results provide the phylogenetic characterization of WNV from mosquito populations in Portugal and reveal previously undocumented viral diversity in the region. This study highlights the importance of integrating entomological surveillance with genomic approaches to better understand the mechanisms underlying WNV introduction, maintenance, and spread in southern Europe.

Introduction

West Nile virus (WNV, *Orthoflavivirus nilense*) is a widely distributed zoonotic arbovirus with important consequences for animal and public health. The virus was first identified in the West Nile district of Uganda in 1937 [1]. WNV is distributed worldwide and causes human infections on every continent except Antarctica [2]. WNV is maintained in nature through an enzootic transmission cycle involving mainly *Culex* mosquitoes and wild birds, which act as the main reservoir hosts [3, 4]. Humans and horses can be susceptible to infection but are considered dead-end hosts due to their low viremia levels [3, 4]. Most human infections are asymptomatic [5, 6], while approximately 20% develop West Nile fever (WNF), a mild flu-like illness. A small proportion of infections progress to West Nile neuroinvasive disease (WNND), which can lead to severe neurological complications or death [7]. In equids, approximately 10% of infections develop neurological signs ranging from mild ataxia to severe paralysis [6]. In birds, susceptibility varies widely across species, and mortality is influenced by viral genotype, bird species, and environmental conditions [8, 10–12].

West Nile virus is a single-stranded, positive-sense RNA virus with a genome of approximately 11 kb [4]. At least eight phylogenetic lineages have been described [11], although most human disease outbreaks have been associated with WNV lineages 1 and 2 [12].

In Portugal, evidence of WNV circulation dates back to 1967, when antibodies were first detected in animals from southern regions [13]. The virus was subsequently isolated from *Anopheles maculipennis* mosquitoes in Alentejo in 1969 [14]. Since then, several surveillance studies have reported WNV antibodies in birds and horses [15–17]. In equids, 59 confirmed cases have been reported between 2015 and 2024 [18]. Additionally, a serosurvey study conducted among blood donors in 2022 estimated a seroprevalence of 1.4% for WNV IgG in mainland Portugal. This study identified southern regions as areas of higher exposure risk but also detected notable seroprevalence in central and northern coastal regions [19].

Despite this evidence of viral circulation, the number of confirmed human cases in Portugal remains low, with only four reported since 2004 [20–22]. Ecological and risk models consistently identified southern Portugal as highly suitable for WNV transmission [23]. In addition, the distribution of key vector species, including *Culex pipiens*, the main vector in Europe, together with other regionally relevant species such as *Cx. modestus* and *Cx. perexiguus/univittatus*, indicates that suitable ecological conditions for WNV transmission extend across large areas of Portugal [24, 25].

In contrast, neighbouring Spain has experienced a marked increase in WNV activity in recent years, with recurrent outbreaks affecting humans, horses, and birds, particularly in southwestern regions [26, 27]. Since 2020, Spain has reported some of the largest WNV outbreaks in Europe, highlighting the Iberian Peninsula as an important hotspot for WNV transmission [28]. Despite the geographic proximity and shared ecological conditions between both countries, it remains unclear whether WNV circulation in Portugal is driven by the same viral lineages and transmission dynamics observed in Spain, or whether distinct epidemiological patterns occur [29, 30].

Molecular data on WNV in Portugal remain scarce. To date, only four genome sequences have been reported, all belonging to lineage 1A. These include the strain PTRoxo, isolated from *Anophleles maculipennis* mosquitoes collected in Alentejo in 1969 (GenBank accession number: AM404308) [14, 31]; two strains were detected in *Cx. pipiens* and *Cx. univittatus* (PT5.2; GenBank accession number: AJ965628, and PT6.16; GenBank accession number: AJ965626), in the Algarve in 2004 [31], near Almancil (Faro district), the same area where human cases were reported, and the recent strain WNV/18665/PT2024 (GenBank accession number: PQ660506) isolated from a brain sample of northern Goshawk (*Astur gentilis*) in Setúbal in 2024 [18].

Given the limited availability of genomic data and the apparent discrepancy between serological evidence, vector distribution, and the low number of reported human cases, this highlights important gaps in our understanding of WNV circulation in Portugal. Furthermore, the contrasting epidemiological situation in Portugal and Spain raises questions about the genetic diversity, origin, and transmission dynamics of WNV strains circulating in the region.

This study aims to investigate WNV circulation in mosquito populations from high-risk wetland areas in Portugal by targeting key *Culex* vector species and genetically characterizing the viruses detected through whole-genome sequencing. Additionally, we aim to situate the Portuguese WNV genomes within the broader Iberian and global genetic diversity, in order to improve our understanding of WNV eco-epidemiology in this region.

Materials and methods

Mosquito collection

Mosquito collections were conducted in two wetland areas in Portugal characterized by extensive rice fields and high densities of wild birds: Alcácer do Sal (38°21'18.31" N, 8°29'27.33" W), and Salvaterra de Magos (39°2'21.83" N, 8°44'22.64" W). These environments were selected due to their ecological suitability for West Nile virus transmission. Adult mosquitoes were collected using Centre for Disease Control miniature light traps (CDC light traps, John W. Hock Company, Gainesville, FL, USA) and BG-Pro traps (Biogents AG, Regensburg, Germany). In each locality, three CDC traps and three BG traps were deployed overnight for a minimum of 12 hours (covering the period between sunset and sunrise). All traps were baited with approximately 1 Kg of dry ice as a CO_2 source. Sampling was conducted one night per week over three consecutive weeks in October 2025. In Salvaterra de Magos, collections took place on 17, 23, and 30 October, while in Alcácer do Sal, sampling was conducted on 23 and 30 October 2025. Collected mosquitoes were transported to the laboratory in dry ice and stored at − 80°C until processing. Morphological identification was carried out on a chill table to maintain the cold chain. Species identification followed standard taxonomic keys [32, 33]. Mosquitoes were pooled according to collection site, date, species and sex, in pools of up to 50 individuals (range: 2–50 mosquitoes).

Virological analyses

Virological analyses were performed on the three most abundant mosquito species with potential vector competence in the study area: *Cx. univittatus*, *Cx. pipiens*, and *Cx. theileri*. Before RNA extraction, mosquito pools were homogenised in 1 ml minimal essential medium (MEM, GIBCO), supplemented with 10% fetal bovine serum (FBS), streptomycin (0.1 mg/ml), and fungizone (1 mg/ml). Homogenisation was performed using a TissueLyser (QIAGEN, Valencia, CA) at 30 cycles/s for 2 min. The homogenates were then clarified by centrifugation at 13,000 rpm for 5 min at 4 ^{o}C. Viral RNA was extracted from clarified supernatant using the MagCore Viral nucleic acid extraction kit in the MagCore® Plus II automated nucleic acids extractor (RBC Bioscience Corp., Taiwan) according to the manufacturer's instructions.

WNV molecular detection

Detection of WNV RNA was performed by real-time RT-PCR [34]. Samples testing positive by qRT-PCR were further confirmed by sequencing. A pan-flavivirus RT-PCR targeting the NS5 gene was performed

using protocols adapted from Briese et al. (1999, 2022) [35, 36] and Patel et al. (2013) [37]. Amplified products were sequenced using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Obtained sequences were compared with previous sequences using BLASTn [38] to confirm WNV identity.

Whole-genome sequencing and bioinformatics analysis

Whole-genome sequencing (WGS) of WNV-positive samples was performed using the Viral Surveillance Panel v.2 (Illumina), following the manufacturer's instructions. This hybrid-capture target enrichment system is optimised for the detection and WGS of approximately 200 viral pathogens. Sequencing libraries were analyzed using paired-end sequencing (2x150 bp) on an Illumina MiSeq, generating an average of approximately 1.5 million reads per sample.

Bioinformatics analyses were performed using the INSaFLU-TELEVIR platform v.2.2.2 (https://insaflu.insa.pt/) with default settings [39, 40]. Briefly, following quality control (QC) and draft *de novo* assembly, QC-passed reads were initially mapped using the INSaFLU module against a closely related WNV complete genome sequence (WNV/18665/PT2024; GenBank PQ660506.1) identified through BLAST analysis of the draft assemblies. Final consensus sequences were then generated and curated by remapping QC-passed reads against highly related consensus sequences representative of the genetic diversity of the studied dataset (Supplementary Table 1). A minimum 10-fold depth of coverage was required for consensus sequence validation (except for one low-coverage sample, PTCulexWNV/SM11/INSA2025, for which a 4-fold cutoff was applied), followed by visual inspection of BAM files using the Integrative Genome Viewer (IGV) embedded in the INSaFLU platform to confirm mutations and recover occasional low-coverage positions (mostly at genome ends). Overall, WNV-mapped reads accounted for a mean of 11% (range: 0.1–20.7%) of the QC-passed read dataset. Mean depth of coverage exceeded 900× for all samples except one (SM11), which showed a very low mean depth (21×). Final curated consensus sequences covered > 99.3% of the expected WNV complete genome length. Detailed WNV sequencing metrics are provided in Supplementary Table 1. Clade classification and further assessment of consensus sequence metrics were performed using Nextclade v 3.18.1 (https://clades.nextstrain.org/; accessed on 2026-02-08) [41].

To complement morphological mosquito identification, cytochrome c oxidase I (COI) sequences were also recovered by mapping sequencing reads against reference COI sequences from *Culex* species.

Phylogenetic and phylogeographic analyses

To phylogenetically place the viral genome sequences obtained in Portugal within the current global genetic diversity of WNV, phylogenetic and geotemporal analyses were conducted using the WNV Nextstrain [42] workflow (https://github.com/nextstrain/wnv), available through the INSaFLU-TELEVIR platform (https://github.com/INSaFLU/WNV, as of 2026-02-12) [39, 40]. The analysis included a total of 3,126 international WNV lineage 1A genome sequences (> 10,000 bp) publicly available, obtained from

the Nextstrain build (as of 2026-02-05), and confirmed to correspond to the same non-redundant sequences available at Pathoplexus (https://pathoplexus.org/, accessed on 2026-02-05). To further refine the phylogenetic and geotemporal analysis, the Nextstrain subtree in which the 2025 Portuguese sequences clustered was zoomed in, including a subset of 34 sequences (eight from Portugal, 24 from Spain, and two from Senegal). For this focused analysis, higher-resolution geographic metadata were used when available, namely NUTS III regions for Portuguese sequences from the present study and Province information for Spanish sequences, as reported in the literature [18, 30] or in https://nextstrain.clinbioinfosspa.es/wnv (accessed as of 2026-02-13). The full phylogenetic trees can be explored interactively at https://auspice.us/ using the JSON files provided as Supplementary Dataset. The subset tree and metadata are also available in Supplementary Dataset.

Results

WNV in mosquito pools

A total of 3,706 female mosquitoes belonging to three *Culex* species were analysed, including *Cx. pipiens* (n = 759, 20.5%), *Cx. theileri* (n = 449, 12.1%), and *Cx. univittatus* (n = 2,498, 67.4%). Most mosquitoes were collected in Salvaterra de Magos (n = 2,489, 67.2%), reflecting the greater sampling effort at this site (three sampling nights) compared with Alcácer do Sal (n = 1,217, 32.8%), where collections were conducted during two nights. These were grouped into 101 pools: 19 pools of *Cx. pipiens*, 22 pools of *Cx. theileri*, and 60 pools of *Cx. univittatus*.

WNV RNA was detected in seven mosquito pools. Four positive pools corresponded to *Cx. univittatus* collected in Salvaterra de Magos (SM11, SM40, SM83 and SM103). Three additional positive pools were detected in Alcácer do Sal, including two *Cx. univittatus* pools (AS25 and AS49) and one *Cx. theileri* pool (AS46) (Table 1). Positive pools were identified in mosquitoes collected on all sampling days (17, 23, and 30 October), indicating continuous viral circulation during the sampling period. Considering both sampling sites, the minimum infection rate (MIR) was estimated at 1.9%. When analysed separately, the MIR was slightly higher in Alcácer do Sal (2.4%) than in Salvaterra de Magos (1.6%).

Table 1
Mosquito pools analysed for West Nile virus RNA in Salvaterra de Magos and Alcácer do Sal (Portugal).

Study site	Mosquito species	No. mosquitoes	No. pools	WNV + pools (ID)	No. genomes	GenBank accession	MIR (%)
Alcácer do Sal	*Cx. univittatus*	739	18	AS25 AS49	2	OZ470012 OZ470015	2.7
Alcácer do Sal	*Cx. pipiens*	186	5		0		
Alcácer do Sal	*Cx. theileri*	292	9	AS46	1	OZ470017	3.4
Salvaterra de Magos	*Cx. univittatus*	1759	42	SM11 SM40 SM83 SM103	4	OZ470018 OZ470013 OZ470016 OZ470014	2.3
Salvaterra de Magos	*Cx. pipiens*	573	15		0		
Salvaterra de Magos	*Cx. theileri*	157	12		0		
Total		**3706**	**101**		**7**		**1.9**

Whole-genome sequencing and phylogenetic analysis

Whole-genome sequencing was successfully performed for all WNV-positive pools, generating seven nearly complete WNV genomes covering more than 99.3% of the expected genome length. Phylogenetic analysis showed that all detected viruses belonged to WNV lineage 1A but formed two distinct genetic clusters (Fig. 1). The WNV strains detected in mosquitoes collected in Alcácer do Sal clustered together with the WNV strain identified in a northern goshawk (*Astur gentilis*) in Setúbal in 2024 [18], as well as with several strains recently reported in Spain, suggesting regional circulation of related viral strains across the southwestern Iberian Peninsula (Fig. 1). In contrast, the cluster detected in Salvaterra do Magos formed a separate cluster that was genetically distinct from recently reported Iberian strains, indicating the presence of a divergent strain that has not been previously detected in Europe (Fig. 1).

Discussion

This study provides new insights into the circulation and genetic diversity of WNV in Portugal through entomological surveillance and phylogenetic characterization of viruses detected in mosquito populations from two wetland areas. The detection of WNV RNA in mosquito pools collected in both

sampling sites confirms active viral circulation in mosquito vectors in Portugal, supporting previous evidence in mosquitoes, birds, horses, and humans [15–22, 31].

The overall MIR observed in this study (1.9%) suggests a relatively intense level of viral circulation in local mosquito populations during the sampling period. Similar infection rates have been reported in entomological surveillance studies conducted in southwestern Spain [26, 43] and other regions of Europe, where WNV transmission occurs regularly [44–46]. Although infection rates can vary widely depending on sampling intensity [47], mosquito species composition [48], and seasonal dynamics [49], the detection of positive pools at both sampling sites and dates in October provides evidence consistent with viral activity in late summer and early autumn of 2025.

Most WNV-positive pools were detected in *Cx. univittatus*, the most abundant mosquito species in the study areas. This finding highlights the potential importance of this species as a main vector of WNV in Portugal. Although *Cx. pipiens* is generally considered the principal WNV vector in Europe [24], other *Culex* species could play an important role in the WNV enzootic transmission cycle. In particular, *Cx. univittatus* and *Cx. perexiguus* are closely related species that are morphologically very similar and often difficult to distinguish without molecular confirmation [50]. While *Cx. perexiguus* has been identified as a key vector driving WNV transmission in southwestern Spain [26, 51], entomological surveys conducted in Portugal have consistently reported the presence of *Cx. univittatus* in similar wetland habitats [52]. These species are typically associated with wetland and rice-field ecosystems, where high densities of avian hosts facilitate enzootic transmission. In such systems, *Cx. pipiens* could act primarily as a bridge vector between birds and humans due to its opportunistic feeding behaviour [53–55]. The predominance of WNV-positive pools in *Cx. univittatus* in the present study, therefore, suggests that this species may play an important role in maintaining local WNV transmission cycles in Portuguese wetlands. The contrasting dominance of *Cx. perexiguus* in southwestern Spain [56] and *Cx. univittatus* in Portugal [52] could reflect subtle ecological or biogeographical differences across the Iberian Peninsula, although further molecular identification studies are required to clarify the distribution and relative abundance of both species. In addition, in our study, WNV RNA was also detected in one pool of *Cx. theileri.* Although the epidemiological role of this species in WNV transmission remains poorly understood, recent laboratory studies have demonstrated that European populations of *Cx. theileri* are competent vectors for WNV lineage 1 [57], suggesting that this species could also contribute to local transmission under suitable ecological conditions.

Genomic analysis revealed that all the WNV strains detected in mosquitoes belonged to WNV lineage 1A, consistent with previous reports from Portugal and Spain [18, 27, 30, 31], and fell within the diverse WMed-1.2 genetic group [30]. However, the phylogenetic analysis identified the presence of two different viral clusters, indicating that multiple WNV strains circulated simultaneously in Portugal in 2025. The viruses detected in Alcácer do Sal clustered with the WNV strain recently identified in a northern goshawk in Setubal, in 2024 [18], as well as with several strains circulating in Spain [30]. This pattern supports the hypothesis that WNV transmission dynamics in southwestern Portugal may be connected to those occurring in neighboring regions of Spain. The Iberian Peninsula has been increasingly

recognized as an important hotspot for WNV circulation in Europe [49, 58], with Spain experiencing large outbreaks in humans and horses [28, 59]. The phylogenetic relationship between Portuguese and Spanish strains therefore suggests regional viral circulation across the southwest Iberian Peninsula, likely facilitated by shared ecological conditions and the movement of migratory birds [60].

In contrast, the strains detected in Salvaterra de Magos formed a separate phylogenetic cluster that was not closely related to recent Iberian WNV strains, suggesting the presence of a divergent group, raising the possibility that this new strain group may represent a recent introduction into the Iberian Peninsula, potentially through migratory birds. Long-distance bird migration between Africa and Europe is known to play an important role in the introduction and dissemination of arboviruses [61, 62], including WNV [60, 63]. The detection of a previously undescribed WNV strain group circulating in Portuguese mosquito populations highlights the Iberian Peninsula as a potential gateway for the introduction of African WNV genetic diversity into Europe [64]. This supports the complex WNV circulation in the Iberian Peninsula, where multiple viral introductions and local transmission cycles coexist [27, 29]. Nonetheless, the limited availability of whole genome sequencing (WGS) data may obscure intermediate evolutionary relationships, making it difficult to draw definitive conclusions about its origin and local transmission dynamics. Continued genomic surveillance will be essential to determine whether this cluster becomes established in local transmission cycles or represents a transient introduction.

Despite evidence of viral circulation in vectors and animals, the number of confirmed human cases in Portugal remains very low [20–22]. This apparent discrepancy between entomological and serological evidence of circulation and the limited number of reported clinical cases could be due to 1) a large proportion of WNV infections in humans are asymptomatic or present with mild symptoms that may go undiagnosed, and/or 2) surveillance intensity and diagnostic awareness may influence case detection [65]. In addition, local ecological factors, including vector feeding behaviour and host community composition, could also reduce human exposure to infected mosquitoes [54, 66].

Overall, our results highlight the importance of entomological and genomic surveillance to better understand WNV circulation in the Iberian Peninsula. The detection of active circulation in mosquito populations, together with the identification of genetically distinct viral clusters, underscores the necessity of continued monitoring of mosquito vectors and viral genetic diversity in the region [67]. Such surveillance efforts are essential to improve early detection of emerging WNV strains, as well as other potential pathogenic arboviruses, to better assess the risk of future outbreaks that could affect public health.

Conclusion

This study provides genomic evidence of WNV presence in mosquito populations from Portuguese wetland ecosystems, consistent with active local circulation during the sampling period. The detection of two distinct lineage 1A clusters, including one genetically related to strains circulating in Spain and another not previously reported, highlights the complex and dynamic nature of WNV circulation in the

Iberian Peninsula. The predominance of WNV-positive pools in *Cx. univittatus* suggests that this species could play a key role in maintaining local enzootic transmission cycles in Portugal. These findings underscore the importance of continued entomological and genomic surveillance to better understand WNV transmission dynamics and assess the risk of future outbreaks in the region.

Declarations

Data availability

WNV reads generated in this study are deposited in the European Nucleotide Archive (ENA) under the BioProject PRJEB87871 and assembled consensus sequences are deposited in Pathoplexus https://pathoplexus.org/ and NCBI (individual accession numbers listed in Supplementary Table 1).

Authors' contributions

RGL, VB and LZZ, wrote the manuscript. RGL, FA, VB, DS and LZZ provided original inputs. RGL, FA, HC and HCO collected the mosquitos. RGL identified the mosquitoes, defined the pools and processed the mosquito specimens. LZZ performed the molecular analysis tests. VB, DS, JPG and LZZ contributed to genomic data analysis. RGL, FA, VB, JPG and LZZ contributed to the conceptualisation and revision of the manuscript. All co-authors contributed to the revision of the manuscript and approved the final version for submission.

Ethics approval and consent to participate

No special permits were required for field studies. Consent was obtained from the property owners for mosquito collection in private areas and with the knowledge and permission of the relevant authority in public areas. No sites were legally protected, and no endangered or protected species were involved in this study.

Competing Interests

The authors confirm that there are no relevant financial or non-financial competing interests to report.

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Figures

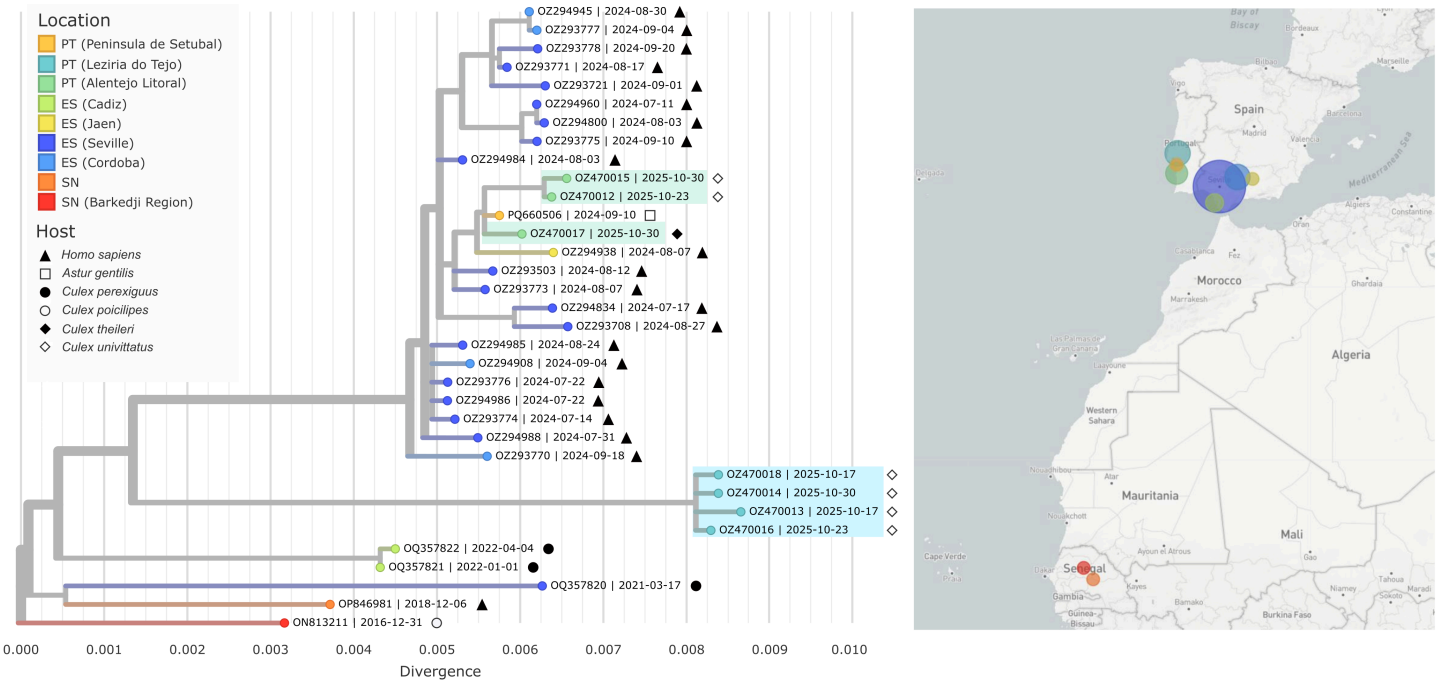


Figure 1

Nextstrain maximum-likelihood phylogeny of WNV strains detected in *Culex univitattus* and *Culex theileri* mosquitoes from Portugal. The figure provides a zoomed-in view of the phylogenetic branch in which the WNV sequences detected in Portugal (highlighted in colored boxes) are positioned. Host species are indicated by symbols next to the sequence identification (NCBI accession | collection date). Tree tips are colored according to geographic location in the map, at the level of NUTS III regions (Portugal) or provinces (Spain), and branch lengths indicate genetic distance (substitutions per site). This phylogenetic sub-branch (34 sequences) was identified through Nextstrain-based analysis using 3126 international WNV lineage 1A complete or near-complete genome sequences (see Methods) and can be delineated by the C4302G mutation (relative to the reference NC_009942.1 used for mutation annotation), which is exclusive to this branch in the full tree. The full phylogenetic trees can be interactively explored via https://auspice.us/ using the JSON files provided in Supplementary Dataset. The corresponding subset tree and metadata are also available in Supplementary Dataset.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- metadatasubtreeFigure1.tsv
- README.pdf
- subtreeFigure1.nwk
- WNVlineage1AfocusPortugal2025.json
- WNVlineage1AfocusPortugal2025tipfrequencies.json