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Introduction and Spread of West Nile Virus in Lazio, Italy, 2025: Molecular Epidemiology and Genomic Characterization

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ABSTRACT

West Nile virus (WNV) is a flavivirus whose cycle involves mosquitoes and avian hosts that occasionally may infect horses and humans as "dead-end hosts." Human infection is mostly asymptomatic, sometimes leading to febrile or neuroinvasive disease. In 2025, Italy experienced its largest epidemic with an unprecedented geographic spread (79 provinces; 52% of cases in central Italy). Here we report whole-genome sequencing and phylogeographic analysis of 39 WNV lineage 2 samples collected in Lazio and Campania regions between July and September 2025 from humans and animal samples. All 2025 strains belonged to a Southern Italian clade that diversified around 2020, with evidence of earlier circulation in 2024. All the 2025 genomes shared a substitution that lies in envelope protein domain III (K399R), potentially influencing interactions with the viral membrane. The same mutation was previously detected in Eastern European strains, leading to the hypothesis of introductions in Italy via migratory birds from these regions. Phylogeography identified strong viral dispersal routes within Central Italy supporting a pattern of short-range spread and local overwintering. Overall, results highlight the roles of ecological conditions and cross-border transmission dynamics in shaping the 2025 Italian epidemic, underscoring the need for integrated genomic/ecological surveillance.

1 | Introduction

West Nile virus (WNV) is a mosquito-borne virus of the Flaviviridae family whose transmission cycle involves mosquitoes and birds. It is mainly transmitted by certain species of the genus *Culex* (mainly *Culex pipiens* in Europe) that become infected when feeding on infected birds that behave as "amplifying hosts." Occasionally infected mosquitoes bite horses or other mammals, including humans, leading to "a dead end" infection. Nevertheless, WNV infection in humans can cause

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clinical disease; mammal hosts are considered accidental hosts, unable to transmit to mosquitoes. This explains the very low risk of human-to-human transmission of the WNV, with the exception from an infected donor to an organ transplant recipient [1] or via blood donation. In addition, mother-to-child transmission during pregnancy, delivery, or breastfeeding has been documented [2, 3]. Accordingly, control strategies focus on reducing vector abundance and preventing WNV circulation in humans and animals, and on preventing transmission trough blood donation and organ/cells transplantation by donors' NAT testing in areas where viral circulation is detected. This includes continuous surveillance of WNV in mosquitoes, birds, and animals to detect transmission hotspots and enable targeted interventions [4].

WNV infection has an incubation time that ranges from 2 to 14 days, but may be more prolonged (up to 21 days) in immunocompromised patients. WNV infection typically causes a self-limiting illness ("West Nile fever") characterized by the abrupt onset of fever, variably associated.

WNV infections in humans are typically asymptomatic, with approximately 80% having no symptoms. The remaining 20% of infections can result in a systemic febrile illness with fever, myalgia and gastrointestinal illness, and approximately 1% of infections resulting in severe neurological disease, including encephalitis, meningoencephalitis, and acute flaccid paralysis. Neuroinvasive infection targets are mostly represented by immunocompromised, aged or comorbid subjects and could range from self-limited confusional states to severe encephalopathy, coma, and death [5, 6].

WNV is now considered one of the most threatening arboviral diseases in Europe. Since the first 1996 outbreak in Romania cause by lineage 1 [7], it has been spreading across European countries due to climatic and environmental conditions that favored its transmission, leading to the co-circulation of both lineage 1 and 2, that first appeared in Hungary in 2004 [8].

In Italy, the first identification dates to 1998, when an outbreak of WNV lineage 1 involved 14 racehorses from nine localities in four provinces of Tuscany (central Italy) [9]. After the first detection, the virus reappeared in 2008 with infections in humans [10]. Since then, there has been a seasonal rise of infections, mainly in the Po valley (Pianura Padana), in the north of the country. Usually, the number of cases increases in July, peaks in August, and declines in September (a median of 197 cases between 2013 and 2024). Temporal variations in incidence rates were observed, with the highest rates recorded in 2018 [11] and 2022 [12].

In the summer-fall period of 2025, Italy faced numerous cases compared to the previous years, resulting in 773 confirmed human cases, including three imported cases; 367 were neuroinvasive forms and 72 resulted in death [13]. The geographic area affected by WNV cases was notably broader, involving 79 provinces of 18 regions spanning from northern to southern regions [14]. While most cases (52%) were concentrated in a few provinces of the Lazio and Campania regions (specifically: Latina, Rome, and Frosinone in Lazio, and Caserta and Naples in Campania), the remaining cases occurred in the endemic areas of the Po Valley and western Sardinia [13, 15].

We performed whole-genome sequencing (WGS) of WNV isolates from humans, animals, and vectors sampled in Lazio and Campania, and applied maximum-likelihood and Bayesian phylogeographic methods to a data set of 2025 Italian strains to reconstruct transmission dynamics underlying the extensive summer–fall 2025 circulation.

2 | Materials and Methods

2.1 | Samples Collection

Samples were collected from human patients, animals, and mosquito pools. Human samples were urine pellets collected between July 18 and September 11, 2025, while animal and vector samples were collected between July 9 and August 9, 2025. Sampling was designed to cover the main affected provinces of Lazio (Latina [LT], Rome [RM] and Frosinone [FR]) and one human sample originated from a patient with putative exposure to WNV infection in Campania. Detailed sample metadata are provided in Tables 1 and 2.

2.2 | Diagnosis of WNV Infection and WGS

WNV RNA was detected by using two different commercially available real-time (rt) PCR assays: the RealStar WNV PCR kit 2.0 PCR kit (Altona Diagnostics GmbH) and the cobas WNV (Roche). Amplification protocols were carried out according to the manufacturer's instructions. For the animal and mosquito samples WNV RNA was detected by using the method described in Del Amo J et al 2013 [16].

The full genome of WNV was amplified by a multiplexed PCR amplicon approach by using two pools of home-made designed primers (totaling 22 overlapping amplicons with a medium length of 600 bps) by using Qiagen One-step enzyme for RT-PCR (primers set are supplied in Table S1). Libraries were then prepared starting from 10 to 100 ng of DNA using the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific) according to the manufacturer's instructions and sequencing was performed on the Gene Studio S5 Prime Sequencer (Thermo Fisher Scientific) to obtain approximately 1 million reads per sample.

2.3 | Bioinformatic NGS Analysis

NGS data filtering and assembly were performed by using the in-house pipeline. Briefly, raw reads were trimmed, removing primer sequences and low-quality bases, using Trimmomatic v.0.39 [17]. A preliminary alignment was performed with BWA v. 0.7.17 [18] to align the filtered reads to lineage 1 and 2 WNV reference sequences (NCBI Accession Numbers, respectively: NC_009942.1 and NC_001563.2). A subsequent alignment was performed to the best-matching genomic reference, using bowtie2 v.2.5.4 [19]. From mapped reads, a variant calling analysis was performed with samtools v.1.23 [20], and a consensus sequence was generated for each sample with a home-made python script [21].

Moreover, all the assembled genomes were checked and corrected manually with Geneious Prime 2025.1.3 software (http://www.geneious.com/).

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TABLE 1 | Characteristics of study samples collected from human patients. NA: data not available.

Patient ID	Collection date	Collection location	Symptomatology
Pt1	July 18, 2025	Latina (LT)	Neuroinvasive
Pt2	July 18, 2025	Sezze (LT)	Neuroinvasive
Pt331	July 18, 2025	Fondi (LT)	Neuroinvasive/exitus
Pt332	July 21, 2025	Latina (LT)	Neuroinvasive
Pt333	July 21, 2025	Latina (LT)	Neuroinvasive
Pt334	July 22, 2025	Latina (LT)	Febrile
Pt336	July 22, 2025	Aprilia (LT)	Neuroinvasive
Pt337	July 23, 2025	Nettuno (RM)	Neuroinvasive
Pt338	July 24, 2025	Cisterna di Latina (LT)	Febrile
Pt341	July 26, 2025	Anguillara Sabbazia (RM)	Febrile
Pt342	July 27, 2025	Baia Domizia (Campania - CE)	Febrile/exitus
Pt340	July 27, 2025	Aprilia (LT)	Febrile
Pt343	July 29, 2025	Fondi (LT)	Febrile
Pt397	August 30, 2025	Cisterna di Latina (LT)	Neuroinvasive
Pt416	September 2, 2025	Latina (LT)	Neuroinvasive
Pt418	September 2, 2025	Terracina (LT)	Neuroinvasive
Pt419	September 2, 2025	Pontinia (LT)	Neuroinvasive
Pt420	September 4, 2025	Sermoneta (LT)	Neuroinvasive
Pt421	September 4, 2025	Velletri (RM)	Neuroinvasive
Pt422	September 4, 2025	Latina (LT)	Febrile
Pt423	September 4, 2025	Latina (LT)	Febrile
Pt424	September 4, 2025	Sabaudia (LT)	Neuroinvasive
Pt425	September 6, 2025	Ausonia (FR)	Febrile
Pt426	September 9, 2025	Boville Ernica (FR)	Neuroinvasive
Pt427	September 9, 2025	Ardea (RM)	NA
Pt428	September 11, 2025	Albano Laziale (RM)	Neuroinvasive

TABLE 2 | Characteristics of study samples collected from animals and vectors.

Sample ID	Collection date	Collection location	Source
IZSLT_CulexPool	July 9, 2025	Pontinia (LT)	Culex spp (pool)
IZSLT_1254	July 20, 2025	Cori (LT)	Horse (encephal)
IZSLT_1254	July 20, 2025	Cori (LT)	Horse (bone marrow)
IZSLT_4471	July 26, 2025	Anzio (RM)	Horse (encephal)
IZSLT_4471	July 26, 2025	Anzio (RM)	Horse (bone marrow)
IZSLT_3678	July 28, 2025	Cassino (FR)	Horse (encephal)
IZSLT_3678	July 28, 2025	Cassino (FR)	Horse (bone marrow)
IZSLT-5679-4	July 30, 2025	Cisterna di Latina (LT)	Corvus (pool)
IZSLT-5679-5	July 30, 2025	Cisterna di Latina (LT)	Corvus (pool)
IZSLT-5679-8	July 30, 2025	Cisterna di Latina (LT)	Corvus (pool)
IZSLT_4199	July 31, 2025	Aprilia (LT)	Corvus (pool)
IZSLT_5680-1	August 2, 2025	Fondi (LT)	Corvus (pool)
IZSLT_5680-6	August 2, 2025	Fondi (LT)	Corvus (pool)

2.4 | Phylogenetic and Bayesian Phylogeographic Analysis

To investigate whether the WNV outbreak in Lazio and Campania regions was the result of a single or multiple introductions, we compiled a curated data set of 2,270 WNV genome sequences representing a broad temporal and geographic distribution worldwide, across Europe and neighboring regions (Table S2). The data set includes publicly available sequences on GenBank

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from south-eastern and central-eastern European countries (Austria, Bulgaria, Greece, Hungaria, Kosovo, Serbia and Slovakia) which share documented bird migration routes with Italy, spanning isolates collected between 2004 and 2025 and our newly generated Italian WNV genomes obtained through national surveillance activities in 2025 from municipalities in the Lazio and Campania region. Each genome was associated with available metadata, including collection date and sampling location. This was used to investigate where our isoaltes sits within the WNV lineages, and whether we had one or more introductions in Italy. Following this analysis, a second maximum-likelihood phylogenetic tree was generated to contextualize the WNV genomes obtained in this study within the diversity of lineage 2 circulating in Europe. The data set included both sequences carrying the K399R substitution in the E gene and sequences without the mutation. Newly generated Italian genomes from 2024 to 2025 (indicated as *Italy 2024–2025* and marked with blue triangles) were analyzed together with available WNV lineage 2 sequences retrieved from the Nexstrain and NCBI databases, representing multiple European countries and years. Sequences were aligned using with Bioedit software (ver. 7.7.1), and phylogenetic analysis was performed with Mega software (v. 12.0.11).

A smaller data set, comprising our newly obtained ones and six sequences from a previous outbreak in Northern Italy with metadata at the county level, added to improve TMRCA accuracy, was used to perform Bayesian phylogeographic analysis (Table S3) in BEAST v1.10.5 [22] after testing for the presence of temporal signal using TempEst v1.5 [23] (Figure S1). Bayesian coalescent framework was implemented using a discrete trait asymmetric diffusion model, enforcing either a strict or Hamiltonian relaxed clock [24], and a constant size or Hamiltonian BSG demographic model [25]. The model was run for 100 million states or until the effective sample size (ESS) values were greater than 200. Testing for the best molecular clock and demographic model was done with sampling (PS) and stepping-stone (SS) method [25]. The best model for this data set was a strict clock with Hamiltonian Monte Carlo SkyGrid (HBSG) demographic model (Table S3). The Maximum Clade Credibility (MCC) tree was generated with a 10% burn-in. The MCC phylogenetic tree was manipulated in R, using the package ggtree, and QGIS v3.28.2-Firenze to graphically display through maps the dispersal events supported by Bayes factor (BF) > 5 as strong transition rate and BF > 100 as very strong transition, constituting the migration graph [26].

2.5 | Selection and Signature Pattern Analyses

We performed the following HyPhy algorithms implemented in the Adaptive Evolution Server (http://www.datamonkey.org/): the fast unconstrained Bayesian approximation (FUBAR) and the mixed effects model of evolution (MEME) to identify episodic selection at individual sites [27–30] on the envelope protein of a data set comprising a total of 66 sequences from Italy that included 26 publicly available sequences from GenBank collected between 2013 and 2024, and 39 sequences from this study (Table S2). Sites were considered to have experienced statistically significant positive or negative selection based on the following cut-offs: posterior probability (PP) > 0.90 for FUBAR and LRT ≤ 0.05 for MEME. Nucleotide and amino acid-specific motifs were analyzed using an open-source computer software for viral epidemiology [31] which detects signature patterns [32].

2.6 | Structural Modeling

The 3D model of the WNV envelope in tetrameric form (composed of two E-M couple proteins) and embedded in a lipid bilayer to model the membrane, was built following the same protocol adopted for the similar dengue virus envelope [33]. In particular, the 7KVA pdb (WNN Kunjin strain) [34] was used as template and the K399R mutation was modeled with UCSF Chimera v1.17.3 [35], using the rotamer tool, and selecting the most probable angle for the amino acid side chain orientation [36]. The whole WNV envelope, composed of 90 copies of E-M tetramers, was obtained with the UCSF Chimera multiscale model option, using the 7KVA pdb [37]. Visualization of the models was performed with VMD v1.9.4a53 [38].

3 | Results

Thirty-nine near full-length sequences of WNV lineage 2 genome were obtained from collected samples: 26 urine pellets from WNV infected patients (61.5% of whom presented neuroinvasive disease) and 13 animal/vector samples (six *Corvus* pools, six from three horses, and one *Culex pipiens* pool). Human and animal samples were collected in a period spanning from July 9, 2025 to September 11, 2026. Sampling covered the main affected provinces of Lazio (Latina [LT], Rome [RM] and Frosinone [FR]) and one human sample originated from a patient resident in Campania. More in details: 27 out of 39 samples were collected in Latina province (LT), three from Frosinone (FR), 6 from Rome (RM) and 1 from Caserta province (CE) in Campania region (Table 1).

3.1 | Phylogenetic and Bayesian Phylogeographic Analysis

Using a curated alignment of 2,270 WNV genomes spanning broad temporal and geographic coverage in Europe, phylogenetic reconstruction placed the 39 Lazio/Campania WNV lineage 2 genomes in a well-supported clade adjacent to seven Northern Italian isolates from 2013 to 2014 (Figure 1). A single non-synonymous substitution in the envelope gene (K399R) was fixed among all 2025 outbreak genomes and was absent from Italian sequences collected between 2013 and 2024. Such mutation was found in sequences from samples collected in South Italy in summer 2024 [39]. Comparable K399R-containing genomes were reported from Eastern Europe (Hungary, Slovakia) since 2017. To illustrate the distribution of the mutation across the phylogeny, we reconstructed a maximum-likelihood tree of the complete polyprotein that explicitly displays both K399R-positive and K399R-negative sequences; K399R status is annotated on the tree to highlight the mutation's phylogenetic distribution (Figure 2). The latter shows that the WNV (collected in 2025 in Lazio and Campania regions) clustered together from a single node with 92% of bootstrap, such node belongs to a cluster with other strains collected in different European region in the previous years (from 2021). An exception is represented by the strain derived from a patient's sample collected in Lazio in summer 2025 (Pt 426). Such

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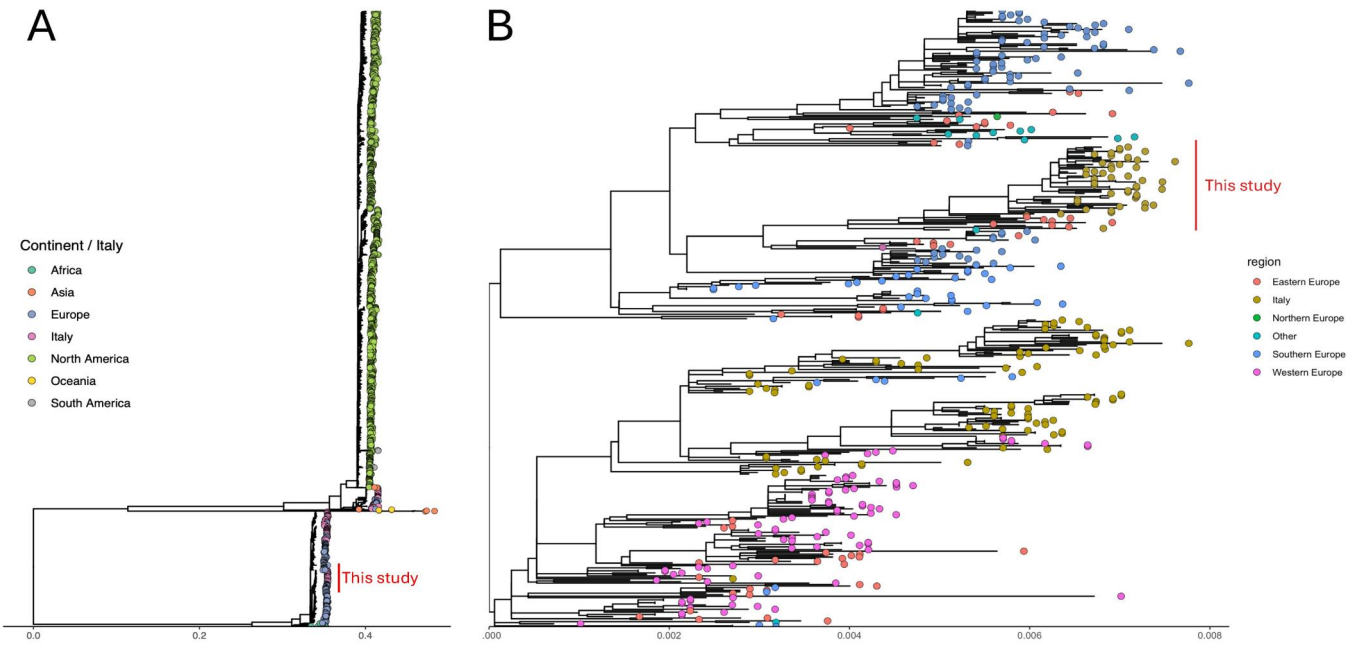


FIGURE 1 | Global phylogeny of West Nile virus (WNV) with placement of Italian sequences generated in this study. (A) Maximum-likelihood phylogeny of 2270 global WNV genomes reconstructed using IQ-TREE. Tips are colored according to continent of sampling (Africa, Asia, Europe, North America, Oceania, and South America) or Italy as origin. The position of the sequences generated in this study is highlighted in red. (B) Zoomed-in view of the clade containing the Italian sequences obtained in this study. Tips are colored by European region (Eastern Europe, Italy, Northern Europe, Southern Europe, Western Europe) and Italy as origin. The position of the sequences generated in this study is highlighted in red. In both trees, branch lengths are proportional to the number of nucleotide substitutions per site.

sequence is separated from the others and belongs to a sub-cluster defined by significative bootstrap (99%) with sequences collected in Hungary and Kosovo between 2022 and 2024.

Furthermore, evolutionary analyses were obtained that indicate that this substitution is currently evolving neutrally, with no evidence of positive selection or functional constraint. Its emergence likely reflects stochastic evolutionary processes rather than an adaptive shift, although continued surveillance will be essential to determine whether this mutation acquires phenotypic relevance as the outbreak progresses.

Phylogeographic analyses revealed two main WNV lineage 2 clades in Italy: a Northern clade circulating previously, between 2013 and 2014, that was not intermixing with the Southern clade. The Southern clade showed two major diversification periods relevant to the current Italian epidemic. The earliest well-supported node showed a time to the most recent common ancestor (TMRCA) in May 2010 (95% highest posterior density [HPD]: November 1996–July 2021), corresponding to the emergence of the Southern clade, with the INMI-Pt426 strain from Boville Ernica separating from the rest of the outbreak and potentially representing a second Southern sub-clade co-circulating with the Southern clade. This is also supported by the genetic distance that indicates that the INMI-Pt426 strain is quite distantly related to all other strains circulating in Lazio and Campania (Table S4). A diversification event inferred around April 2020 (95% HPD: August 2015–December 2023) gave rise to the Southern clade, which includes the majority of recently generated Italian sequences, responsible for the 2025 outbreak in Southern Italy (Figure 3A). Italian genomes from Lazio and Campania clustered within this 2020-emergent clade, with well-supported posterior values and clear spatial structuring across provinces. Discrete phylogeographic analysis showed strong evidence of viral movement among Italian provinces, with Bayes Factor (BF) > 100 for several transition routes (Figure 3B). The most strongly supported dispersal events included Caserta to Frosinone, Caserta to Latina, and Latina to Rome, indicating repeated southern to central region disseminations within the 2023–2025 transmission seasons (Table S5). Additional transitions with moderate support (BF 5–100) linked northern provinces of Rovigo to Cremona to Pavia, suggesting an interconnected transmission network in the Po Valley.

3.2 | Structural Impact of the K399R Variant in the E Protein

We evaluated the structural impact of the unique K399R variant found in the Southern clade. Figure 4 shows the 3D model of WNV (in the INMI sequence of patient #1, Acc. Number PXO24932) in tetramereric conformation (composed by two E-M proteins). The K399R variant is located at the C-terminal region of β7 (G391-K399, yellow in Figure 4), part of an anti-parallel β-sheet composed also by strands β3 (I340-V343, pink in Figure 4), and β6 (G380-R388, green in Figure 4), with β6 at the center [40]. Together with a second β-sheet composed by β1, β2, β4 and β5, they form the Envelope Domain III (EDIII) of WN, the receptor-binding domain and the main target of neutralizing antibodies [41]. An enlargement of the β-sheet in which K399R is located is shown in Figure 4, panel B, with R399 represented in Van der Waals (VdW) surface.

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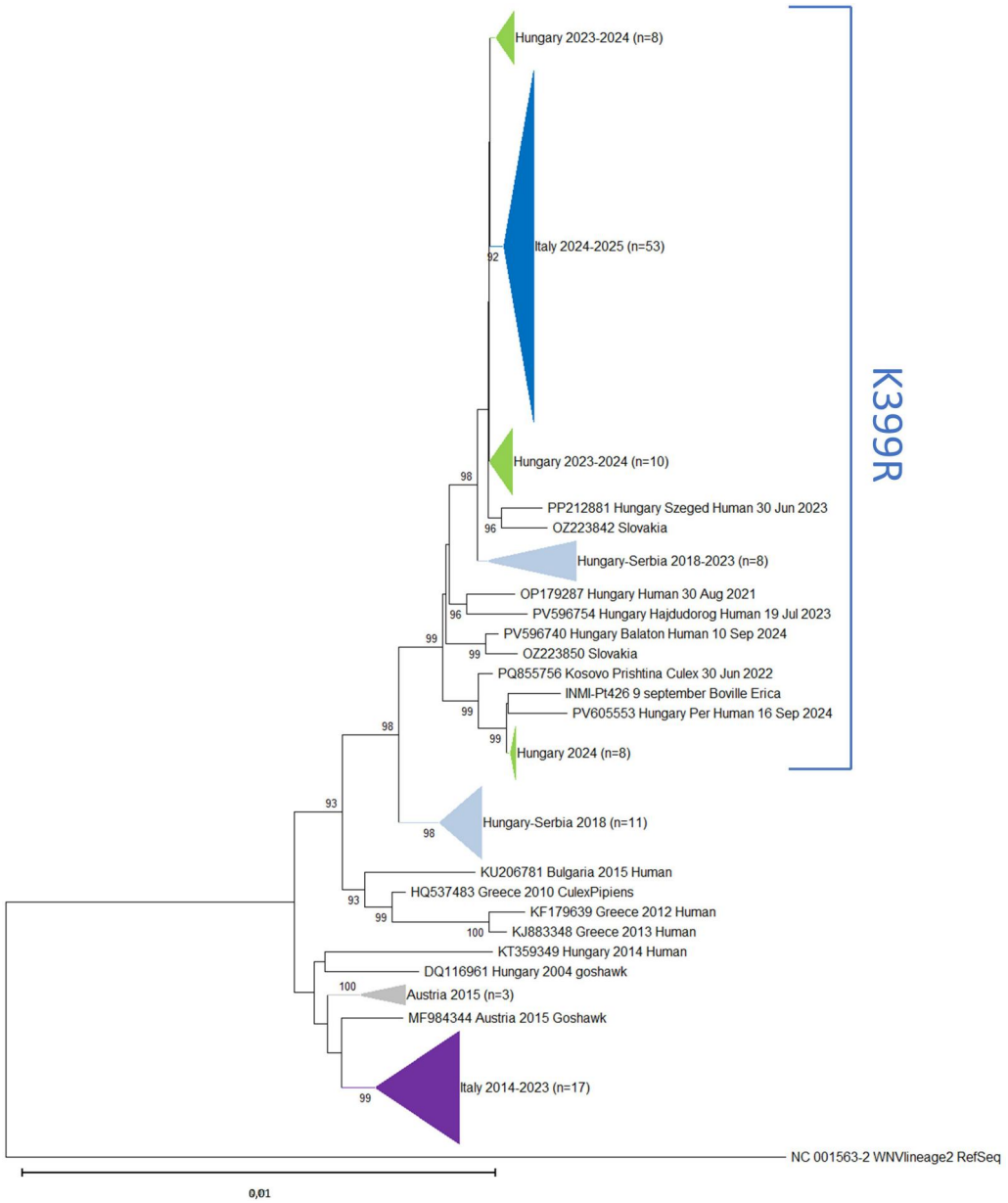


FIGURE 2 | Maximum Likelihood phylogenetic tree. 135 sequence Maximum Likelihood phylogenetic tree, rooted on the reference genome *NC_001563.2*. Node support was assessed with 1000 bootstrap replicates, and bootstrap values ≥ 90 are reported at the main nodes. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. Geographically and temporally coherent clusters are highlighted with colored triangles and annotated with origin and the number of isolates.

We also modeled the whole E-M envelope (composed of 90 E-M tetramers) and, again, highlighted the position of R399 in the VdW surface (see Figure 4C).

Both models show how the positively charged residue 399 is in a key position to interact with the negatively charged phospholipid heads of the membrane, potentially influencing indirectly the structure and dynamic properties of EDIII, a domain that plays a role in receptor and antibody binding.

4 | Discussion

The 2025 WNV epidemic in Italy represents the largest episode recorded in the country to date in terms of both number of human cases and geographic spread, surpassing the previous peak observed in 2018, which was mainly concentrated in Northern Italy. Genomic and phylogeographic analyses here state that the strains circulating in Lazio and Campania belong to a Italian Southern clade that diversified around 2020, with evidence of earlier circulation in 2024 but without triggering detectable human transmission. Our phylogeographic findings defined Central Italy as an important node for sustained WNV lineage 2 spread. The combined phylogenetic and phylogeographic results point to ongoing clades co-circulation and recurrent short-distance dispersal pattern within Italy. This would suggest a distinct epidemiological shift, characterized by the intense involvement of the Central Italian Tyrrhenian side. Thus, changes in the pattern of WNV lineage 2 cases consisted mainly of a broader geographical distribution of human cases,

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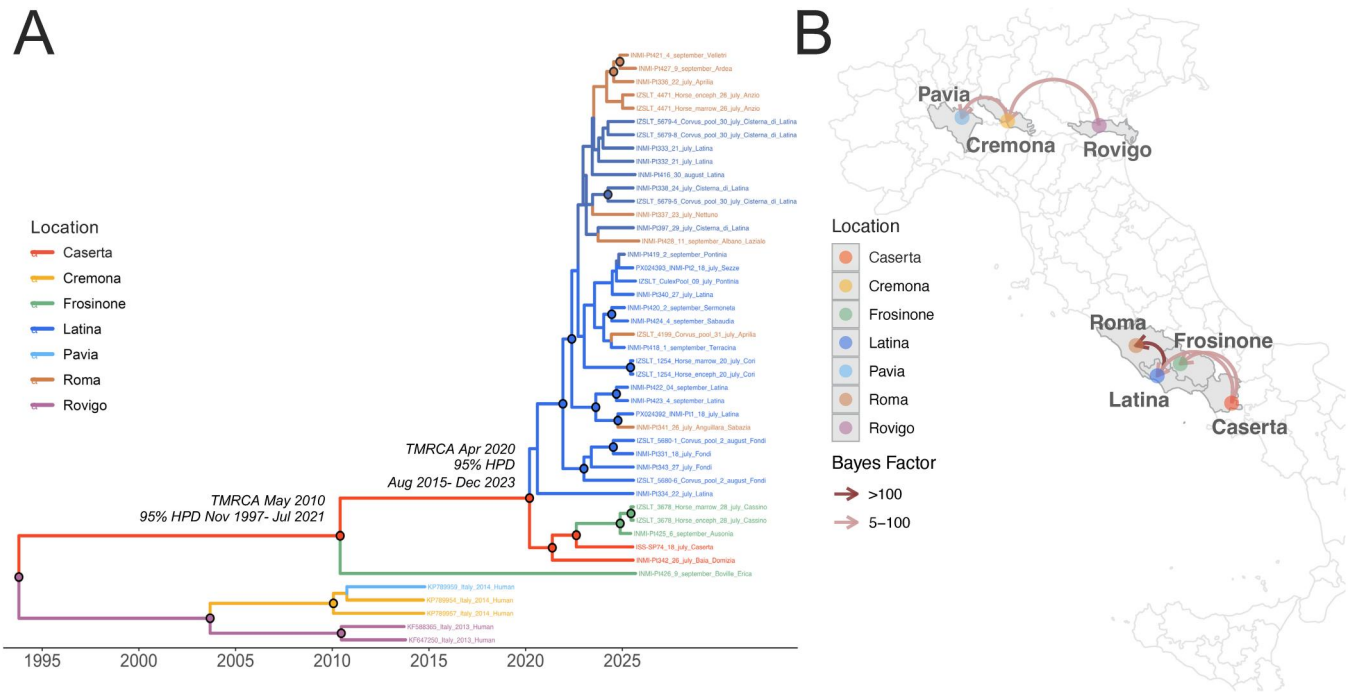


FIGURE 3 | Time-scaled phylogeny and phylogeographic reconstruction of the WNV outbreak in Italy. (A) MCC tree reconstructed using a Bayesian phylodynamic framework, showing the temporal evolution and geographic structure of the Italian WNV outbreak in reference to closest sequences from a previous outbreak in Northern Italy. Branches are colored according to the inferred province as sampling location (Caserta, Cremona, Frosinone, Latina, Pavia, Roma, and Rovigo). Node circles represent posterior probability support > 0.9 and are colored by inferred province of origin. The time to the most recent common ancestor (TMRCA) of the Italian outbreak cluster is estimated around January 2020 (95% highest posterior density (HPD) interval: June 2015–December 2023), while deeper ancestry within the data set traces back to approximately February 2010 (95% HPD: May 1996–April 2021). (B) Spatial reconstruction of WNV phylogeographic transitions among Italian provinces inferred using a discrete trait phylogeographic model. Arrows represent statistically supported viral movements between locations, with line thickness and color indicating the strength of support measured by Bayes factors (BF > 100 or BF = 5–100). The map highlights the inferred dispersal routes linking northern and central Italian regions, including Rovigo, Cremona, and Pavia in northern Italy and Roma, Latina, Frosinone, and Caserta in central–southern Italy.

also confirmed by the detection of the virus in mosquitoes and animal species [15].

Within this framework, the identification of the K399R substitution provides additional insight into the evolutionary context of the outbreak. The mutation lies at the C-terminal end of the WNV envelope protein domain III [40], a region involved in receptor engagement and targeted by neutralizing antibodies [41], although this specific residue has not been implicated in functional or antigenic changes. The substitution is shared by all strains from the recent outbreak but shows no evidence of positive selection, consistent with neutral evolutionary drift. The same mutation was detected in strains circulating in southeastern and central-eastern European countries in previous years (2018 and 2024), suggesting that the Italian 2025 Southern clade belong to a broader regional viral continuum. This continuum forms a geographical corridor extending from the Eastern to Central Europe and is compatible with repeated introductions into Italy through established migratory flyways of wild birds, which remain the primary long-distance dispersal mechanism for WNV across Europe [42]. The K399R substitution is shared among all strains from the Southern clade outbreak but shows no evidence of positive selection, consistent with neutral evolutionary drift. Continued surveillance is foreseen to help determine whether this mutation may confer relevance in antibodies and/or receptor interactions for vectors or hosts virus propagation and monitor the eventual emergence of other mutations with possible impact on viral fitness.

The persistence of WNV in temperate regions is well documented [43–45] and the combination of our genomic data with the spatial clustering of cases is consistent with the hypothesis of overwintering in diapausing *Culex* mosquitoes, which likely bridged the 2024 and 2025 transmission seasons. The previous detection of the same variant in 2024 Italian sequences [39] further reinforces this scenario, indicating that the virus was already established locally, as highlighted by the detection in 2024 for the first time in Campania of cases of neuroinvasive disease and in blood donors, but did not encounter the ecological conditions necessary for efficient amplification.

The sudden emergence of human cases in 2025 therefore appears to reflect a convergence of factors: the presence of an established viral clade capable of overwintering, the ecological suitability of the 2025 season (likely involving temperature anomalies, vector abundance, and avian host dynamics) and repeated viral introductions along migratory routes that may have contributed additional seeding events. These conditions collectively enabled the first autochthonous cases in Lazio and sustained transmission across a wider portion of the country than previously observed.

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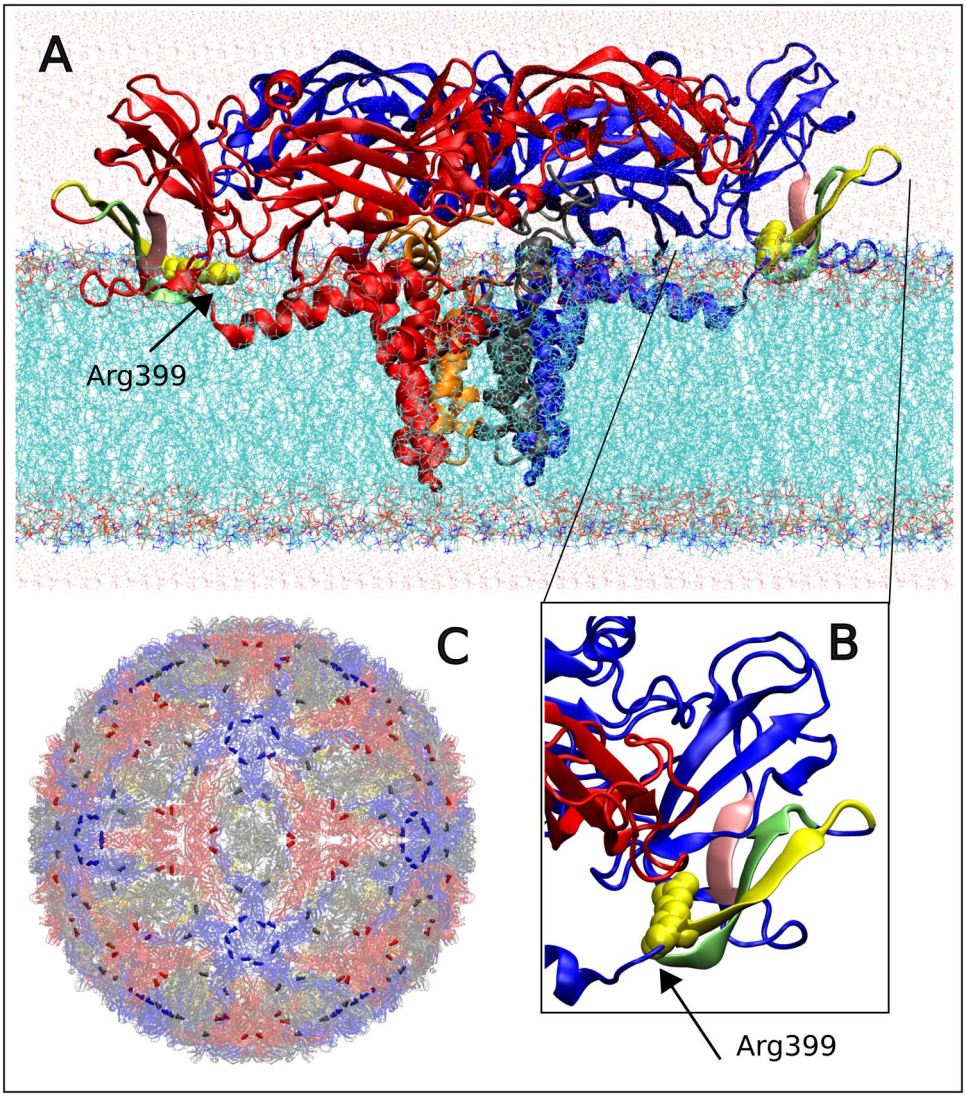


FIGURE 4 | Model of the WNV envelope. The envelope, as in dengue virus, is composed of 360 E-M proteins, organized in tetramers. (A) E-M tetramer for the WNV INMI variant, immersed in a lipid bilayer, modeling its interaction with the virus membrane. The two E proteins are represented in red and blue, while the two M proteins are in black and orange. The anti-parallel β-sheet composed by β3 (Ile340-Val343), β6 (Gly380-Arg388), and β7 (Gln391-Lys399) is shown in pink, green, and yellow, respectively. The Arg399 variant, at the C-term of β7, is highlighted in VdW surface. (B) enlargement of the anti-parallel β-sheet region containing Arg399. (C) Model of the whole WNV envelope. The 360 E and M proteins, organized in 90 tetramers, are colored as in panels A and B. Arg399 is represented in the VdW surface.

Overall, our findings highlight the importance of integrating genomic surveillance, ecological monitoring, and vector–host dynamics investigations, to understand and anticipate WNV spread in Italy. The detection of a mutation shared with Eastern and Central European strains underscores the transboundary nature of WNV circulation, while the evidence for over-wintering emphasizes the need for continuous, year-round surveillance. As climate variability continues to shape vector ecology, such integrated approaches will be essential to mitigate the impact of future WNV circulation seasons.

Author Contributions

Conceptualization: M.R., CN.M., F.C. Methodology: M.R., CEM.G., G.DeC., I.M., CN.M., G.M., D.LaR., JG.PJ., E.S., Fr.C., F.S., A.N. Formal analysis and investigation: M.R., CEM.G., CN.M., F.C.; Writing – original draft preparation: M.R., CN.M., F.C. Writing – review and editing: CEM.G., G.C., C.DeL., A.S., F.V., F.M. Funding acquisition: G.C., F.V., F.M. Resources: C.M., F.R., MT.S., G.V., MG.S., F.V., F.M.; Supervision: G.C., F.V., F.M., F.C. All authors read and approved the final manuscript.

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Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki; human and animals' samples were collected as part of surveillance activities.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Complete genome sequences of WNV from human samples have been submitted to NCBI Nucleotide database with the following accession numbers: PX024392, PX024393, and from PZ212485 to PZ212521.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: jmv71188-sup-0001-Table_S1.xlsx.
Supporting File 2: jmv71188-sup-0002-Table_S2.csv.
Supporting File 3: jmv71188-sup-0003-Table_S3.xlsx.
Supporting File 4: jmv71188-sup-0004-Table_S4.csv.
Supporting File 5: jmv71188-sup-0005-Table_S5.txt.
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