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West Nile virus in Italy: beyond the bird routes

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To my grandmother Ada

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LIST OF SYMBOLS AND ABBREVIATIONS

ABD	Arthropod Borne Diseases
AIC	Akaike Information Criterion
BIC	Bayesian Information Criterion
C	Capsid protein
CAR	Central African Republic
CEC	Central European Clade
CFT	Complement fixation test
CHIKV	Chikungunya virus
CSF	Cerebrospinal fluid
DENV-2	Dengue 2 virus
DRC	Democratic Republic of Congo
E	Envelope protein
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FEM	Edmund Mach Foundation
HIA	Haemagglutination-inhibition test
IFA	Immunofluorescent assay
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IPD	Institut Pasteur de Dakar
IZSAM	Istituto Zooprofilattico of Abruzzo and Molise, Teramo
Kb	Kilobases

KDGV	Kedougou virus
KOUTV	Koutango virus
KUNJ	Kunjin virus
JEV	Japanese Encephalitis virus
L	Lineage
L1a	Lineage 1 clade 1a
M	Membrane protein
NGS	Next Generation Sequencing technology
mNGS	Nucleic acids metagenomic
NS	Non-structural proteins
nt	Nucleotides
ORF	Open reading frame
PrM	Pre-membrane protein
PRNTs	Plaque reduction neutralization tests
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription polymerase chain reaction
RVFV	Rift Valley Fever virus
SoHO	substance of human origin
TDR	Special Programme for Research and Training in Tropical Diseases
WHO	World Health Organization
W-Med	Western Mediterranean
WNF	West Nile fever
WNND	West Nile neurological disease
WNV	West Nile virus
WSLV	Wesselsbron virus

USA	United States of America
USUV	Usutu virus
UTR	Untranslated region
YFV	Yellow Fever virus
ZIKV	Zika virus

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ABSTRACT

Context: West Nile virus (WNV) is an arthropod-borne virus considered a One Health challenge because of its increasing impact on human and animal health. It is one the most widely distributed viruses of the encephalitic *Flaviviruses*. It may cause severe neurological symptoms in humans and animals and is recognized as a serious public health problem also because of its impact on blood transfusion and organ transplantation. First identified in Africa in 1937, it was later introduced and spread in Italy, where in many regions it is now endemic, due to the increasingly favorable climatic and environmental conditions.

Aim: The main objectives of this study, based on an interdisciplinary One Health approach, were: (1) to characterize the geographical distribution within specific host and vector populations in Africa; (2) to describe its phylogeographical patterns between Africa and Europe; (3) to define the genetic structure and epidemiology of Italian WNV strains, giving an insight of the viral circulation dynamics in the Italian territory.

Methodology: Ecological and epidemiological studies were combined with molecular and phylogenetic analyses, carrying out field sampling activities, cellular culture, viral infection, immunofluorescent assay, multiplexed RT-PCR, sequencing, data analysis, and novel technique design. These activities were carried out both in Italy and in Senegal.

Results: Our study evidences: (i) the circulation of several WNV lineages [Lineage 1 (L1), 2 (L2), 7 (L7), and 8 (L8)] in the African Continent; (ii) the presence of diverse competent mosquito vectors in Africa, mainly belonging to the *Culex* genus; (iii) the lack of vector competence studies for several other mosquito species found naturally infected with WNV in Africa; (iv) the need of more vector

competence studies on ticks; (v) the circulation of WNV among humans, animals and vectors in at least 28 African countries; (vi) the lack of knowledge on the epidemiological situation of WNV for 19 African countries, and (vii) the importance of carrying out specific serological surveys in order to avoid possible bias on WNV circulation in Africa (**objective 1**). Furthermore, a new set of WNV L1 and L2 genome-specific primers for tiled-amplicon sequencing have been designed and a consistent dataset of 64 WNV L2 and 31 WNV L1 Italian genomes and of 3 WNV L2 and 7 WNV L1 Senegalese genome sequences from samples collected in Italy and Senegal between 2006 and 2022 has been produced. Twenty more WNV L1 and L2 Senegalese sequences obtained from samples collected in Senegal between 1985 and 2018 have been shared by the Institut Pasteur Dakar of Senegal and added to the dataset. This allowed the conduction of phylogenetic and phylogeographic analyses, evidencing: (viii) the presence of a strong viral connection between Africa and Europe, with intercontinental circulation supported by birds crossing international boundaries while migrating through the African-Eurasian flyways; (ix) the WNV L1 Western-Mediterranean cluster probable spread from Senegal, where the virus was first reported in 1979, to Italy, where the lineage first appeared in Europe in 1998, and to France in 2000, and the presence of back re-introductory events from Italy, Spain, and France to North and West Africa from the 2010s; and (x) the first African introduction of WNV L2 in Europe in Hungary in 2004, possibly from South African countries (**objective 2**). Our study also gives an insight of the dynamics of the viral circulation in Italy, demonstrating: (xi) the endemic presence of WNV L1 and L2 in part of Italy supported by resident wild birds and vector competent mosquitoes mainly belonging to the *Culex* genus; (xii) the current existence of two diverse WNV L1 strains circulating in Italy, one in the North-East, and one circulating intra-regionally in the Campania region; (xiii) suggested characteristic silent periods observed for WNV L1 in the country, with unnoticed circulation lasting sometimes for more than 10 years; (xiv) the 2022 WNV L1 increasing incidence of neurological disease cases in humans; (xv) the presence of genetically stable WNV L2 strains in Italy

with continuous circulation throughout the time; (xvi) the presence of overwintering mechanisms supported by bird-to bird, rodent-to bird, or mosquito-to bird transmission routes; (xvii) the existence of WNV L1 and L2 co-infections in birds and mosquitoes; (xviii) the existence of a continuous transmission of the two strains between Western Mediterranean countries, supported by short distance migratory birds; and (xix) the crucial importance of the surveillance system other than the strategic role of wildlife rescue centers in monitoring both the introduction and circulation of avian emerging zoonotic diseases in Italy (**objective 3**).

Conclusion: Our work points out the existence of high genetic diversity of WNV strains in Africa, the spread of L1 and L2 strains from Africa to Europe, and the existence of continuous transmission episodes among several Western-Mediterranean countries, with few recently suspected back introductory events from Europe to Africa. The progressive increase of the WNV L2 circulation both temporally and spatially in the Mediterranean countries and the WNV L1 re-appearance in Europe, both associated with a significant impact on humans and animal health, other than the strong WNV incidence in Italy and its endemization in part of its territory, evidence a solid WNV epidemic risk for Italy and a persistent threat for WNV spread into new areas. To predict and control future epidemics, it is crucial to constantly monitor the circulation and evolution of WNV in Europe and Africa, and to implement coordinated surveillance plans in both Continents, even in areas which are not currently affected.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Arthropod-Borne Diseases

"Arthropod-Borne Diseases" (ABD) are intended as a group of human and animal infections caused by a series of pathogenic microorganisms transmitted by the bite of hematophagous arthropods or by few other transmission routes [1]. It is commonly known that the infectious diseases transmitted by arthropods are mainly affecting tropical and subtropical regions, nevertheless some of them are still common also in many temperate regions, where, despite being often maintained under surveillance and control, they are becoming a source of rising concern [2]. Indeed, ABD incidence are increasing worldwide, due to several factors such as climate change, population growth, urbanization, and the intensification of global travels [3]. These factors act as drivers for the rapid spread of infections across new areas over their natural range, emerging in countries and continents which have never experienced these diseases before [3]. The threat from new emerging and re-emerging viruses has markedly increased in recent decades. At present, they account for about 50% of the global infectious diseases reported every year to the WHO/TDR (https://tdr.who.int/), comprising 17% of all communicable diseases in humans, and causing one billion cases and one million deaths per year [4]. The "heavy" economic and public health impact of ABD is expected to continue and increase shortly, profoundly restricting the socio-economic status and development of the poorest tropical and subtropical countries. On the other hand, a sudden epidemic of an inexperienced tropical disease occurring in a large city of a developed country could have a devastating impact [2].

One of the most widespread ABD worldwide is West Nile virus (WNV) [5], which was first isolated in 1937 in Uganda [6] and then spread throughout the world [7].

1.2 West Nile virus

WNV is the most widely distributed virus of the encephalitic flaviviruses, belonging to the genus *Flavivirus*, family *Flaviviridae*, and member of the Japanese Encephalitis virus (JEV) serocomplex, that includes other viruses such as Usutu virus, Kokobera, Stratford, Saint Louis encephalitis, Alfuy encephalitis, Murray Valley, and Japanese encephalitis [5,8–11]. It is a vector-borne pathogen of global importance, affecting human and animal populations [12]. WNV was isolated for the first time in Africa in 1937 in the West Nile district of the Northern province of Uganda, following a campaign aimed at monitoring the circulation of the Yellow Fever virus [6]. In the beginning, it was not considered a highly pathogenic virus, but it soon started being detected in many other African countries [11,13,14], then spreading outside East Africa since the 1950s, and soon reaching Europe, America, Asia, and Oceania, becoming a serious public health concern worldwide [15].

1.2.1 West Nile virus transmission cycle

The virus is maintained in nature among vector-competent mosquitoes and a wide variety of reservoir host bird species [16]. The main mosquito vectors belong to the *Culex* complex in Europe [17,18] and in Africa [14,19–21]. Other mosquitoes, such as *Aedes* or *Ochlerotatus* can also act as bridge vectors, although they are considered less efficient transmitters [22,23]. Adult female mosquitoes might take an infected blood meal from a viremic host, contracting the virus horizontally. Mosquitoes can also contract the virus through vertical transmission, during which the adult female mosquito transmits the virus to its offspring, although at low rate [24–26]. Evidence for vertical transmission is proved by the detection of adult male mosquitoes naturally infected by WNV [26]. Once inside the mosquito body, the virus replicates in the midgut cells and moves to the salivary glands, from where it can be transmitted to other hosts during a new mosquito blood meal [22]. Other WNV vectors include ticks, although their vectorial capacity is considered low [14,27,28].

Birds are the main reservoir hosts and ensure the spread of the virus from affected to unaffected regions through their migratory flows [29]. Bird species most involved in the viral cycle are the ones belonging to the order Passeriformes [16], but the virus has been detected also in the orders of Piciformes, Columbiformes, Charadriiformes, Falconiformes, Strigiformes, Anseriformes, Psittaciformes, and Galliformes [5]. Even though the majority of WNV infections in birds are usually mild or asymptomatic [30], some species, such as birds of prey, jays, and crows, are highly susceptible and can develop severe and even fatal encephalitis [16,30,31]. Clinical symptoms associated with WNV infection have been mainly reported in the orders of Passeriformes (corvids, blue jays, magpies) and Falconiformes (birds of prey) in Europe and the USA [32,33]. In Europe, fatal infections have been for example described in European eagles in Spain [34,35], and geese and poultry in Hungary [36]. In Africa, no clinical signs have been reported in birds so far, except one pigeon in Egypt in 1953 [37].

Many other vertebrate hosts, such as reptiles, amphibians, and mammals are susceptible to the virus [38]. Humans and horses are considered dead-end hosts, meaning that they are susceptible to the infection but with a low level of viraemia, being unable to further infect mosquitoes [5,39]. For this reason, the WNV epidemiology is primarily driven by avian hosts and vector mosquitoes, and not by humans, as in the case of other flaviviruses or parasites such as Dengue or Malaria [12,14].

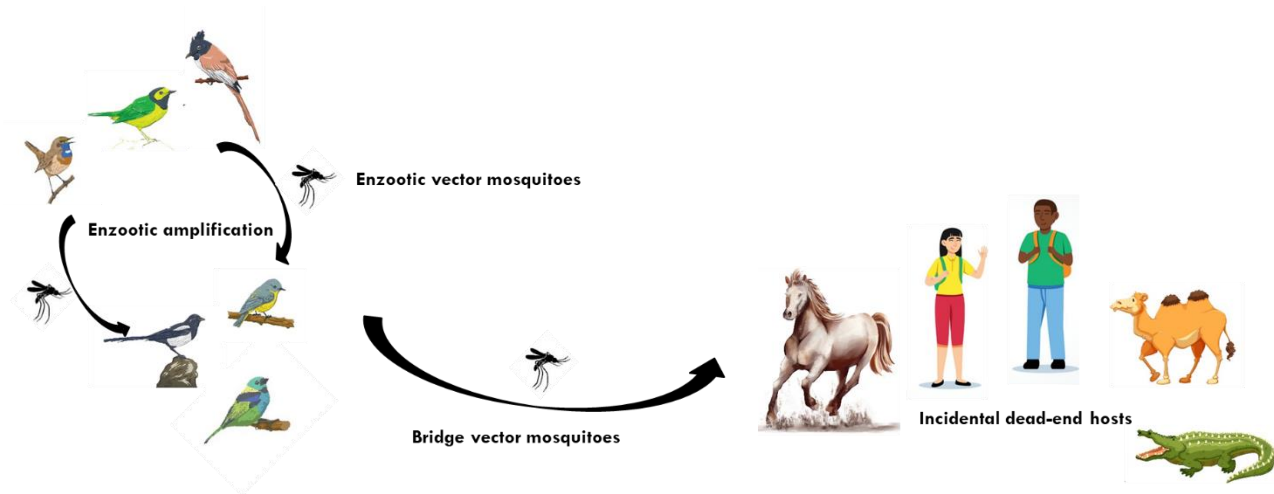

Figure 1.1 West Nile virus transmission cycle.

1.2.2 WNV in humans

WNV is often asymptomatic in humans (80%), however, some infections (20%) can lead to febrile illness (West Nile Fever, WNF), characterized by fevers and maculopapular rash (50% of cases), headache, myalgia, arthralgia, malaise, anorexia, nausea, vomiting, diarrhea, abdominal pain, retro-orbital pain, pharyngitis, and lymphadenopathy [40–44]. In 1% of these cases, mainly in elderly or immunocompromised people, it can lead to neurological disease (West Nile Neuroinvasive Disease WNND) characterized by encephalitis, meningitis, and acute flaccid paralysis [40–44]. In 0.1% of people, the virus can lead to fatal outcomes [45]. Pancreatitis, orchitis, myocarditis, hemorrhagic fever, nephritis, hepatitis, and rhabdomyolysis may be rare non-neurological infections associated with WNV [42]. A biphasic fever, usually helpful for WNV diagnosis, is an additional characteristic feature [46]. After recovery, patients may present long-term sequelae, such as persistent movement disorders, functional disabilities, weakness, cognitive impairments, and, in some cases, early death [47,48]. WNV is mostly transmitted to humans naturally through mosquito bites. Other transmission routes include the possibility of infection through substances of human origin (SoHO) such as blood

transfusion, organ, tissues, or cell transplantation from an infected donor. Breastfeeding and intrauterine transmission have also been documented [49–51]. Technician and laboratory workers may also be infected by WNV during manipulations of infected material through percutaneous injections or potentially through the inhalation of droplets [50–55]. Human behavior can influence the likelihood of contracting WNV disease [14]. In fact, the exposure to mosquito bites, when coupled with the presence of infected mosquitoes and birds, can result in an increased probability of getting infected [14]. The incubation period may vary between 2 and 15 days after exposure to the virus which can last for 3 to 6 more days [14,42]. Acute form of WNV may persist for up to 60 days and can be characterized by a biphasic condition [14]. No treatment or specific prophylaxis has been proven to be effective for WNV infection. The only available therapy is supportive (intravenous fluids, nursing care and pain medication), solely affecting WNV clinical manifestations [43]. Despite the presence of treatments and vaccination in horses, vaccines are still not available in humans [5,52,56].

1.2.3 WNV genetic organization and viral replication

The West Nile virion has an icosahedral symmetry and a diameter of about 50 nm. Its structure is made of an envelope with a nucleocapsid enclosing the genome [57]. Like other Flaviviruses, WNV is a single-stranded, positive-sense RNA virus, constituted of about 11 kilobases (kb), containing one long single open reading frame (ORF) confined at each side by one 5' and one 3' untranslated-region (UTR), respectively [12,58,59]. The WNV 5′UTR region, made of 96 nucleotides (nt) and highly conserved compared to other members of the flavivirus family, contains two stem-loops, the SLA and SLB, involved in viral cyclization, replication, and virulence [60,61]. The single ORF encodes for a polyprotein that is processed in three structural proteins - capsid (C), pre-membrane (prM) and envelop (E) - and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) [12,62]. Each of the viral proteins, either structural or non-structural, play a different and specific role

in the biology and/or the pathogenesis of WNV infections. The three structural proteins make up the mature virion and are responsible for host specificity, tissue tropism, replication capacity, and cells T and B response induction [63–65]. The non-structural proteins are multifunctional and play critical roles in the viral RNA synthesis, virion assembly, cellular signaling, and in mechanisms of host's immune response evasion [12].

A summary of the structural and non-structural protein functions is shown in Table 1.1.

Table 1.1 West Nile virus structural and non-structural proteins and their functions

Proteins	Type of protein	Main functions	References
Capsid (C)	Structural	Genomic RNA packaging into nucleocapsid structures; prM protein translocation into the endoplasmic reticulum (ER).	[61,64,66]
Pre-membrane (prM)/Membrane (M)	Structural	prM: promotes the correct folding of the E protein during transit through the secretory pathway and is involved in the virion assembly; M: small proteolytic fragment of the precursor prM, protects the immature virion from a	[12,61]

Proteins	Type of protein	Main functions	References
		premature fusion with the host cell.	
Envelop (E)	Structural	WNV cell tropism determination through the binding to entry receptors and the fusion with target cells.	[63–65]
NS1	Non-structural	Virus replication and maturation; innate immune system regulation and evasion; viral stability in the host cells; and complement system down-regulation.	[12,67–73]

Proteins	Type of protein	Main functions	References
NS2A	Non-structural	RNA replication; host immune modulation; induction of inhibition of the host α/β interferon; virus virulence regulation.	[61,74]
NS2B	Non-structural	NS2B/NS3 stable complex formation; cleavage of the flavivirus polyprotein necessary for viral infection; viral replication.	[61,75]
NS3	Non-structural	Protease activity through cleaving the other non-structural proteins from the polyprotein (NS2B/NS3);	[61,62,76–80]

Proteins	Type of protein	Main functions	References
		antiviral target (NS2B/NS3); RNA helicase (NS2B/NS4); and NTPase (NS2B/NS5).	
NS4A	Non-structural	Proteolytic processes; virus replication; viral RNA synthesis; immune evasion; viral membrane rearrangement; interferon α/β host response inhibition; NS3 helicase ATPase activity regulation.	[62,76,81–83]
NS4B	Non-structural	Interferon α/β antagonist; Immune evasion; virus replication.	[84]

Proteins	Type of protein	Main functions	References
NS5	Non-structural	Virus replication; RNA capping; evasion of the innate immune response and virulence determinant; interferon α/β antagonist	[61,64,76,85–88]

1.2.4 West Nile virus genetic diversity and distribution

WNV is characterized by high genetic diversity, with a single serotype and at least eight different lineages: Lineage 1 (L1) to Lineage 8 (L8) [89]. The most widespread and pathogenic are L1 and Lineage 2 (L2), responsible for numerous outbreaks and deaths in humans and animals [5,90,91].

L1 is characterized by three clades: a, b, and c [9]. Clade 1a (L1a) is the most widespread in Africa, America, and Europe [9]. Clade 1b, also known as Kundjin virus, has been registered only in Australia [92], while clade 1c is mainly distributed in India [93], showing only one endemic genotype for each of these two countries, probably due to one single successful introduction [11].

L1a has a wide geographical distribution. It was first detected in Northern African countries, causing numerous neurological disorders and deaths in Egypt (1951), Algeria (1994), Morocco (1996), and Tunisia (1997) [36,94], the latter reporting a big number of outbreaks and severe human encephalitis and deaths [41,52,95,96]. It has also been detected in other African countries, such as South Africa [97], Senegal [5,21], Central African Republic (CAR), Kenya, and Cote d'Ivoire, but a significant impact has not been observed in these countries [8,98]. It spread to the Middle East in 1998 (Israel) and to the USA (state of New York) in 1999 [99].

In Europe, WNV L1a was observed for the first time in the middle 1960s in France, Portugal, and Cyprus [100–102]. It re-appeared more than 30 years later in Italy in 1998 and in France in 2000, causing numerous infections among humans and animals [103,104]. The clade responsible for these epidemics belonged to the Western-Mediterranean (W-Med) clade [104]. In the same years, another clade, the Eastern-European clade, was responsible for human infections in Romania (1996) and Russia (1999) [105,106]. In the early 2000s, WNV L1 infections belonging to the W-Med clade were reported again in France and Romania [107,108]. Since 2005, WNV L2 appeared in Europe and co-circulation of both lineages was observed [36,109], with WNV L2 exponentially increasing, and WNV

L1 being reported only sporadically in Spain, France, Portugal, Romania, Bulgaria, Turkey, Cyprus, and Serbia [64,102,110–113].

In Italy, after its first appearance in 1998, WNV L1 re-appeared in 2008 in North-Eastern regions, causing severe infections in humans and horses [114,115], and spread to Southern Italian regions in 2010-11 [116]. Progressively, it started being reported only sporadically in the country in North-Eastern regions (2012–2014, 2017), Sardinia (2015–2016), and Campania (2020) until 2021-22, when it re-emerged causing severe neurological cases and deaths in humans and horses, and infections in wild birds and mosquitoes [117].

L2 emerged around 1936 in Africa, with uncertainties on the exact area of its origin: the most quoted hypothesis is Uganda with few studies addressing South Africa, where it is often detected, probably due to its high circulation and to the efficiency of the public health system [118]. It is characterized by several clades. Clade a, probably originated in Uganda, was first detected in the 1960s and includes a sequence isolated in Uganda in 1963, and two sequences isolated in Senegal in the 1990s. Clade b, probably originated in South Africa in 1949, includes several European and Russian sequences, other than other subclades with sequences from Madagascar and South Africa. Besides clade a and b, an outgroup is formed by a sequence isolated in Uganda in 1937 [118].

In Africa, L2 is endemic in Southern Africa and Madagascar, other than being detected in Mozambique, Botswana, Namibia, Uganda, Senegal, Kenya, and CAR [21,39,119–121]. It was for a long time regarded as less pathogenic than L1, until it provoked severe pathological conditions in South Africa and encephalitis among birds and humans in Europe [5], where it was detected for its first time in Hungary in 2004 [36,122].

The strain, belonging to the Central European clade (CEC), was again reported in Hungary and Austria between 2008 and 2009 [123], in Italy and Greece in 2010 and 2011 [123–126], and in Serbia in 2012 [127], spreading faster to many other European countries [39,94,123]. At the same time, a different

clade, the Russian/Romanian clade, emerged in Russia in 2004-07 [128], spreading to Romania in 2010 [129]. The strain belonging to CEC kept circulating in Europe where an exceptional number of infections occurred in 2018, considered the year with the largest L2 outbreak observed in Central and Southern Europe, with over 2000 symptomatic human cases, most of them reported in Italy [130–132]. More recently, cases were detected in France [102], Germany [133], Czech Republic [134], Netherlands [135], Spain [89], and Italy, the latter being again one of the most affected countries, reporting co-circulation with the L1 strain, and numerous infections in humans and animals [117]. Nowadays, L2 is the most prevalent lineage circulating in the European Continent [102,136].

Other less widespread lineages are L3, also known as Rabensburg virus, present in southern Moravia and Czech Republic [137]; L4, isolated and reported in Russia from ticks, mosquitoes and frogs [138]; L5, isolated in India [93], L6, based on a small gene fragment, isolated in Spain and probably evolving from distinct introductions into the Northern Hemisphere [112], and putative L9, isolated in *Uranotaenia unguiculata* mosquitoes in Austria [139].

While six lineages have been so far identified in Europe [140], in Africa four different lineages have been detected until now: L1, L2, L7, and L8 [147,5]. L7, isolated in ticks and rodents in Senegal [141], and in sandflies in Niger [142], was considered a separate lineage, but it is nowadays classified as a different virus: the Koutango virus (KOUTV) [143].

All lineages are phylogenetically very similar among them, suggesting local and long-range WNV circulation through migratory and resident birds [98,103].

1.2.5 WNV diagnosis and genetic characterization

WNV can be diagnosed by different molecular and serological tests. Serological tests are not able to distinguish WNV different lineages, while rapid molecular tools can be used to recognize the great genetic diversity of the virus [144]. In particular, the reverse transcription polymerase chain reaction

(RT-qPCR) assay is a molecular test that allows viral detection (WNA RNA) in the acute phase of the disease. WNV RNA can be present in the urine of patients for several weeks after the symptoms onset while in sera and cerebrospinal fluid (CSF) is not always detectable [43].

Serological analyses include i) immunoglobulin M (IgM) antibody capture enzyme-linked immunosorbent assay (ELISA), used to investigate a WNV recent infection, and ii) immunoglobulin G (IgG) antibody sero-conversion (ELISA), used to examine the presence of previous infections [43,46,145]. IgM antibodies appear during the first week of illness (2-8 days), peak at the second week of infection, and slowly decrease, persisting for up to two months in the 50% of patients. IgG antibodies appear after IgM antibodies (generally at 12 days from the beginning of symptoms) and persist for many years after symptomatic or asymptomatic infections [145,146]. Serological tests are not 100% specific and reliable due cross-reactions between diverse flaviviruses [40,46]. Consequently, plaque reduction neutralization tests (PRNTs) are used, only in reference laboratories, to confirm the presence of anti-WNV specific antibodies, detected by ELISA assays, as well as titration method [146]. Besides that, hemagglutination-inhibition test (HIA), complement fixation test (CFT), micro-sphere immunoassay, indirect immunofluorescent (IFA) assays, and virus isolation in Vero, mosquito cell lines, or mice brain tissues are used to detect WNV. However, they are not very convenient because they are time-consuming, characterized by low sensitivity to early infections, and technically difficult [14,146].

Nowadays, many studies within the One Health context are focusing on the design of novel techniques for the characterization of emergent virus diverse strains. The genetic characterization might help to track the routes of viral introduction, being critical in epidemic management. Many approaches can be used to obtain viral whole genomes [147–149]. Among them, the multiplex PCR-based target enrichment or amplicon-based protocol appears to be characterized by high specificity and sensibility,

allowing to directly sequence clinical samples also with low input titers and under resource-limited conditions, as shown during the COVID-19 pandemic, when it was used for the SARS-CoV-2 genomic surveillance [150–153]. Since WNV has a broad range of hosts and a profound impact on human and animal health, it might be the right target for the development of a fast, reliable, and cost-effective sequencing tool.

1.2.6 Aims and objectives:

The main objectives of this study were: (1) to characterize the geographical distribution of WNV within specific host and vector populations in Africa (Chapter 2); (2) to describe WNV phylogeographical patterns between Africa and Europe (Chapter 3 & 4); and (3) to define the genetic structure and epidemiology of Italian WNV strains, giving an insight of the viral circulation dynamics in Italy (Chapters 5 – 9).

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CHAPTER 2. EPIDEMIOLOGY OF WEST NILE VIRUS IN AFRICA: AN UNDERESTIMATED THREAT

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by

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2.1 Abstract

2.1.1 Background

West Nile virus is a mosquito borne *Flavivirus* which has been posing continuous challenges to public health worldwide due to the identification of new lineages and clades and its ability to invade and establish in an increasing number of countries. Its current distribution, genetic variability, ecology, and epidemiological pattern in the African continent are only partially known despite the general consensus on the urgency to obtain such information for quantifying the actual disease burden in Africa other than to predict future threats at global scale.

2.1.2 Methodology and principal findings

References were searched in PubMed and Google Scholar electronic databases on January 21, 2020, using selected keywords, without language and date restriction. Additional manual searches of reference list were carried out. Further references have been later added accordingly to experts' opinion. We included 153 scientific papers published between 1940 and 2021.

This review highlights: (i) the co-circulation of WNV-lineages 1, 2, and 8 in the African continent; (ii) the presence of diverse WNV competent vectors in Africa, mainly belonging to the *Culex* genus; (iii) the lack of vector competence studies for several other mosquito species found naturally infected with WNV in Africa; (iv) the need of more competence studies to be addressed on ticks; (iv) evidence of circulation of WNV among humans, animals and vectors in at least 28 Countries; (v) the lack of knowledge on the epidemiological situation of WNV for 19 Countries, and (vii) the importance of carrying out specific serological surveys in order to avoid possible bias on WNV circulation in Africa.

2.1.3 Conclusions

This study provides the state of art on WNV investigation carried out in Africa, highlighting several knowledge gaps regarding i) the current WNV distribution and genetic diversity, ii) its ecology and transmission chains including the role of different arthropods and vertebrate species as competent reservoirs, and iii) the real disease burden for humans and animals. This review highlights the needs for further research and coordinated surveillance efforts on WNV in Africa.

2.2 Author summary

Since its discovery in the African continent in 1937, West Nile virus expansion and invasion into new regions represent a serious concern today for an increasing number of countries worldwide. Although about 80% of infected individuals are asymptomatic, this zoonotic virus is pathogenic for humans other than for some animal species, displaying a range of clinical manifestation spanning from influenza-like symptoms to severe neurological complication and death. This study provides an updated overview on the current knowledge of WNV epidemiology in each African country, summarizing available data on incidence of the infection in humans and animals, the circulating lineages and clades, other than an updated list of the principal arthropod vectors found naturally infected and the availability of vector competence studies. However, this review highlights also the lack of knowledge regarding the occurrence and intensity of circulation of WNV in many African countries. Therefore, considering the sensitivity of WNV transmission system to climate and other environmental changes, along with the increasing level of interconnections among continents due to globalization, the intensification of the research activities on WNV and a promotion of a coordinated surveillance actions across African and European countries would provide the information of utility for a better evaluation of the actual risk of WNV and disease burden at trans-continental scale.

2.3 Introduction

West Nile virus (WNV) is a mosquito-borne virus, part of the genus *Flavivirus*, family *Flaviviridae* and member of the Japanese Encephalitis virus serocomplex which includes other closely related viruses such as Saint Louis encephalitis, Usutu, Kunjin, Kookaburra, Stratford, Alfuy and Murray Valley encephalitis [1,2]. WNV is endemo-epidemic in Africa, Europe, Middle East, Asia, and the New World, representing an emerging threat for public and animal health due to the continuous expansion of its range [2,3]. The first description of WNV dates back to 1937 when it was reported from Omogo, in the West Nile district of the Northern province of Uganda following a campaign aimed at monitoring the circulation of Yellow Fever virus [4]. The principal vectors of WNV are mosquitoes, mostly belonging to *Culex* spp. and *Aedes* spp. [5]. Other arthropods found naturally infected with WNV are ticks, although their role as competent vector is still not well understood [6,7]. WNV has been identified in several vertebrate species, especially birds belonging to the order Passeriformes [8]. Other species in which WNV has been reported include Piciformes, Columbiformes, Charadriiformes, Falconiformes, Strigiformes, Anseriformes, Psittaciformes, and Galliformes [2]. The role of these species as real competent reservoir hosts has been proved only for a limited subset [7]. Humans, horses, and other vertebrate hosts are considered WNV dead-end hosts, since they are susceptible to the infection but unable to transmit the virus to mosquitoes [7]. WNV infection is mostly asymptomatic, but a range of clinical forms and symptoms are reported for humans, horses, and birds [7]. In humans, around 20% of cases develop influenza-like symptoms (West Nile fever, WNF), while less than 1% develop the West Nile Neuroinvasive Disease (WNND) with encephalitis, meningitis, acute flaccid paralysis, and occasionally death [2]. The severity of symptoms generally depends on WNV strains involved other than to the general physical conditions of the patients [5]. In domestic animals, such as horses, only 20% of WNV-infected individuals show mild

symptomatic infections while 1–10% are characterized by severe neurological disease with a mortality rate of about 33% [7]. Among birds, corvids and raptors appear highly susceptible to WNV infection, resulting in higher incidence with severe neurological signs that lead the individuals to death [9,10]. WNV currently includes up to nine phylogenetic lineages, identified through phylogenetic analyses: WNV lineage 1 (WNV-L1) to lineage 9 (WNV-L9) [11]. WNV-L7 has been recently classified as a distinct flavivirus, the Koutango virus (KOUTV) [2,12]. Among all these observed lineages, only WNV-L1, L2, and L8, other than the KOUTV, have been detected in Africa [2]. WNV-L1 and L2 are the most important from the public health point of view because they are most pathogenic and widespread, and implicated in several outbreaks worldwide [3,7]. WNV-L1, mainly diffused in Central and Northern Africa, emerged in Europe in the 1960s [3]. After 30 years of silence, it started causing epidemics in North America, Northern African, Western, and Eastern European countries [3]. The main actor of the European scenario was WNV-L1 up to 2004 when WNV-L2, considered endemic in Southern Africa and Madagascar, was reported for the first time in Hungary [3]. Since 2010, it started causing several outbreaks in central Europe and it is nowadays one of the main lineages responsible for WNV infections in Europe [3,7]. Other less widespread lineages are WNV-L3, also known as Rabensburg virus, present in Czech Republic; WNV-L4, isolated and reported in Russia; WNV-L5, isolated in India and often considered as the clade 1c of WNV-L1, and WNV-L6, based on a small gene fragment, isolated in Spain [1,2]. Finally, putative lineage 9, often considered a sub lineage of WNV-L4, has been isolated from *Uranotaenia unguiculata* mosquitoes in Austria [11]. These lineages, never isolated in Africa, might have evolved from distinct introductions into the Northern Hemisphere [13]. Translocation of diverse WNV lineages from the original ecological niches to new geographic areas is generally thought to occur mainly through migratory birds, although the final chain of events that lead to the introduction or reintroduction of the virus into new continents needs further explanations [7]. For example, phylogenetic analyses revealed that all European WNV-

L1 and 2 strains are derived from a limited number of initial independent introductions, most likely directly from Africa, followed by local amplification and spread [14,15]. WNV current distribution, genetic variability, ecology, and epidemiological pattern in the African continent are only partially known despite the general consensus on the urgency to obtain such information for quantifying the actual disease burden in Africa other than to predict future threats at continental and global scale. Therefore, we performed a systematic review with the aim to provide an updated overview of the current knowledge regarding WNV epidemiology in Africa, its major features in terms of geographical distribution, molecular diversity and phylogeography, principal vectors and hosts, human and animal epidemiological patterns. This information would provide an updated overview and data of utility for better quantifying the actual risk and disease burden in Africa other than predicting future threats at global scale.

2.4 Material and methods

2.4.1 Search strategy and selection criteria

Pertinent articles were searched, screened, and incorporated in the Systematic Review according to PRISMA and QUORUM criteria [16]. Relevant background information was obtained by searching on the PubMed and the Google Scholar electronic databases on January 17, 2020 (n = 375), using the search terms "West Nile virus" and "Africa" with no restrictions on the earliest date of the articles returned. Additional records have been identified through contact with experts (n = 33). Studies were classified by topic (West Nile vector-borne disease) and Continent (Africa). Each search was conducted with common variations of the virus name, specifically: West Nile virus, WNV; and the geographic region intended to be studied, specifically: Africa. Full-text original articles were searched. After removal of duplicates, two reviewers independently screened articles by title and abstract. Finally, pertinent records were selected for full-text screening and, if relevant, included in

the review (for details see S2.1 Fig). Documents were included if containing the following information: i) general overview of WNV features and distribution; ii) WNV phylogenetics, including a description of the biology, phylogenetic and phylogeography of WNV lineages over all continents but focusing mainly on Africa; iii) WNV main vectors and animal hosts; and iv) human epidemiology, with all the information related to the virus and the human infection along with a detailed report of molecular and serological studies. Two reviewers processed the document evaluation based on articles designed for full-text review.

2.5 Results

Article's selection process

We identified 408 articles. After duplicates were removed, the remaining 395 records were screened by title, abstract, and full text, resulting in 84 studies which were finally included into the review. Reference lists of the included studies were further screened for relevant research. Following the same eligibility criteria, 69 citations were incorporated in the study. Finally, a total of 153 studies, including 84 full-text reviewed articles and 69 citations were considered.

2.5.1 Genomics and phylogeography

WNV is a biologically diverse virus, characterized by several genotypic and phenotypic changes [2,7]. Phylogenetic analysis, performed through the construction of evolutionary trees, predicted the time for the WNV most common ancestor (tMRCA) to be between the 16th and the 17th century in Africa [2]. The virus evolution led to the formation of two new branches, one characterized by WNV-L1 and L5, and the other by WNV-L2, L3, L4, L8 and L9 (not enough information is available regarding WNV-L6). WNV-L1 was successfully introduced into Europe in the 1960s while it appeared for the first time in North America in 1999, subsequently becoming endemic across both continents [1].

WNV-L2, after its first appearance in Hungary in 2004, showed multiple introductions into Europe [1,2,17]. WNV-L1 is widespread and frequently associated with symptomatic infections in humans and horses [3]. It includes 3 clades (A, B, and C) and several sub-clades [1,2,15,18]. Only Clade A is widespread in the African continent. Clade A is composed by 6 sub-clusters [14]. Among them, the sub-clusters 1, 2, 3, 5 and 6 have been detected in different areas of the Continent [1]. WNV-L1-Clade A strains belonging to the diverse sub-clusters are all phylogenetically very similar to each other, suggesting local and long-range WNV circulation probably through migratory birds [19]. Following the introduction of WNV-L1 in the New World, a huge number of sequences have been obtained overtime [3,14,20]. Interestingly, phylogenetic analysis demonstrated that the strain *PaH001* isolated in Tunisia in 1997 roots the tree of WNV-L1 circulating in North America [14,21]. Furthermore, phylogenetic and genetic distance studies evidenced that the Tunisian strain *PaH001* is closely related to a group of highly conserved viruses collected in America and Israel between 1998 and 2000, suggesting that viruses circulating in the Middle East / North Africa are related to those circulating in North America [14]. A possible introduction of WNV-L1 in Europe from Morocco is also suspected: the closest ancestor of the European strains could be a Moroccan strain which appears to be closely genetically related to French and Italian isolates (France: 2000, 2006; Italy: 1998, 2008) [3]. Up to date, WNV-L1 has been reported in the following African countries: Algeria, Central African Republic, Egypt, Côte d'Ivoire, Kenya, Morocco, Tunisia, Senegal, and South Africa [1,2,14,15,18,19,21–23]. WNV-L2 was considered for a long time to be less pathogenic than WNV-L1, until it evolved [six amino acid substitutions at the level of the E (V159I), NS1 (L338T), NS2A (A126S), NS3 (N421S), NS4B (L20P) and NS5 (Y254F) proteins] becoming more virulent and causing also severe disease forms in South Africa other than among humans and birds in Europe [2,17,18]. It is now endemic, and it is the most prevalent lineage circulating in several African and European countries [18,24]. In Africa, WNV-L2 circulation has been reported in Botswana, Central

African Republic, Congo, Djibouti, Madagascar, Mozambique, Namibia, Senegal, South Africa, Tanzania, and Uganda [1,2,13,17–19,22,23,25–27]. WNV-L2 includes 4 clades (A-D) all circulating in Africa [17,18,27]. Clade A is characterized by strains circulating in Madagascar, Senegal, and Uganda. Clade B is composed of three main subclades: Cluster 1, 2 and 3. Among them only cluster 3 occurs in Africa (Madagascar, Namibia, and South Africa) [17,27]. Clade C is characterized by strains circulating in Madagascar while Clade D, the most widespread, is composed of strains circulating in Central African Republic, Congo, Namibia, Senegal, South Africa, and Uganda [17,27]. The exact origin of WNV-L2 strains and the following route of introduction into Europe are not clear [3,28]. Detected for the first time in Hungary in 2004, WNV-L2 then spread into many European wetland areas, such as the Aliakmonas Delta in northern Greece (2010), and the Po Delta in north-east Italy (2011) [28]. These areas are along the major flyways of birds migrating from Africa, thus supporting the hypothesis of a possible role of migratory birds for the introduction of WNV from African countries into Europe [12,28]. Additional lineages were discovered in Africa as WNV-L7 (KOUTV) and putative Lineage 8 [12,22]. WNV-L7, reported for many years as a separate lineage of WNV [14], has been recently classified as the KOUTV, which is now considered a distinct flavivirus (https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/flaviviridae/360/genus- flavivirus). KOUTV, discovered in the Koutango district of the Kaolack region of Senegal, has been isolated from ticks, rodents, and sandflies [12]. Important studies showed a high virulence of Koutango virus in mice and a potential risk for humans has been highlighted following a severe accidental infection in a Senegalese lab worker [2,22]. KOUTV is exclusively present in Africa, circulating in Senegal, Gabon, Somalia, and Niger [12]. Its invasion into other Continents could represent a possible future threat worldwide. Putative lineage 8 has been isolated from *Culex perfuscus* mosquitoes in 1992 in the Kedougou region of Senegal [22]. It is characterized by low virulence, and this might represent a good feature for making the lineage a good

candidate for a new WNV vaccine [2]. Fig 2.1 shows the currently known WNV lineages reported for 17 countries in Africa.

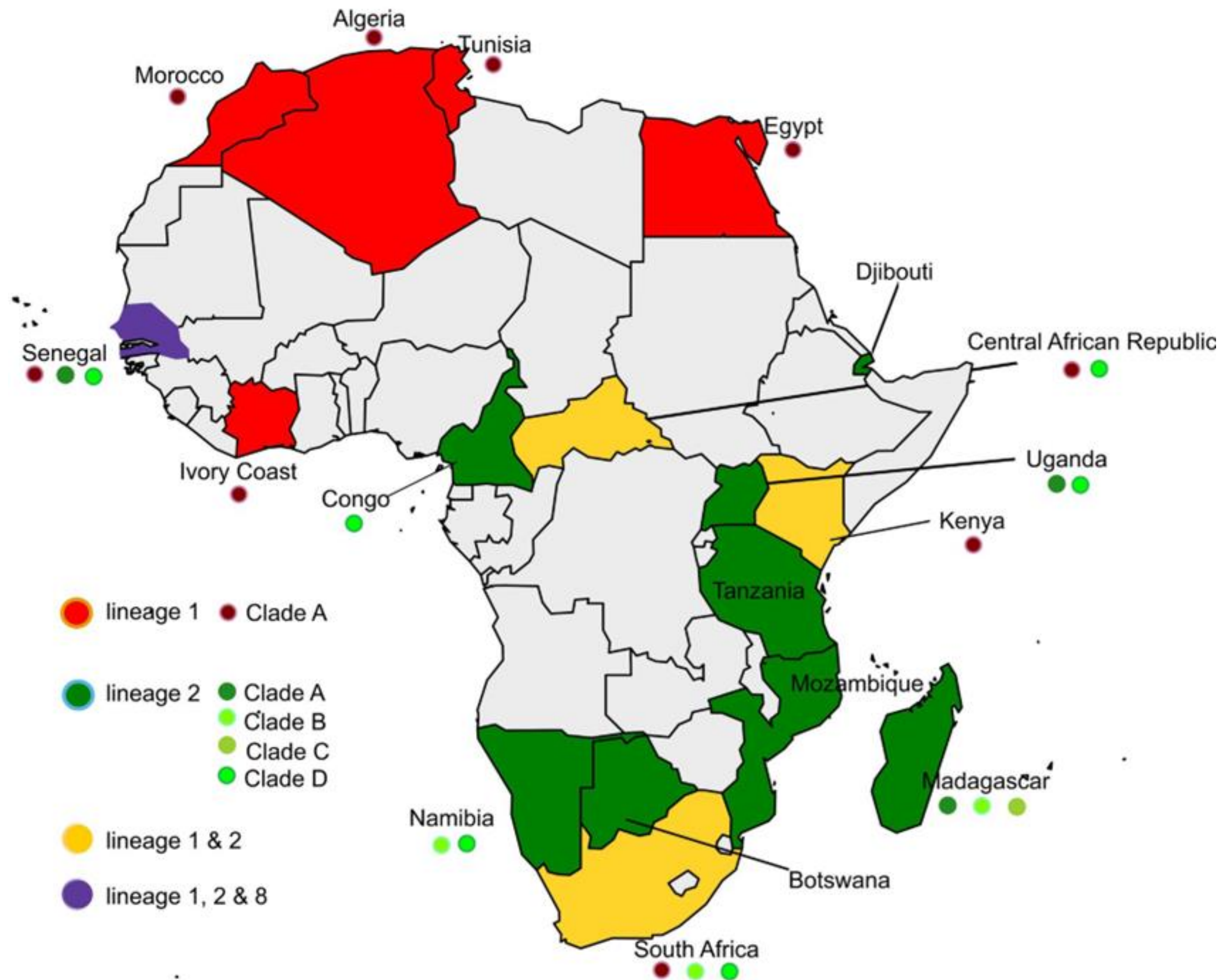

Figure 2.1 West Nile virus lineages reported for 17 African countries.

Map was generated using publicly available shapefiles, https:// smart.servier.com/category/general-items/world-maps/. https://doi.org/10.1371/journal.pntd.0010075.g001

2.5.2 West Nile virus vectors in Africa

In Africa, the most widespread WNV vectors mainly belong to the *Culex pipiens* complex (*Cx. pipiens* s.l.) [7,29–31] which includes *Cx. p. pipiens* Linnaeus, *Cx. p. pipiens* biotype molestus Forskal, *Cx. p. quinquefasciatus* Say, *Cx. p. pallens* and *Cx. torrentium* Martini [32,33]. As summarized in Table 2.1, WNV has been isolated from at least 46 mosquito species but studies to assess their vectorial competence have been carried out, between 1972 and 2016, only for 8 mosquito species (*Cx. quinquefasciatus*, *Cx. univittatus*, *Cx. vansomereni* Edwards, *Ma. uniformis*, *Ma. Africana*, *Cx. pipiens*, *Cx. theileri*, and *Cx. neavei*) in Madagascar, the Maghreb region (Algeria, Morocco, Tunisia), Senegal, Kenya, and South Africa (Table 2.2) [19,22,23,26,27,29,34–50]. These studies provide evidence of the vector competence of *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. vansomereni*, *Cx. univittatus*, *Cx. theileri*, and *Cx. neavei* mosquitoes in Africa [22,29,33–35,48,51].

Table 2.1 West Nile virus isolation and availability of vector competence studies for different mosquito species in Africa

Species	Country	Virus isolation	Vector competence experiment	References
Anopheles brunnipes	Madagascar	+	-	[36[c],51[c]]
Anopheles coustani	Madagascar	+	-	[56[b]]
Anopheles maculipalpis	Madagascar	+	-	[36[c],51[c]]
Anopheles pauliani	Madagascar	+	-	[56[b]]
Anopheles scotti	Madagascar	+	-	[36[c],51[c]]
Anopheles spp.	Kenya	+	-	[37[b]]
Aedeomyia africana	Senegal	+	-	[36[c],38[a]]
Aedeomyia madagascarica	Madagascar	+	-	[56[b]]
Aedes albocephalus	Madagascar	+	-	[36[c]]
Aedes (Aedimorphus) dalzieli	Madagascar	+	-	[56[b]]

Aedes juppi + caballus	South Africa	+	-	[34[c]]
Aedes vexans	Senegal	+	-	[36[c],39[a]]
Aedes madagascariensis	Madagascar	+	-	[36[c],56[b]]
Aedes albothorax	Kenya	+	-	[36[c]]
Aedes circumluteolus	Madagascar, South Africa	+	-	[34[c],36[c],56[b]]
Aedes aegypti	Madagascar	+	-	[12,35[a],36[c],51[c],56[b]]
Aedes aegypti	Senegal	-	+ (KOUTV)	[12,54,55]
Aedes africanus	Central African Republic	+	-	[36[c]]
Aedes spp.	Kenya	+	-	[37[b]]
Aedes spp.	Senegal	+	-	[12[c]]
Culex antennatus	Egypt, Madagascar, Senegal	+	-	[36[c],38[a],40[a],48[c]]
Culex decens group	Madagascar	+	-	[36[c],56[b]]
Culex ethiopicus	Ethiopia, Senegal	+	-	[36[c],38[a]]

Culex guiarti	Ivory Coast	+	-	[35[a],36[c]]
Culex neavei	Senegal, South Africa	+	+	[22[a],34[c],38[a],40[a]]
Culex nigripes	Central African Republic	+	-	[36[c]]
Culex perfuscus	Ivory Coast, Central African Republic, Senegal	+	-	[36[c]]
Culex perexiguus	Algeria	+	-	[23[b]]
Culex pipiens	South Africa, Egypt, Algeria, Morocco, Tunisia	+	+	[21[b],29,31[c],34[c],36[c],41[b],42[b],45[c],48[c],49[c]]
Culex pipiens spp. torridus	Djibouti	+	-	[26[b]]
Culex poicilipes	Senegal	+	-	[36[c],38[a],43[a]]
Culex pruina	Central African Republic	+	-	[36[c]]
Culex quinquefasciatus	Djibouti, Madagascar	+	+	[26,35[a],36[c],51[a]]
Culex scottii	Madagascar	+	-	[36[c]]
Culex spp.	Algeria, Kenya	+	-	[19,37[b],44[a,b]]

Culex theileri	South Africa	+	-	[34[c]]
Culex tritaeniorhynchus	Madagascar	+	+	[35[a],36[c],38[a],51[a]]
Culex univittatus	Madagascar, Egypt, Kenya, Namibia, South Africa	+	+	[27[a,b],34[c],36[c],45[c],46[a],47[a,b],48[c],49[c],51[a]]
Cx. vansomereni Edwards	Kenya	-	+	[50]
Culex weschei	Central African Republic	+	-	[36[c]]
Coquillettidia metallica	Uganda	+	-	[36[c]]
Coquillettidia microannulata	South Africa	+	-	[34[c],36[c]]
Coquillettidia richiardii	South Africa	+	-	[34[c],36[c]]
Mansonia africana	Senegal	+	+	[35[a],38[a]]
Mansonia uniformis	Senegal, Ethiopia, Madagascar	+	+	[35[a],36[c],38[a],40[a],43[a],51[a],56[b]]
Mimomyia hispida	Senegal	+	-	[36[c],38[a]]
Mimomyia lacustris	Senegal	+	-	[36[c],38[a]]

Mimomyia splendens	Senegal	+	-	[36[c],38[a]]
Mimomyia sp.	Senegal, Kenya	+	-	[36[c],38[a]]

+ At least one study report with positive results found; — no available studies; [a] = "virus isolation on cell cultures / injection into suckling mice. Viruses detected by immunofluorescence assay using specific mouse immune ascitic fluids"; [b] = "RNA molecular detection"; [c] = "viral isolation, not specified if [a] and/or [b]"; no apical letters = studies indicating only vector competent experiments. https://doi.org/10.1371/journal.pntd.0010075.t001

Table 2.2 Quantitative information related to WNV vector competence studies carried out on different mosquito species in Africa

Country	Days post infection	Infection rate	Transmission rate	Dissemination rate	Species [lineage; virus titer]	References
Madagascar	(··)	16%	< 16%	(··)	*Aedes aegypti*	[35,50]
Madagascar	(··)	8-86%	2-52%	(··)	*Culex quinquefasciatus*	[35,50]
Madagascar	(··)	10-90%	100%	(··)	*Culex tritaeniorhynchus*	[35,50]
Madagascar	(··)	51%	100%	(··)	*Culex univittatus*	[35,50]
Madagascar	(··)	43%	(··)	(··)	*Mansonia uniformis*	[35,50]
Algeria, Morocco, Tunisia	3	(··)	5%	(··)	*Culex pipiens*	[29]
Algeria, Morocco, Tunisia	14	(··)	40%	59·1% - 100%	*Culex pipiens*	[29]
Algeria, Morocco, Tunisia	21	(··)	80%	(··)	*Culex pipiens*	[29]
Senegal	4	14·28%	(··)	0%	*Culex neavei* [L1, Titer 10^6]	[22]

Senegal	8	14·28%	(··)	0%	*Culex neavei* [L1, Titer 10^6]	[22]
Senegal	12	25%	(··)	0%	*Culex neavei* [L1, Titer 10^6]	[22]
Senegal	15	55%	83·3%	54·5%	*Culex neavei* [L1, Titer 10^6]	[22]
Senegal	4	0%	(··)	(··)	*Culex neavei* [L2, Titer: 10^5]	[22]
Senegal	8	0%	(··)	(··)	*Culex neavei* [L2, Titer: 10^5]	[22]
Senegal	12	0%	(··)	(··)	*Culex neavei* [L2, Titer: 10^5]	[22]
Senegal	15	6·67%	(··)	50%	*Culex neavei* [L2, Titer: 10^5]	[22]
Senegal	4	0%	(··)	(··)	*Culex neavei* [L8, Titer: 10^5]	[22]
Senegal	8	0%	(··)	(··)	*Culex neavei* [L8, Titer: 10^5]	[22]
Senegal	12	0%	(··)	(··)	*Culex neavei* [L8, Titer: 10^5]	[22]
Senegal	15	5·55%	(··)	100%	*Culex neavei* [L8, Titer: 10^5]	[22]
Senegal	4	25%	(··)	0%	*Culex quinquefasciatus* [L1, Titer 10^6]	[22]

Senegal	8	25%	(··)	0%	*Culex quinquefasciatus* [L1, Titer 10^6]	[22]
Senegal	12	25%	(··)	0%	*Culex quinquefasciatus* [L1, Titer 10^6]	[22]
Senegal	15	75·86%	(··)	18·18%	*Culex quinquefasciatus* [L1, Titer 10^6]	[22]
Senegal	4	0%	(··)	(··)	*Culex quinquefasciatus* [L2, Titer 10^5]	[22]
Senegal	8	0%	(··)	(··)	*Culex quinquefasciatus* [L2, Titer 10^5]	[22]
Senegal	12	0%	(··)	(··)	*Culex quinquefasciatus* [L2, Titer 10^5]	[22]
Senegal	15	5·26%	(··)	0%	*Culex quinquefasciatus* [L2, Titer 10^5]	[22]
Senegal	4	0%	(··)	(··)	*Culex quinquefasciatus* [L8, Titer 10^6]	[22]
Senegal	8	0%	(··)	(··)	*Culex quinquefasciatus* [L8, Titer 10^6]	[34,53]

Senegal	12	0%	(··)	(··)	*Culex quinquefasciatus* [L8, Titer 10^6]	[34,53]
Senegal	15	0%	(··)	(··)	*Culex quinquefasciatus* [L8, Titer 10^6]	[34,53]
Senegal	15	0%	(··)	(··)	*Culex quinquefasciatus* [L8, Titer 10^5]	[34,53]
South Africa	21	97%	(··)	(··)	*Culex neavei*	[34,53]
South Africa	13-29	24%	(··)	(··)	*Culex neavei*	[34,53]
South Africa	15-18	4%	(··)	(··)	*Culex neavei*	[34,53]
South Africa	15-18	8%	(··)	(··)	*Culex neavei*	[34,53]
South Africa	17	100%	97%	(··)	*Culex univittatus*	[34,53]
South Africa	(··)	100%	33%	(··)	*Culex univittatus*	[34]
South Africa	(··)	84%	(··)	(··)	*Culex univittatus*	[34]
South Africa	(··)	41%	(··)	(··)	*Culex univittatus*	[34,52]
South Africa	(··)	(··)	0%	(··)	*Culex theileri*	[34,52]
South Africa	(··)	(··)	25%	(··)	*Culex theileri*	[34,52]

Infection rate (number of infected mosquito bodies per 100 mosquitoes tested); Dissemination rate (number of mosquitoes with infected legs/wings per 100 mosquitoes infected); Transmission rate (number of mosquitoes with infected saliva per 100 mosquitoes with infected legs/wings); (··) Not defined; [ ] Lineage and virus titer (PFU/mL).

Cx. pipiens, characterized by high dissemination and transmission rates, are indicated by numerous reports as the most important vector species of WNV [29,31,34,36,41,42,45,48,49]. High ornithophilic and low anthropophilic *Cx. univittatus* mosquitoes are considered both highly susceptible and efficient transmitters of WNV [34]. WNV vertical transmission has been described under field conditions in *Cx. univittatus* males in the Rift Valley province of Kenya [38]. *Cx. theileri* is a less efficient WNV vector: despite being highly susceptible to the virus it has a lower transmission rate [34,52]. Vector competent experiments highlight that this mosquito species can be infected as much as *Cx. univittatus*, but probably due to its host preferences (less ornithophilic than *Cx. univittatus* and feeding mostly at ground level) there are very few WNV isolates obtained from wild populations, classifying this species as a poor vector [52].

Cx. quinquefasciatus, shown to be a competent species for WNV-L1 and -L8, is widespread in urban environments and active all year-round, and might be considered as another important WNV vector, especially in urban settings [22]. Furthermore, vector competence studies highlighted that *Cx. quinquefasciatus* mosquitoes were not competent for KOUTV [22].

Cx. neavei, attracted by both horses and birds, and widespread in different types of lands, might have a possible role as bridge vectors in the sylvatic transmission cycle [22]. This mosquito species has been shown to be more efficient vectors for WNV-L1 than L8, and to be susceptible to WNV-L2 and KOUTV infections [22]. However, WNV transmission has not been observed for WNV-L2 and KOUTV [22]. As shown in Table 2.2, experimental infections conducted on *Cx. neavei* in South Africa [34,53] showed high transmission rate but a 50% infection threshold, observed after exposing birds with different viraemias to this mosquito species, of 4.4 logs per ml, that was higher than those observed in *Cx. univittatus* mosquitoes (2.1 logs per ml) [53]. Furthermore, the time taken by *Cx. neavei* and *Cx. quinquefasciatus* mosquitoes to develop WNV after infection (extrinsic incubation

period, EIP) has been estimated to last 15 days at 27˚C [22]. However, the infective life survival rate has been estimated to be comprised between 0.75 and 0.88 for *Cx. neavei* and between 0.87 and 0.88 for *Cx. quinquefasciatus* mosquitoes [22]. Based on these data, only 1.3% to 10.4% of *Cx. neavei* and 12.59% to 15.45% of *Cx. quinquefasciatus* mosquitoes have been estimated to survive at 15 days post infection by the authors of the study [22]. Despite *Cx. quinquefasciatus* and *Cx. neavei* mosquitoes being widely distributed in the African continent and their proven competence for distinct lineages of WNV, such findings imply a low probability of WNV transmission to new hosts in the African continent and they might indicate a low impact of *Cx. neavei* and *Cx. quinquefasciatus* mosquitoes in WNV circulation in Africa [22]. However, the small number of mosquitoes tested, as reported in reference [22], and the lack of experimental infection at different temperatures would require further studies. Interestingly, KOUTV viral dissemination and vertical transmission have been observed in *Ae. aegypti* mosquitoes in Senegal [12,54,55] while WNV vector competence experiments have never been conducted for this mosquito species in the African continent. The ability of different mosquito species to transmit specific lineages of WNV highlights a direct correlation between vector competence and genetic variability [22]. Besides mosquitoes, ticks have been suggested as possible WNV hosts in Africa even though they are generally considered less competent as vectors compared to mosquitoes [6,7]. The role of ticks in WNV ecology and transmission is still an open question due to the little number of studies carried out so far on this topic [6,7,36,45,57–62]. Ticks are characterized by a protracted life cycle, holding the virus for a long time [61]. Furthermore, the transstadial maintenance of WNV in hard and soft ticks has been demonstrated [60,63,64]. Particular attention should be given to *Ornithodoros savignyi* and *Argas arboreus* ticks as potential vectors of WNV in the African continent. In Egypt, experimental infections of adult soft tick *O. savignyi*, using a local strain of WNV (Ar-248), showed that the species got infected without being competent. However,

after parenteral infection, *O. savignyi* could transmit the virus to infant mice and WNV isolation could be obtained from its coxal fluid [57].

Argas arboreus ticks are also shown to be WNV competent vectors and vertical and horizontal transmission has been observed for this species. The virus titer was detected to be 10^4 PFU/mL at 4 days post infection (pi), remaining constant at 10^3 PFU/mL from day 6 to day 50 pi. After 20 days from experimental infections, *A. arboreus* adults could transmit the virus to uninfected chickens. Furthermore, F1 *A. arboreus* larvae from WNV experimentally infected females could also transmit the virus to uninfected chickens. WNV was isolated from *A. arboreus* salivary glands, synganglia, and coxal fluids [59]. A large number of ticks are carried around the world by mammals and by birds during their migration paths [61]. Further studies are needed to assess the possible contribution of ticks on the maintenance, transmission, and spread of WNV over long distances and extensive periods of time. Considering that WNV competent hosts are characterized by relatively short viremic duration periods, the role of ticks as WNV vectors should be further evaluated especially for better explaining the biological mechanism which favors virus translocation from the African continent into the others [6,65]. Results of laboratory vector competence experiments and WNV isolation in hard and soft ticks are shown in Table 2.3.

Table 2.3 Viral isolation and laboratory vector competence experiments on hard and soft African ticks

Year	Species	Country	Viral Isolation	Competence experiments	WNV Strain / Titer	Infection	Transmission	References
(··)	*Ornithodoros capensis*	Egypt	+	+	-	Yes	No	[7,36[c]]
(··)	*Ornithodoros erraticus*	Egypt	-	+	(··)	Yes	No	[36[c],45[c],60[a]]
1950s	*Ornithodoros savignyi*	Egypt	-	+	Ar-248 strain	Yes	No	[45[c],60[a]]
1950s	*Ornithodoros savignyi*	Egypt	+	+	(··)	Yes	Yes	[57[a]]
1993, 2003	*Argas hermanni*	Egypt, Senegal	+	+	$10^{5.5}$ TCID50/mL	Yes	No	[7,36[c],45[c],59[a],61]
1993, 2003	*Argas hermanni*	Egypt, Senegal	+	+	$10^{6.2}$ TCID50/mL	Yes	No	[7,36[c],45[c],59[a],61]
1993, 2003	*Argas persicurs*	Egypt, Senegal	-	+	$10^{5.5}$ TCID50/mL	Yes	No	[57[a],59[a],61]

1993, 2003	*Argas persicurs*	Egypt, Senegal	-	+	$10^{6\cdot2}$ TCID50/mL	Yes	No	[57[a],59[a],61]
1993, 2003	*Argas arboreus*	Egypt	-	+	$10^{5\cdot5}$ TCID50/mL	Yes	No	[57[a],59[a]]
1993, 2003	*Argas arboreus*	Egypt	-	+	$10^{6\cdot2}$ TCID50/mL	Yes	Yes	[59[a]]
(··)	*Rhipicephalus turanicus*	Central African Republic	+	-	-	No	No	[36[c],66[a]]
(··)	*Rhipicephalus lunulatus*	Central African Republic	+	-	-	-	-	[66[a]],IPD[c]
(··)	*Rhipicephalus muhsamae*	Central African Republic	+	-	-	-	-	[12[a],66[a]],IPD[c]
(··)	*Amblyomma variegatum*	Central African Republic, Ivory Coast	+	-	-	No	No	[7[c],36[c],66[a]],IPD
2010-2012	*Rhipicephalus pulchellus*	Kenya	+	-	-	No	No	[62[a,b]]

(··)	*Hyalomma*	Africa	+	-	-	No	No	[7[c]]
2010-2012	*Amblyomma gemma*	Kenya	+	-	-	No	No	[7[c],62[a,b]]
(··)	*Dermacentor marginatus*	Africa	+	+	-	No	No	[7[c],62[a,b]]

2.5.3 West Nile virus epidemiology in African vertebrates

Several avian species are exposed to WNV infection as ascertained through sero-epidemiological studies or virus isolation but only for a subset of species their role as competent reservoir has been tested [7,8]. Susceptibility of birds to WNV infection is dependent on bird species other than to the viral strain involved [10]. In Europe and the United States, Passeriformes and Falconiformes appear to be highly susceptible to WNV-L1 and L2, showing neurological symptoms and variable mortality rates [7,10,67]. On the contrary, clinical signs have been rarely reported in birds in the African continent, with exception of one moribund pigeon affected by WNND in Egypt in 1953 [45].

However, the high viral circulation among birds in Africa is documented by seroprevalence studies carried out in Algeria, Egypt, Morocco, Tunisia, Southern Sudan, Senegal, Madagascar, and South Africa, as summarized in Fig 2.2 and Table 2.4 [34,41,45,56,68–76].

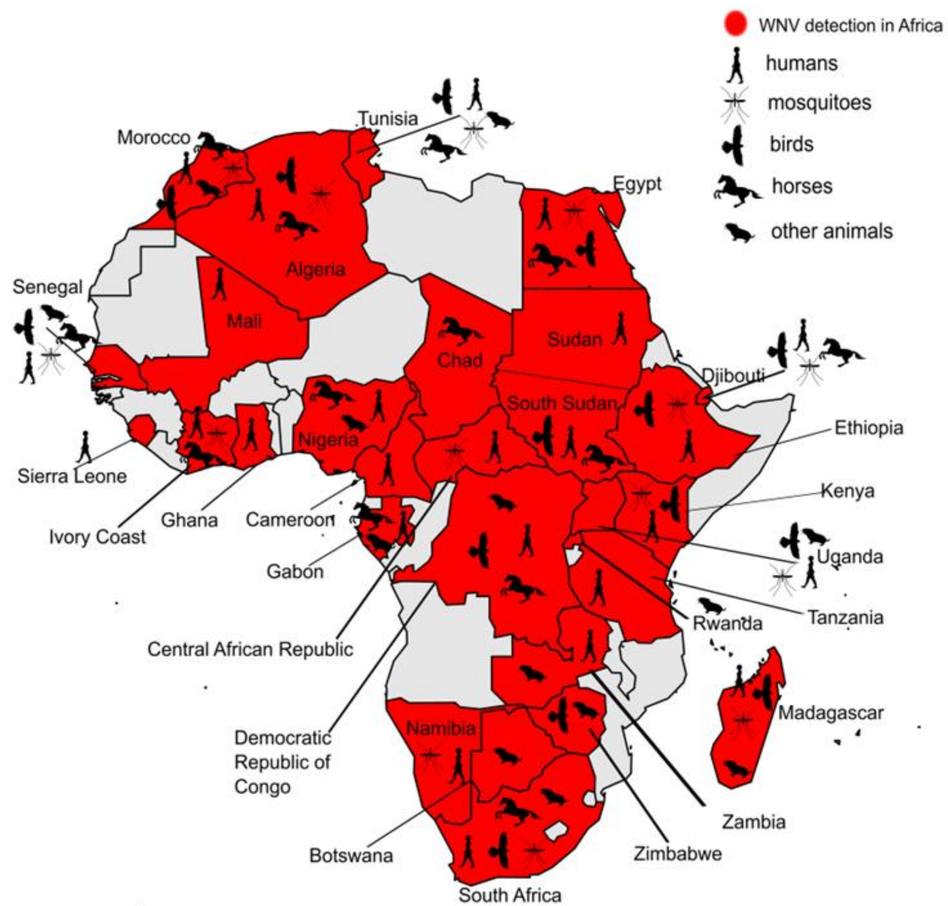

Figure 2.2 Map of West Nile virus distribution in Africa based on sero-epidemiological surveys carried out on humans and animals, and viral isolation in mosquitoes.

Map was generated using publicly available shapefiles, https://smart.servier.com/

Table 2.4 West Nile virus records in avian species in Africa

Species	Country	Antibodies detection	Viral detection	Case of illness	Experimental infection	References
Acrocephalus gracilirostris	South Africa	+	-	-	-	[34[d]]
Anas erythrorhyncha	South Africa	+	-	-	-	[34[d]]
Anas platyrhynchos domesticus	Egypt, Madagascar	+	-	-	-	[45[e],56[f]]
Anas undulata	South Africa	+	-	-	-	[34[d]]
Anas platyrhynchos	Tunisia	+	-	-	-	[41[f]]
Anthus trivialis	Senegal	+	-	-	-	[68[f]]
Antichromus minutus	Central African Republic	-	+	-	-	IPD[c]
Bubulcus ibis	Egypt, Southern Sudan, Nile Delta, South Africa	+	-	-	+	[34[d],45[e],70[g]]
Cercotrichas podobe	Senegal	+	-	-	-	[68[f]]

Cercotrichas galactotes	Senegal	+	-	-	-	[68[f]]
Cettia cetti	Morocco	+	-	-	-	[71[h]]
Columba livia	Egypt	+	+	+	+	[45[c,e],70[g]]
Coracopsis vasa	Madagascar	-	+	-	-	[72[c]]
Corvus corone sardonius	Egypt, Southern Sudan, Nile Delta	+	+	-	+	[45[c,e],70[g]]
Anas platyrhynchos	Tunisia	-	+	-	-	[41[b]]
Egretta garzetta Linnaeus	Madagascar	-	+	-	-	[72[c]]
Estrilda melpoda	Central African Republic	-	+	-	-	IPD[c]
Euplectes orix	South Africa	+	-	-	-	[34[d]]
Falco tinnunculus	Egypt, Southern Sudan, Nile Delta	+	-	-	+	[45[e],70[g]]
Fulica cristata	South Africa	+	-	-	+	[34[d]]

Gallus gallus	Egypt; Madagascar; Tunisia	+	+	-	+	[21[b],45[c,e],56[b,f],73[b]]
Goose [Anatidae]	Egypt, Madagascar	+	-	-	-	[45[e],56[f]]
Guinea fowl [Numididae]	Madagascar	+	-	-	-	[56[f]]
Hippolais opaca	Senegal	+	-	-	-	[68[f]]
Hirundo rustica	Zimbabwe	-	+	-	-	[74[b]]
Jynx torquilla	Senegal	+	-	-	-	[68[f]]
Lanius senator	Senegal	+	-	-	-	[68[f]]
Meleagris	Madagascar	+	-	-	-	[56[f]]
Milvus migrans aegyptius	Egypt	+	-	-	-	[45[e]]
Oena Capensis	Senegal	+	-	-	-	[68[f]]
Passer domesticus	Egypt, Southern Sudan, Nile Delta, Morocco, Algeria	+	-	-	+	[45[e],70[g],71[h],75[e]]

Pelecanus onocrotalus	Senegal	+	-	-	-	[76[f]]
Ploceus cucullatus	Senegal	+	-	-	-	[68[f]]
Ploceus velatus	Senegal, South Africa	+	-	-	+	[34,68[f]]
Quelea quelea	South Africa	+	-	-	+	[34[d]]
Riparia paludicola	Zimbabwe	-	+	-	-	[74[b]]
Streptopelia vinacea	Senegal	+	-	-	-	[68[f]]
Streptopelia senegalensis	Egypt, Southern Sudan, Nile Delta, Senegal, South Africa	+	-	-	+	[34[d],45[e],68[f],70[g]]
Threskiornis aethiopicus	South Africa	+	-	-	+	[34[d]]
Tchagra australis	Central African Republic	-	+	-	-	IPD[c]
Turdus merula	Morocco	+	-	-	-	[71[h]]
Turdus philomelos	Algeria	+	-	-	-	[75[e]]

Urocolius macrourus	Senegal	+	-	-	-	[68[f]]

+ At least one study report with positive results found; — no available studies; IPD: Institut Pasteur de Dakar, Senegal, personal communication. [a] = "virus isolation on cell cultures / injection into suckling mice. Viruses detected by immunofluorescence assay using specific mouse immune ascitic fluids", [b] = "RNA molecular detection", [c] = "viral isolation, not specified if [a] and/or [b]"; antibodies detection via [d] = "hemagglutination-inhibition test (HIT)", [e] = "serological surveys, type of antibodies detection tests non specified", [f] = "epitope-blocking enzyme-linked immunosorbent assay (ELISA)", [g] = "virus-neutralization test (VNT)", [h] = "micro virus-neutralization test (micro-VNT)", [i] = "immunoglobulin M (IgM)-specific ELISA", [l] = "plaque reduction neutralization test (PRNT)", m = "Flavivirus microsphere immunoassay (MIA)". Reference numbers with no apical letters refer to experimental infections or cases of illness. https://doi.org/10.1371/journal.pntd.0010075.t004

Among equids, symptomatic infections and fatalities have been reported in Morocco [WNV-L1] and South Africa (WNV-L2) [7,77–79]. In South Africa, WNV-L1 is rare and was detected only once in a lethal neurological case involving a mare and its aborted fetus during an eight yearlong observational study [79].

Seroprevalence studies carried out on horses and other equids and aimed at assessing the circulation of WNV infection have been carried out in Morocco, Tunisia, Egypt, Algeria, Nigeria, South Sudan, Democratic Republic of Congo, Chad, South Africa, Gabon, Côte d'Ivoire, Senegal, and Djibouti, as shown in Fig 2.2 and Table 2.5 [7,13,42,44,61,78–93].

Table 2.5 West Nile virus records in equids in Africa (period 1975–2015)

Country	Year of the study	Viral Isolation	Antibody detection	Seroprevalence rate	References
Algeria	1975	-	+	96.6%	[44[e]]
Chad	2003	-	+	97%	[78[f,n]]
Djibouti	2004–2005	-	+	9%	[78[f,n]]
Democratic Republic of Congo	2004	-	+	30%	[78[f,n]]
Egypt	1963	-	+	54%	[80[g]]
Gabon	2004	-	+	3%	[78[f]]
Ivory Coast	2003	-	+	79%	[81[f]]
Ivory Coast	2003–2004–2005	-	+	28%	[78[f]]
Morocco	1996 (42 deaths)	+	-	-	[7[b]]
Morocco	After the epizootic of 1996	-	+	42–57%	[82[e]]
Morocco	2003 (5 deaths)	+	-	-	[83[f]]

Morocco	2010 (8 deaths)	+	-	-	[44[f]]
Morocco	2011	-	+	31%	[84[f,g]]
Morocco	2018	-	+	33.7%	[42[f,h,m]]
Nigeria	2011–2012	-	+	90.3%	[85[f,i]]
Nigeria	2014	-	+	11.5%	[86[g]]
Nigeria	2014	-	+	8.5% (donkeys)	[86[g]]
Senegal	2002–2003, Dakar	-	+	92%	[78[f,l]]
Senegal	2003, Ferlo area	-	+	78.3%	[61[l]]
Senegal	2005, Senegal river basin	-	+	85%	[87[f,g]]
Senegal	2014, North-west Senegal	-	+	68.7%	[88[f]]
Senegal	2014, Keur Momar Sarr	-	+	86.2%	[88[f]]
South Africa	2001	-	+	15% (foals)	[89[l]]
South Africa	2001	-	+	11% (yearlings)	[89[l]]

South Africa	2001	-	+	75% (dams)	[89[l]]
South Africa	2007–2008	-	+	21.8%	[13[f,g]]
South Africa	2008–2015	-	+	7.4%	[79[f,g]]
Tunisia	1980	-	+	0.35%	[90[e]]
Tunisia	2005	-	+	25%	[91[e]]
Tunisia	2005	-	+	37% (donkeys & mules)	[91[e]]
Tunisia	2007	-	+	IgG 30%	[92[f]]
Tunisia	2007	-	+	IgM 0.78%	[92[f]]
Tunisia	2008	-	+	27.1%	[93[f]]

+ At least one study report with positive results found; — no available studies; () specified when not horses [a] = “virus isolation on cell cultures / injection into suckling mice. Viruses detected by immunofluorescence assay using specific mouse immune ascitic fluids”, [b] = “RNA molecular detection”, [c] = “viral isolation, not specified if [a] and/or [b]”; antibodies detection via [d] = “hemagglutination-inhibition test (HIT)”, [e] = “serological surveys, type of antibodies detection tests non specified”, [f] = “epitope-blocking enzyme-linked immunosorbent assay (ELISA)”, [g] = “virus-neutralization test (VNT)”, [h] = “micro virus-neutralization test (micro-VNT)”, [i] = “immunoglobulin M (IgM)-specific ELISA”, [l] = “plaque reduction neutralization test (PRNT)”, [m] = “flavivirus microsphere immunoassay (MIA)”, [n] = “immunoblotting method (WB).” https://doi.org/10.1371/journal.pntd.0010075.t005.

Besides in birds and equids, WNV has been reported in other animal species [94,95]. Fig 2.2 and Table 2.6 summarize the recording of WNV exposure in other vertebrates in African countries although their role in the transmission cycle is not well understood yet [15,19,34,66,77,79,86,88,94–102].

Table 2.6 West Nile Virus records in other vertebrate species

Species	Country	Antibodies detection	Viral detection	Case of illness	Experimental infection	References
African forest buffalo, *Syncerus caffer nanus*	Democratic Republic of Congo, Gabon, South Africa	+	+	-	-	[95[l],96[b]]
African elephant, *Loxodonta*	Zambia	+	-	-	-	[95[l]]
Calves, domestic bovid, Bovidae	South Africa	+	+	-	+	[34[q],66[q],96[b],98[d],103[n]]
Domestic dog, *Canis lupus familiaris*	South Africa, Senegal, Botswana	+	+	+	+	[7[p,d,g],19[c,r],34[q],88f,94[r],96[b],97[d,g,o,p,q]]
Donkey, *Equus asinus*	Algeria, Senegal, Nigeria	+	-	-	-	[44[e],86[g],88[f]]
Fallow deer, *Dama dama*	South Africa	-	+	-	-	[96[b]]
Giraffe, *Giraffa*	South Africa	-	+	-	-	[96[b]]
Goat, *Capra aegagrus hircus*	Senegal, Nigeria, South Africa	+	+	-	+	[34[q],88[f],96[b],98[d]]

Humped camel, *Camelus bactrianus*	Morocco, Nigeria	+	-	-	-	[86[d],88[f], 98[d],99[f,g],103[n]]
Lemur, *Galago senegalensis*	Senegal	-	+	-	-	[66[c]]
Lion, *Panthera leo*	South Africa	-	+	-	-	[96[b]]
Livestock	South Africa	+	+	+	-	[79[b,f,g,r]]
Mountain gorillas, *Gorilla beringei beringei*	Uganda, Rwanda, DRC	+	-	-	-	[95[l]]
Oxen, *Bos*	Madagascar	+	-	-	-	[7[e]]
Pigs, *Sus*	South Africa	-	-	-	+	[7[q],66[q]]
Roan antelope, *Hippotragus equinus*	South Africa	-	+	-	-	[96[b]]
Small rodents, *Rodentia*	Nigeria, Morocco, Tunisia, South Africa	+	+	-	+	[77[c],94[d,n,q],100[p,q], 102[d]]
Wild rodents	Senegal, Somalia, Central African Republic	-	+ (KOUTV)	-	-	[12[c]]

Sheep, *Ovis aries*	South Africa, Nigeria	+	-	+	+	[66[o,p,q],98[d],101[r,q]]
Kuhl's pipistrelle, *Pipistrellus kuhli*	Tunisia	+	-	-	-	[102[d]]
Wildlife	South Africa	+	+	+	-	[79[b,f,g]]
White rhinoceros *Ceratotherium simum*	South Africa	+				[96[f]]

+ At least one study report with positive results found; — no available studies [a] = "virus isolation on cell cultures / injection into suckling mice. Viruses detected by immunofluorescence assay using specific mouse immune ascitic fluids", [b] = "RNA molecular detection", [c] = "viral isolation, not specified if [a] and/or [b]"; antibodies detection via [d] = "hemagglutination-inhibition test (HIT)", [e] = "serological surveys, type of antibodies detection tests non specified", [f] = "epitope-blocking enzyme-linked immunosorbent assay (ELISA)", [g] = "virus-neutralization test (VNT)", [h] = "micro virus-neutralization test (micro-VNT)", [i] = "immunoglobulin M (IgM)-specific ELISA", [l] = "plaque reduction neutralization test (PRNT)", [m] = "flavivirus microsphere immunoassay (MIA)", [n] = "complement fixation test (CFT)"; "Experimental infection" means: [o] = "disease" [p] = "antibodies" or [q] = "attempt without any clinical signs/development of viraemia" [r] = "case of illness". https://doi.org/10.1371/journal.pntd.0010075.t006.

2.5.4 West Nile virus distribution in humans

Several WNV outbreaks in humans were registered in the African continent starting from the 1950s [2,13,21,34,41,44,46,49,104–106]. Neurological cases and fatalities related to WNV-L2 were reported in South Africa (1976, 1980, 1984) while in the Mediterranean basin, hundreds of cases of encephalitis and deaths related to WNV-L1 (clade A) were registered in Tunisia between 1997 and 2018 (1997, 2003, 2007, 2010, 2011, 2012, 2016, 2018) [21,41,44,104,107]. In addition, WNV-L1 human infections were recorded for the first time in 1994 and 1996 in Algeria and Morocco, respectively [44,82,105]. Real time PCR analysis confirmed the WNV circulation in the Central African Republic, Guinea, Ghana, Gabon, Nigeria, Senegal, and Sierra Leone between 1983 and 2020 [26,61,108– 112] while serological surveys reported WNV circulation in humans in Algeria, Central African Republic, Democratic Republic of Congo, Egypt, Ethiopia, Gabon, Ghana, Kenya, Madagascar, Mali, Morocco, Mozambique, Namibia, Nigeria, Senegal, Sierra Leone, South Africa, South Sudan, Sudan, Tanzania, Tunisia, Uganda, and Zambia, as shown in Fig 2.2 and Table 2.7 [7,19,21,34,44,45,49,51,61,65,66,69,90,91,103–107,109–147].

Table 2.7 West Nile virus seroprevalence studies carried out in humans between 1950 and 2019

Country	Year of the study	Seroprevalence rate	References
Algeria	1965	0%	[44[e],113[e]]
Algeria	1973, 1975	14.6%	[44[e],113[e]]
Algeria	1973, 1975	58.3%	[44[e],113[e]]
Algeria	1973, 1975	3.5%	[44[e],113[e]]
Algeria	1976	37.5%	[44[e],113[e]]
Algeria	1976	19%	[44[e],113[e]]
Algeria	1994	83.3%	[44[e]]
Burundi	1980–1982	0%	[114[e]]
Cameroon	1990	0%	[115[f,o]]
Cameroon	2000–2003	6.6%	[116[l]]
Central African Republic	1964	High arbovirus circulation (··)	[117[e]]

Central African Republic	1975–1976	2.3%	[117[e]]
Central African Republic	1979	WNV-positive results (··)	[118[d,n]]
Democratic Republic of Congo	1998	66%	[119[i]]
Egypt	1950	70%	[105[g,n]]
Egypt	1951–1954	61% (44% < 15 years old; 72% > 15 years old)	[105[g,n]]
Egypt	1952	(··)	[148[e]]
Egypt	1999–2002	35% Upper Egypt	[106[f,l]]
Egypt	1999–2002	27% Middle Egypt	[106[f,l]]
Egypt	1999–2002	14% Lower Egypt	[106[f,l]]
Egypt	1999–2002	1% North Sinai	[106[f,l]]
Egypt	1999–2002	7% South Sinai	[106[f,l]]
Ethiopia	1959–1962	(··)	[149[e]]

Gabon	1975	(··)	[121[f]]
Gabon	1975	KOUTV (··)	[12[e]]
Gabon	21st century	27.2%	[121[f]]
Ghana	2008	Children: 1.4% IgM, 4.8% IgG; Adults: 27.9%	[109[f]]
Kenya	1959–1962	(··)	[149[e]]
Kenya	1966–1968	3.2% Central Nyanza	[122[d]]
Kenya	1966–1968	13.8% Kitui District	[122[d]]
Kenya	1966–1968	65.3% Malindi district	[122[d]]
Kenya	1987	0.9%	[123[p]]
Kenya	2009–2012	12.4%	[124[f],125[f,l]]
Kenya	2016–2017	10.2% Turkana	[126[l]]
Madagascar	Since 1975	(··)	[51[d]]
Madagascar	1996	2.1%	[127[e]]

Madagascar	1999	10.6%	[127[e]]
Madagascar	2011	IgM antibodies	[128[l,f]]
Mali	2009–20013	0.27% IgM	[129[f]]
Mali	2009–20013	39.1% IgG	[129[f]]
Morocco	2011	11.8% (4.5% Meknès; 12% Rabat; 18.8% Kenitra)	[130[l]]
Morocco	2019	4.39% positive to flaviviruses (75% of which confirmed WNV + by VNT)	[69[f,g]]
Mozambique	2012–2013	0%	[131[f,o]]
Mozambique	(··)	(··)	[34[e]]
Namibia	1983	%	[132[e]]
Nigeria	1970s	28%	[133[d]]
Nigeria	1990s	65%	[133[d]]
Nigeria	2008	25%	[107[f]]
Nigeria	2011–2012	73.2%	[134[f,l]]

Nigeria	2013	0% IgM	[135[i]]
Nigeria	2016	7.5% IgM	[136[i]]
Nigeria	21st century	1.2% IgM; 80.16% IgG	[7f,l]
Nigeria	21st century	40%	[7d,f]
Senegal	1972–1975	(··)	[120[d,]]
Senegal	1988–1990	IgM < 15 years old (4.6% out of 456 and 3.5% out of 396 children tested)	[137[f]]
Senegal	1989	80% (5–15 years old; 45% < 5 years old; 98% > 15 years old)	[61[l],66[f]]
Senegal	1991	22.7% of adults; 18% < than 15 years old	[66[f]]
Sierra Leone	2006–2008	IgG in 50% of patients presenting severe symptoms, IgM 1/4 of them	[111[f]]
Sierra Leone	2006–2008	1.2% IgM	[138[i]]
South Africa	1950	2.6%	[139[e]]
South Africa	1960s	4.7%	[140[d]]

South Africa	(··)	1%	[141[d,g]]
South Africa	1962–1964	10.22%	[140[d]]
South Africa	1970s	7% Central Highveld Region; 17% Karoo; 2% Kwazulu Natal	[7f,o]
South Africa	1974	55% - 85%	[19[e],49[e]]
South Africa	2009	17.47%	[142[f,g]]
South Africa	2017	woman (IgM positive—2 weeks later IgG positive), man (IgM and IgG at 5 days after the beginning of the symptoms)	[143[f]]
South Sudan	1951–1954	40%	[45[e],105[g,n]]
Sudan	After the epidemics of 1998	59% IgG antibodies	[144[p]]
Tanzania	1971	17.4%	[145[g]]
Tunisia	1968	1.80%	[90[d,f]]
Tunisia	1970s	4.7% (3.8% Djerba region; 7.8% Tunis; 7% Gabes; 9% other Tunisian regions)	[91[g,o]]

Tunisia	1997	86%, including 5 fatalities	[7[f,i],105[n,n],107[f]]
Tunisia	1997	9 IgM positive results	[7[f,i],105[n,n],107[f]]
Tunisia	2007	12.5% (27.7% Kerouan, 7.5% Sfax, 0.7% Bizerte)	[91[g,o],104[f,l]]
Tunisia	2018	24%	[21[i]]
Uganda	1984	16% of anti-flavivirus antibodies (probably due to WNV)	[146[d]]
Zambia	2010	10.3%	[147[f]]

(··) Not defined; antibodies detection via [d] = “hemagglutination-inhibition test (HIT)”, [e] = “serological surveys, type of antibodies detection tests non specified”, [f] = “epitope-blocking enzyme-linked immunosorbent assay (ELISA)”, [g] = “virus-neutralization test (VNT)”, [h] = “micro virus-neutralization test (micro-VNT)”, [i] = “immunoglobulin M (IgM)-specific ELISA”, [l] = “plaque reduction neutralization test (PRNT)”, [m] = “flavivirus microsphere immunoassay (MIA)”, n = “complement fixation test (CFT)”, o = “indirect immunofluorescent assays (IFA)”, p = “enzyme immunoassay (EIA)”.
https://doi.org/10.1371/journal.pntd.0010075.t007.

On the contrary, no WNV antibodies were detected in sero-surveys conducted in Algeria [113], Burundi [114], Cameroon [115], Mozambique [131], and Nigeria [135]. In more than 20 countries (Angola, Benin, Botswana, Burkina Faso, Chad, Congo Brazzaville, Eritrea, Equatorial Guinea, Guinea, Guinea-Bissau, Côte d'Ivoire, Lesotho, Liberia, Malawi, Libya, Mauritania, Niger, Rwanda, Somalia, Swaziland, The Gambia, Togo, Western Sahara, and Zimbabwe) no WNV seroprevalence studies on humans have been conducted so far. Therefore, the real disease burden for the African human population is currently largely underestimated.

2.6 Discussion

This study, based on the analysis of 153 scientific papers published between 1940 and 2021, provides updated knowledge and data on the state of art on WNV investigation carried out in Africa, highlighting several knowledge gaps related to fundamental aspects of WNV ecology and epidemiology. They include the partial knowledge on the actual WNV distribution and genetic diversity, its ecology and transmission chains including the role of different arthropods and vertebrate species as competent reservoirs, and the real disease burden for humans and animals, therefore emphasizing the needs for further research studies to be addressed with high priority in this Continent. Numerous reports highlight the circulation of WNV-L1, 2, and 8 in the African continent, where the most common ancestor originated between the 16th and 17th century, followed by the introduction of WNV-L1 into Europe and the Americas, and WNV-L2 into Europe [2,3]. The KOUV, highly virulent in mice and associated with a symptomatic infection in a clinical laboratory worker, is also occurring in the African continent. Its potential spread into Europe and the Americas, and a possible impact on human and animal health should be considered [2,12,22,150].

Nowadays, little is revealed about the spatio-temporal epidemiology of WNV, and genetic relationships between African, European, and American strains are mostly unknown [14]. All the strains circulating in America seem to be derived from a single introduction of WNV-L1, detected in North America in the 1999 [5]. Phylogenetic analyses support the hypothesis that this introduction was originated from Israel, as highlighted by genetic similarity of American strains with certain Israeli strains [14]. These strains, grouped in the Israeli-American cluster, are characterized by wild bird mortality and fatal encephalitis in humans and horses [5,8]. A close similarity has been observed between the Israeli-American strains (1998–2000) and the *PaH001* Tunisian strain of 1997, supporting the hypothesis of a possible flow of WNV between Africa and the New World via the Middle East [14]. This hypothesis is corroborated by i) the enormous avian biodiversity of Tunisia, considered an important flyway for birds migrating from Africa to northern countries [73]; ii) the circulation of WNV in *Cx. pipiens* competent mosquitoes, birds, horses, and humans in the country [41,91] and iii) the WNV-L1 meningo-encephalitis outbreak, characterized by 173 human cases and 8 deaths, occurred in Tunisia in 1997, one and three years before the first detection of WNV in Israel and United States, respectively [44,104,107].

In Europe, WNV-L1, first detected in the 1960s, re-emerged in the Continent in the 1990s [5]. Since then, WNV-L1 strains, belonging to the Western-Mediterranean clade (Morocco 1996, Italy 1998, France 2000) and to the Eastern-European clade (Romania 1996, Russia 1999), caused numerous outbreaks in European countries and North Africa [3]. These strains were characterized by moderate pathogenicity for horses and humans and limited or no pathogenicity for birds [5]. A possible European WNV-L1 introduction from Morocco is suggested: the closest ancestor of the European strains may be a Moroccan strain which appears to be genetically related to French and Italian isolates (France: 2000, 2006; Italy: 1998, 2008) [3,6,20,83].

WNV-L2, for long time believed to be restricted to Sub-Saharan Africa and considered not pathogenic, is nowadays endemic and the most prevalent in several African and European countries, provoking clinical symptoms (main neurologic signs of infection include ataxia, weakness, recumbence, seizures and muscle fasciculation) among horses in South Africa [7,79], and pathogenesis among horses, humans and birds in Europe [3]. The exact origin of WNV-L2 strains and the following route of introduction into Europe is not clear [3,6]. In Europe, WNV-L2 was reported for the first time in Hungary in 2004 [3,15,151]. Since 2008, an increase in its transmission has been observed in many European countries (Austria, Greece, Italy, Serbia, Bulgaria, Romania, Spain, and Germany) [3]. Interestingly, in Africa WNV-L2 has been reported in Sub-Saharan African countries (Botswana, Central African Republic, Congo, Djibouti, Madagascar, Mozambique, Namibia, Senegal, South Africa, Tanzania, and Uganda) but never in Northern African countries, suggesting a possible flow between Sub-Saharan Africa and Europe, via the Nile Delta and the Mediterranean Sea through migratory birds. These reports evidence the active circulation of WNV-L1 and L2 in Africa and the possible viral spread into Europe and the Americas, further emphasizing the need of a coordinated surveillance in Africa and Europe and the necessity of intensifying WNV research.

In Africa, WNV isolation on cell cultures and RNA molecular detection have been obtained from 46 mosquito species, as shown in Table 2.1. Many of these mosquito species have not been tested for their competence for WNV (Table 2.2) and therefore they should be the future subject for WNV laboratory vector competence experiments. Vector competence experiments conducted in Madagascar, Algeria, Morocco, Tunisia, Senegal, Kenya, and South Africa highlight the vector competence of *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. univittatus*, *Cx. theileri*, *Cx. vansomereni*, and *Cx. neavei* mosquitoes in Africa [c]. In particular, our review highlights the high competence of *Cx. pipiens* and *Cx. univittatus* mosquitoes while transmission rates in *Cx. neavei* and *Cx. quinquefasciatus* seem to be lower, due to estimated short longevity and long EIP [22]. Further

research is needed to confirm these findings and assess their impact on WNV circulation in Africa. Particular attention should be paid towards *Ae. aegypti* mosquitoes in Africa. Although WNV has been isolated in wild specimens belonging to this species [51], which was also found to be capable of KOUTV transmission in Senegal [22,54,55], *Ae. aegypti* African populations have never been tested for WNV vectorial competence so far. However, laboratory studies conducted in the USA demonstrated the vector competence of local *Ae. aegypti* strains for WNV, although the species was found to be less efficient than *Cx. pipiens* [152]. Interestingly, unusual cases of WNV transmission have been highlighted in *O. savignyi* and *A. arboreus* ticks in Egypt [57,59]. In particular, *O. savignyi* has been proven to be competent after parenteral infection while vertical and horizontal transmission have been shown for *A. arboreus* tick species. The role of ticks in transmission and maintenance of WNV should therefore be further explored.

Several WNV seroprevalence studies carried out on humans and animals have been reported, providing evidence of an intense WNV circulation in the Continent. In particular, as illustrated by Fig 2.2 and reported by the studies of Tables 2.4, 2.5, 2.6 and 2.7, seropositivity in humans and animals to WNV has been reported in Morocco, Tunisia, Algeria, Egypt, Mali, Senegal, Sierra Leone, Ivory Coast, Gabon, Ghana, Cameroon, Nigeria, Chad, Sudan, South Sudan, Djibouti, Ethiopia, Kenya, Tanzania, Central African Republic, Rwanda, Uganda, Zambia, Namibia, Botswana, South Africa, and Madagascar. Diverse serological methods have been used in different countries, ranging from HIT, ELISA, VNT, micro-VNT, PRNT, MIA, CFT, IFA, WB, and EIA. For 19 out of 99 serological studies (19.19%) [41,56,57,66,68,76,88,92,93,96,107,109,111,121,124,129,137,143,147] only ELISA test was carried out without a specific WNV neutralization test, and therefore potential of cross-reactivity with closely related pathogens, such as Usutu virus, St. Louis encephalitis virus, or Japanese Encephalitis virus cannot be excluded [91]. Finally, detailed information on the serological

tests carried out are not available for 18 of these studies (18.18%) [12,19,34,44,45,49,75,82,90,91,113,114,117,127,132,139,148,149]. It would be extremely important, in the next future surveys, to carry out WNV confirmation tests with standard methods such as micro-VNT or PRNT, in order to obtain specific serological responses, particularly in areas characterized by circulation of several Flaviviruses [121]. Unfortunately, many times the low volume of collected samples does not allow to perform further laboratory tests. Despite these limitations, these serologic findings associated with viral isolation through cell culture and RNA molecular detection in mosquitoes, few ticks, birds, horses, humans, and other mammals indicate that WNV is actively circulating in many areas of the African continent. There are no studies available on WNV for Angola, Benin, Burkina Faso, Congo Brazzaville, Eritrea, Equatorial Guinea, Guinea, Guinea-Bissau, Lesotho, Liberia, Malawi, Libya, Mauritania, Niger, Somalia, Swaziland, The Gambia, Togo, and Western Sahara, highlighting the lack of information for several African countries. For this reason, the real burden of WNV infections in Africa may differ from what is currently reported by the published literature. However, despite the limitations due to a lack of observational and clinical data for a number of countries, the available information summarized in this review contribute to fill an existent knowledge gap on the phylogeography, ecology and epidemiology of WNV in Africa.

2.7 Conclusions

Since its discovery in 1937 in Uganda, WNV has spread beyond its original ecological niches, becoming one of the most widespread viruses in the world and a serious public and veterinary health concern. Given the global burden of the virus, a deepened knowledge of its phylogeny, epidemiology and circulation in the African continent acquires increasing importance to predict, identify and control WNV, but also other viruses as KOUTV future epidemics. The actual epidemiological situation in most countries of the African continent is unknown, due to: i) the non-specificity of clinical signs of

WNV infection with respect to other arboviruses, very often indistinguishable from each other and from other tropical diseases such malaria, typhoid fever or undifferentiated febrile illness [7,126]; ii) the poor information management and sharing of public health data system of most African countries [153]; iii) the possible bias obtained by serological analysis due to the utilization of non-specific WNV neutralization assays, where the risk of cross-reactivity among closely related pathogens cannot be excluded, and iv) the restricted availability of diagnostic capacity, lack of awareness and inconsistent surveillance, with most investigations performed only during outbreak periods [7,78,125]. All these factors make quantification of the yearly WNV circulation at continental level difficult, with only limited accurate data available in North and Sub-Saharan Africa. New surveillance strategies for preventing and control WNV in Africa need to be implemented not only to better assess the current health impact but also to prevent and control future outbreaks, and therefore limit disease transmission. National and district levels should cooperate with Ministries of Health, and other international partners such as the WHO and European public and veterinary health bodies, to implement national surveillance programs in African countries and to coordinate surveillance actions between Africa and Europe. These programs core objectives should i) minimize the suffering and damage, ii) prevent national and international spread, and iii) contain outbreaks through strong surveillance for early detection and rapid response, as done before for other viruses, as the Yellow Fever and Poliomyelitis viruses.

The ability to accurately identify pathogens in a timely and accurate way has grown in the last few decades, and it has become increasingly clear that the implementation of a collaborative international and multidisciplinary One Health action, based on the analyses of the interconnection among environmental, humans' and animals' health factors in different Continents, will be crucial to allow a more accurate risk assessment and thus an early response to West Nile virus but also to other emerging zoonotic pathogens by public human and veterinary health actors.

2.8 Supporting information

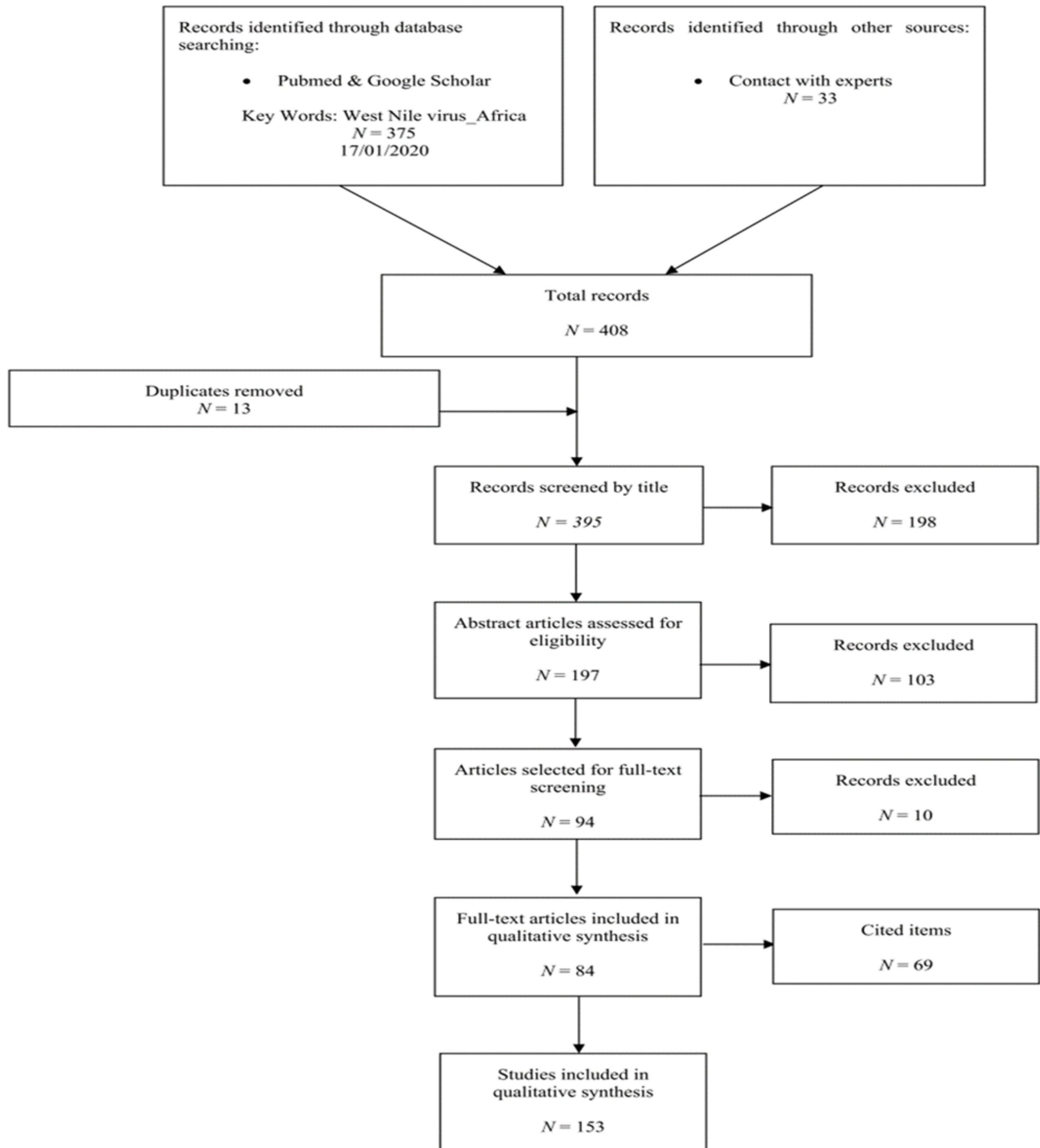

Figure S2. 1 Flow chart of the study selection process

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2.10 Author Contributions

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2.12 Competing interests

The authors have declared that no competing interests exist.

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CHAPTER 3. NOVEL WEST NILE VIRUS AMPLICON-BASED SEQUENCING APPROACH

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by

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3.1 Abstract

West Nile virus is a re-emerging arbovirus whose impact on public health is increasingly important as more and more epidemics and epizootics occur, particularly in America and Europe, with evidence of active circulation in Africa. Because birds constitute the main reservoirs, migratory movements allow the diffusion of various lineages in the world. It is therefore crucial to properly control the dispersion of these lineages, especially because some have a greater health impact on public health than others. This work describes the development and validation of a novel whole genome amplicon-based sequencing approach to West Nile virus. This study was carried out on different strains from lineage 1 and 2 from Senegal and Italy. The presented protocol/approach showed good coverage using samples derived from several vertebrate hosts and may be valuable for West Nile genomic surveillance.

3.2 Keywords

West Nile virus, lineages, Nest-Generation-Sequencing, Amplicon-based sequencing.

3.3 Introduction

The threat from new re-emerging viruses has markedly increased in recent decades due to population growth, urbanization, and the expansion of global travel, facilitating the rapid spread of infection during an outbreak. West Nile virus (WNV), an arbovirus belonging to the flavivirus genus, was firstly isolated in 1937 in Uganda [1] before spreading throughout the world [2]. The enzootic cycle includes mosquitoes and several vertebrate species including birds, allowing long-distance viral spread during migratory seasons [3,4]. Humans are considered WNV dead-end hosts since no human-to-mosquito transmission is reported up to now [5]. If most of WNV infection are either asymptomatic or self-

limiting febrile illness, a very few percentages of cases can develop neuroinvasive disease with a broad range of symptoms and occasionally death [6, 7].

Before 1990, WNV disease was considered a minor public health impact with only sporadic human cases. Since the first outbreaks reported in Algeria and Romania in 1994 and 1996, the virus diffused to cause large epidemics in North America, Northern African, Western and Eastern European countries [7]. In Italy, areas with either proven active asymptomatic WNV circulation or high probability of human infection were previously reported [8, 9], and an increasing number of neuroinvasive human infections is described [10, 11]. In Africa, only few evidence of WNV epidemics were detected. In Senegal where WNV was firstly isolated from an acute human case in 1970, the virus was also detected from mosquitoes, birds, horses, and humans. From 2012 to 2021, active WNV circulation among mosquitoes and humans was documented after a re-introduction event from Europe [12].

WNV exhibits a great genetic diversity with now 8 different lineages (excluding Koutango virus) circulating in the world [13]. The lineages 1 (WNV-L1) and 2 (WNV-L2) are the ones causing the main public health concern [7, 12]. Genetic characterization of the strains detected allow to potentially track the routes of introduction of viruses with a particular interest for public health authorities for designing surveillance and countermeasures plans.

Genome sequencing of viruses has proven to be critical in the management of epidemics. Many approaches can be used to obtain viral whole genomes: i) propagation with cell cultures followed by nucleic acids metagenomic (mNGS), ii) hybrid capture using specific biotinylated probes and iii) a multiplex PCR-based target enrichment or amplicon-based protocol. This last approach became the most used one for the SARS-CoV-2 genomic surveillance during the COVID-19 pandemic due to its

robustness to a large range of input titers allowing to directly sequence clinical samples, as well as its high specificity and scalability under resource-limited conditions with lower cost [15, 16, 17, 18].

Due to the broad range of WNV hosts, many studies within the One Health context are focusing on WNV. Since genomic data is a key information for understanding the mechanisms of emergence and circulation of this virus, it is crucial to develop a fast, reliable, and cost-effective sequencing tool more accessible than isolation methods or mNGS.

We describe here the development and evaluation of a whole genome amplicon-based sequencing approach for WNV-L1 and WNV-L2 by Illumina technology in different types of vertebrate and mosquito species from Senegal and Italy.

3.4 Methods

3.4.1 Primers Design for WNV tiled amplicons-based Sequencing systems

Primer design was made in IPD using a web-based tool entitled Primal Scheme [18] in order to obtain two non-overlapping pools of WNV targeting primers to perform multiplexed PCR reactions, generating approximately 400 bp amplicons tiled along the targeted genome. A WNV reference genome (accession number: NC009942) was chosen as the template. An alignment of WNV whole-genome sequences available on Genbank representative of all WNV lineages in both Africa and Europe was then used to identify nucleotide mismatches for potential correction at ambiguous sites of each primer to ensure both good coverage and high specificity for diverse WNV lineages. Overall, the approach used was a two-pool multiplex amplicon-based sequencing.

3.4.2 WNV primer pools validation

Validation of the primer sets followed several steps: (i) inclusivity test by sequencing attempts on several WNV-L1 and WNV-L2 strains; (ii) specificity and sensitivity tests by sequencing attempts on several flaviviruses and other arboviruses, as well as serial dilutions of WNV-L1 and WNV-L2 culture isolates; and (iii) final validation by sequencing confirmed positive WNV samples derived from different species of vertebrates and mosquitoes from Italy and Senegal.

3.4.2.1 Sequencing of WNV-L1 and WNV-L2 isolates

The designed primer systems were challenged for amplicon-based whole-genome sequencing of well-characterized WNV-L1 and WNV-L2 isolates from Senegal and Italy. The experiments were undertaken by both the teams in Senegal and Italy with their local isolates. WNV-L1 (n = 10) and WNV-L2 (n = 8) well-characterized viral isolates from both countries were used to assess the ability of the designed primer pools for whole-genome amplicon-based sequencing. WNV strains from Senegal were obtained after infection of C6/36 monolayer cells with homogenized mosquito pools as previously described [12]. Isolates from Italy were obtained from birds' internal organ homogenates after two to three passages on Vero monolayer cell lines, followed by an infection on C6/36 cell lines. A genome coverage of 95% and above was targeted.

3.4.2.2 Specificity and sensitivity of the WNV amplicon based-sequencing systems

The second step was to assess specificity by performing the experiment on several other arboviruses: Rift Valley fever virus (RVFV); Yellow fever virus (YFV); Zika virus (ZIKV); Dengue 2 virus (DENV-2); Wesselsbron virus (WSLV); Kedougou virus (KDGV); Usutu virus (USUV); and

Chikungunya virus (CHIKV). The sensitivity of the approach was evaluated using serial dilutions of WNV-L1 and WNV-L2 culture isolates at different concentrations (10^6–10^2 RNA copy/µL). Each concentration was sequenced in triplicate.

3.4.2.3 Validation on confirmed WNV samples

Finally, sequencing attempts on both WNV-L1 and WNV-L2 positive samples from mosquitoes, birds, and horses from Italy and Senegal were conducted. The CT values of the samples were confirmed by RT-qPCR using a consensus WNV assay [6] in Senegal and a molecular WNV sub-typing assay [19] in Italy, prior to proceeding to the sequencing.

3.4.2.4 Next Generation Sequencing and Genome Assembly

Viral RNAs were extracted using the QIAamp viral RNA mini-kit (QIAGEN, Hilden, Germany) and were reverse-transcribed into cDNAs using the Superscript IV Reverse Transcriptase enzyme (ThermoFisher Scientific, Waltham, MA, USA). The synthesized cDNAs served as templates for direct amplification to generate approximately 400 bp amplicons tiled along the genome using two non-overlapping pools of WNV targeting primers at 10 nM and Q5® High-Fidelity 2X Master Mix (New England Biolabs) with the following thermal cycling protocol: 98 ◦C for 30 s; 35 cycles of 95 ◦C for 15 s and 65 ◦C for 5 min; and a final cooling step at 4 ◦C. In Senegal, libraries were then synthesized by tagmentation using the Illumina DNA Prep kit and the IDT® for Illumina PCR Unique Dual Indexes. After a cleaning step with the Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN), libraries were quantified using a Qubit 3.0 fluorometer (Invitrogen Inc., Waltham, MA, USA) for manual normalization before pooling in the sequencer. Cluster generation and sequencing were conducted with ab Illumina MiSeq instrument with 2×300 nt read length. Consensus genomes were generated using the nextflow-based nf-core viral reconstruction pipeline

(https://github.com/nf-core/viralrecon, accessed on 20 January 2023) from the standardized nf-core pipelines [20,21]. The versions of nextflow and viralrecon used were v21.10.6 and v2.5, respectively. In Italy, amplified DNA was diluted to obtain a concentration of 100–500 ng, then used for library preparation with an Illumina DNA prep kit, and sequenced with a NextSeq 500 (Illumina Inc., San Diego, CA, USA) using a NextSeq 500/550 Mid Output Reagent Cartridge v2 for 300 cycles with standard 150 bp paired-end reads. After quality control and trimming with the Trimmomatic v0.36 (Usadellab, Düsseldorf, Germany) [22] and FastQC tool v0.11.5 (Bioinformatics Group, Babraham Institute, Cambridge, UK) [23,24], reads were de novo assembled using SPADES v3.11.1 (Algorithmic Biology Lab, St Petersburg, Russia) [25]. The contigs obtained were analyzed with BLASTn to identify the best match reference. Mapping of the trimmed reads was then performed using the iVar computational tool [26] to obtain a consensus sequence.

3.5 Results

3.5.1 WNV oligonucleotide primers sets

A first multiplex primer system was designed based on a WNV-L1 reference genome (accession number: NC009942), generating a set of 35 oligonucleotide primer pairs that amplify overlapping products spanning almost the whole WNV genome. The primers set (set A) was subsequently compared to an alignment of 15 sequences representing the different WNV lineages (Table S3.1). Degeneration was then added in relevant ambiguous sites on each primer in order to cover a maximum of lineages while trying to maintain a balance for specificity. The list of WNV primers in set A can be found in Table 3.1. We should notice that two extra primers (KOUV_2_RIGHT and KOUV_7_LEFT) were incorporated into set A to potentially extend the sequencing to Koutango virus, even if this work was not carried out in this study.

Table 3.1 Sequences of the West Nile virus primers sets A and B

WNV Primers Set A		WNV Primers Set B	
WNV_1_LEFT	GCCTGTGTGAGCTGACAAACTT	WNV-L2_1_LEFT	GCCTGTGTGAGCTGACAAACTT
WNV_1_RIGHT	TTCTTTTGTTTTGAGCTCCKCC	WNV-L2_1_RIGHT	TTCTTTTGTTTTGTGCTCCGGC
WNV_2_LEFT	ACAGCGATGAAACACCTTCTGA	WNV-L2_2_LEFT	ACAGCGATGAAGCATCTCTTGA
WNV_2_RIGHT	CGTGTCTTGGTGCATCTTCCAT	WNV-L2_2_RIGHT	GBCGDGTYTTDGTGCATCTYCC
KOUV_2_RIGHT	TTYCCTCTGATGCATCTTCCAT	WNV-L2_3_LEFT	GTSYTRGCTGCTGGAAATGAYC
WNV_3_LEFT	CCRGTACTGTCGGCTGGTAATG	WNV-L2_3_RIGHT	CMACCCATGTAGCTCCAGAYAC
WNV_3_RIGHT	CVAGAACCAAATCCACCCAWGT	WNV-L2_4_LEFT	ATNCTATTGCTCCTGGTRGCA
WNV_4_LEFT	ACAGCTTCAACTGCCTTGGAAT	WNV-L2_4_RIGHT	TCCADCCAGTTGCTTTGGTKGW
WNV_4_RIGHT	TGRTTCTTCCTATTGCCTTGGT	WNV-L2_5_LEFT	GTRGACAGRGGATGGGGAAAYG
WNV_5_LEFT	GGYTGCGGACTATTTGGMAAA	WNV-L2_5_RIGHT	GRTTCCTCCAHGYGGTGCTT
WNV_5_RIGHT	CTCCACACAGTACTTCCAGCAC	WNV-L2_6_LEFT	CCTTCCTGGTYCACCGAGARTG
WNV_6_LEFT	GGHACAAAGACGTTCTTGGTYC	WNV-L2_6_RIGHT	KGAVGAAATGGGCACYTTRCAR
WNV_6_RIGHT	GMACTTTGCAAGGTCCATCYGT	WNV-L2_7_LEFT	GCDTTYAAATTCGCYGGGACTC
WNV_7_LEFT	AYGCTTTCAAGTTTCTTGGGACT	WNV-L2_7_RIGHT	GTGTATRGCTTTCCCYACYGAG
KOUV_7_LEFT	AYGCTTTCAAGTTTCTTGGGCAT	WNV-L2_8_LEFT	ACTCAGAGGAGCTCAACGACTC
WNV_7_RIGHT	AASACTTGATGGACAGCCTTCC	WNV-L2_8_RIGHT	ATTYTTGCTAGGCCTTGTGGHG
WNV_8_LEFT	ARMGACTAGCCGCTCTAGGAGA	WNV-L2_9_LEFT	CGGTGYGGAAGTGGAGTGTTYA
WNV_8_RIGHT	TGDGCTTTCTGAATGATCTTGGCT	WNV-L2_9_RIGHT	TRYTRTTCCATGCTCGGTTSR

WNV Primers Set A		WNV Primers Set B	
WNV_9_LEFT	AAYGATGTGGAGGCTTGGATGG	WNV-L2_10_LEFT	YGCDCCAGARCTAGCTAACAAYA
WNV_9_RIGHT	AWRCTATTCCAAGCGCGATTSK	WNV-L2_10_RIGHT	CCCTGGYCTCCTGTTRTGRTTGC
WNV_10_LEFT	TMTTTGCACCAGAACTCGCYAA	WNV-L2_11_LEFT	CACHYTGTGGGGTGATGGAGTT
WNV_10_RIGHT	CWGGTCTCCGATTGTGATTGCT	WNV-L2_11_RIGHT	CAGAAGGCCCAACTGAAAAGGA
WNV_11_LEFT	AYACCTTGTGGGGCGATGGART	WNV-L2_12_LEFT	GAYGAAAAGACCCTCGTGCA
WNV_11_RIGHT	GGCCCAACTGAAAAGGGTCAAT	WNV-L2_12_RIGHT	CCATTTGRAAGAAAGCAGCTGCR
WNV_12_LEFT	TGGARATCAGACCACAGAGRCA	WNV-L2_13_LEFT	CAGTCTTTCTGGTGGCTTCBTT
WNV_12_RIGHT	TTGRAAGAAAACAGCCGCCARC	WNV-L2_13_RIGHT	YCCAGCKGCAAGTATCATBGGA
WNV_13_LEFT	CCAGTGTTTATGGTGGCWTCGT	WNV-L2_14_LEFT	MRGTTGGAAGCYTCATCAARGARA
WNV_13_RIGHT	CHGCAAGGATCATGGGGTTGAA	WNV-L2_14_RIGHT	TGAAAATTTCCATCRTCATCCARCC
WNV_14_LEFT	GVAGCTTGATCAGGGAGAARAG	WNV-L2_15_LEFT	GGACRGCTGAYATYACYTGGGA
WNV_14_RIGHT	TCCBGGATCATTCATGARCTGG	WNV-L2_15_RIGHT	RCTCATGAGAGCRGCTCCCTTR
WNV_15_LEFT	CKGACATTTCCTGGGAAAGTGA	WNV-L2_16_LEFT	ATHATGACTCGAGGTCTGCTYG
WNV_15_RIGHT	TCATCAAAGCGGCTCCTTTWGT	WNV-L2_16_RIGHT	MRCCATTDGGCATGATGACKCC
WNV_16_LEFT	CTYGGCAGTTATCAAGCAGGAG	WNV-L2_17_LEFT	RGACTATCCCACYGGAACRTCA
WNV_16_RIGHT	WATBGCGCTTATGTATGAHCCR	WNV-L2_17_RIGHT	TGGCACATGACATCAACGATYT
WNV_17_LEFT	CMATAGTGGACAAAAACGGTGATGT	WNV-L2_18_LEFT	TKAGAGGACTTCCCATYCGGTA
WNV_17_RIGHT	DGTGAGGGTAGCATGACACATG	WNV-L2_18_RIGHT	TTBCCCATTTTCACACTTGGAACR
WNV_18_LEFT	ATGKCTGAAGCACTGAGRGGA	WNV-L2_19_LEFT	ACMGAGCCTGGAACACTGGVTA
WNV_18_RIGHT	CCCATCTTGACACTAGGCACAA	WNV-L2_19_RIGHT	CCTCCATAGCAATACTCATCACCA

WNV Primers Set A		WNV Primers Set B	
WNV_19_LEFT	CGRGCTTGGAACTCTGGATAYG	WNV-L2_20_LEFT	TGATGGAAGAGTCATCCTGGGV
WNV_19_RIGHT	TACTCATCACCAACTTGCGAYG	WNV-L2_20_RIGHT	GTGTTGGTTCGAGGTCCATCAA
WNV_20_LEFT	GGAGAACCATCTGCAGTGACAG	WNV-L2_21_LEFT	CWGTCTGGCTCGCTTACAAAGT
WNV_20_RIGHT	CMACTTCGTTGTTGTCTTCTAAAATTG	WNV-L2_21_RIGHT	ARGCTATTGTCTGAAGGGCRTC
WNV_21_LEFT	CYTACAAGGTTGCAGCRGCT	WNV-L2_22_LEFT	TTKGACACGATGTATGTGGTKG
WNV_21_RIGHT	ACTCCCATGGTCATCACACWCA	WNV-L2_22_RIGHT	CAYTCTTGGTCTTGTCCAGCCA
WNV_22_LEFT	GAGGMAGAGCTCACAGAATGGC	WNV-L2_23_LEFT	YCAGCTYGCCGTGTTTTTGATY
WNV_22_RIGHT	CCTTGACCTCAATTCTTTGCCC	WNV-L2_23_RIGHT	CYGCAGTCACAGTCACAGTCAG
WNV_23_LEFT	TTGTGTCATGACCCTTGTSAGC	WNV-L2_24_LEFT	YTTTGTVGACGTTGGTGTGTCA
WNV_23_RIGHT	GGDACCATGTAGGCATAGTGGC	WNV-L2_24_RIGHT	TYGTTGCRTTCCACACTGARCT
WNV_24_LEFT	TTYGTCGATGTTGGAGTKTCA	WNV-L2_25_LEFT	ARRACTGTCAGAGAGGCYGGAA
WNV_24_RIGHT	GTYGTTGCGTTCCAMACWGAGC	WNV-L2_25_RIGHT	GCCTTTCCACTAACCACCGYAR
WNV_25_LEFT	RGACHGTVCGAGAAGCYGGAAT	WNV-L2_26_LEFT	RHGCCAGGAGAGAGGGAAAYRT
WNV_25_RIGHT	GRTCGAGGAAACBCCGTTCGAC	WNV-L2_26_RIGHT	CCARTCTTCCACCATCTCCARR
WNV_26_LEFT	RAGAAGGCAAYRTCACYGGAGG	WNV-L2_27_LEFT	CACACTGCTCTGTGACATTGGA
WNV_26_RIGHT	ARAATTCCCTTGGCCCTCGG	WNV-L2_27_RIGHT	AGAATTGAGGAGAGGCTTCCCY
WNV_27_LEFT	TCAAGTGCTGAGGTTGAAGAGC	WNV-L2_28_LEFT	AAGAAAACVTGGAAGGGACCYC
WNV_27_RIGHT	GCCTGAGTCGTTCAATCCTGTT	WNV-L2_28_RIGHT	TBGTGGTCTCATTGAGGACGTR
WNV_28_LEFT	TGTAAACTTGGGAAGTGGAACCA	WNV-L2_29_LEFT	CTCCTTTCGGHCAACAACGRGT
WNV_28_RIGHT	TTTWTCKCTGGCCAYAAAVGCC	WNV-L2_29_RIGHT	CYCCBAGCCACATGAACCADAT

WNV Primers Set A		WNV Primers Set B	
WNV_29_LEFT	GTGGAYACGAAAGCTCCTGARC	WNV-L2_30_LEFT	ACYTGCATCTACAACATGATGGG
WNV_29_RIGHT	ATTGAGAAAACCSAGAGCTTCG	WNV-L2_30_RIGHT	GGBCTCATCACTTTCACGACYT
WNV_30_LEFT	MAARGCCAARGGMAGCAGAG	WNV-L2_31_LEFT	CDAAGGTBCTTGARCTGCTDGR
WNV_30_RIGHT	CCYCTCTGATCTTCTCTGGAGA	WNV-L2_31_RIGHT	GGACCTTTGACATGGCATTBAGR
WNV_31_LEFT	TGAGCTCACCTATCGWCACAAA	WNV-L2_32_LEFT	GGHGATGACTGYGTGGTDAA
WNV_31_RIGHT	CAYCCAGTTGACGGTTTCCACT	WNV-L2_32_RIGHT	GRACCCAGTTVACAGGCACA
WNV_32_LEFT	TGGTRAAGCCCCTGGAYGAY	WNV-L2_33_LEFT	GCAGATGTGGCTGYTGCTTTAT
WNV_32_RIGHT	TCTCCTCCTGCATGGATSGA	WNV-L2_33_RIGHT	YRTCTTCATACCTCCTCARDGA
WNV_33_LEFT	AGWAGAGACCTGMGGYTCAT	WNV-L2_34_LEFT	GCGCHACTTGGGCTGAAAAYAT
WNV_33_RIGHT	TCTACAAAACTGTGTCCTCAACCA	WNV-L2_34_RIGHT	MYCTTCCGAGACGGTTCTGA
WNV_34_LEFT	AGTCAGWKCAATCATCGGRGAWG	WNV-L2_35_LEFT	GGAAGTTGAGTAGACGGTGCTG
WNV_34_RIGHT	CACTATCGCAGACTGCACTCTC	WNV-L2_35_RIGHT	TCCCAGGTGTCAATATGCTGTT
WNV_35_LEFT	CAGGAGGACTGGGTGAACAAAG		
WNV_35_RIGHT	TGGTTGTGCAGAGCAGAAGATC		

A second primer set (set B) was designed based on a WNV-L2 reference genome (accession number: MH021189) and was compared with an alignment of 82 WNV-L2 sequences (Table S3.2) to capture the diversity within the lineage. The list of WNV primers in set B can be found in Table 3.1.

3.5.2 WNV primers sets validation

3.5.2.1 Validation of set A

3.5.2.1.1 Inclusivity test

After the design of set A, seven WNV-L1 and three WNV-L2 isolates from Senegal were selected, and three viral culture supernatants for each lineage from three different Italian regions were processed for amplicon-based sequencing in triplicate. Overall, tiled amplicon whole-genome sequencing undertaken on both strains from Senegal and Italy yielded 99–100% horizontal coverage with genome length between 10,961 nt and 11,018 nt for WNV-L1 and between 10,914 nt and 10,926 nt for WNV-L2 (Table 3.2).

Table 3.2 Inclusivity test of the West Nile virus set A primers.

RT-PCR Ct value		# Total Trimmate Reads	# WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
Viral strain WNV L1 Italy	14	2.369.723	649.022	99%	5802.67	10.969
	14	1.922.532	581.215	99%	5346.85	10.966
	13	2.715.830	754.081	99%	6244.36	10.966
	N of replicates with Coverage ≥ 95 %			3/3 (100 %)		
Viral strain WNV L1 Senegal	25	623.535	238.259	99%	7997	10.961
	28	5.034.151	1.035.983	99%	6862.12	10.965
	16	5.034.151	547.775	99%	5183.83	10.966
	19	810.906	327.395	100%	3971.4	11.018
	17	924.142	363.236	99%	4412.7	10.963
	17	899.818	342.133	99%	3851.34	10.966
	17	819.552	358.631	99%	4306.94	10.961
	N of replicates with Coverage ≥ 95 %			7/7 (100%)		
Viral strain WNV L2 Italy	18	2.607.185	546.249	100%	3607.99	10.926
	18.71	2.466.682	511.381	100%	3488.1	10.926
	18.13	2.061.961	465.844	100%	3445.55	10.926
	N of replicates with Coverage ≥ 95 %			3/3 (100 %)		
	14	4.861.644	706.114	100%	3519.51	10.914

	RT-PCR Ct value	# Total Trimmate Reads	# WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
Viral strain WNV L2 Senegal	14	4.087.737	835.098	100%	5593.58	10.914
	14	4.750.250	885.595	100%	5609.34	10.914
	N of replicates with Coverage ≥ 95 %			3/3 (100 %)		
Viral strain USUV Italy	10	1.997.126	56.4902	100%	6053.7	10.837
	20	1.909.064	482.135	100%	5360.97	10.835
	16	2.056.663	553.137	100%	6041.88	10.837
	N of replicates with Coverage ≥ 95 %			3/3 (100 %)		
Viral strain USUV Senegal	33	694.838	225.462	95%	3041.56	10.494
	28	854.870	304.085	97%	4033.3	10.494
	27	556.582	223.863	97%	3024.04	10.499
	31	523.901	197.085	95%	2715.43	10.494
	29	769.757	247.559	95%	3073.1	10.499
	32	3.250.194	605.236	97%	5460.49	10.498
	13	628.539	281.283	99%	3729.34	10.695
	13	679.204	289.399	100%	3803.38	10.825
	14	379.968	188.337	100%	2479.05	10.836
	14	445.022	221.901	99%	2975.98	10.699
	N of replicates with Coverage ≥ 95 %			10/10 (100%)		

3.5.2.1.2 Sensitivity test

One representative isolate of each lineage, i.e., WNV 15217 (accession number: FJ483548) and WNV Thessaloniki_MC82m/2018 (accession number: MN652880) for WNV-L1 and WNV-L2, respectively, was selected to evaluate the detection limit of the set A primers under optimal conditions. Serial dilutions from 10^6 to 10^2 cp/µL were processed in triplicate for sequencing. The set A primers were able to detect more than 95% of the total WNV-L1 genome up to 10^4 cp/µL. At 10^3 cp/µL, the horizontal coverage was between 91% and 94%, while at 10^2 cp/µL, 80 to 82% of the WNV-L1 sequence was completed. However, poor coverage was observed in the WNV-L2 samples (between 17% and 35% completeness) as shown in Table 3.3.

Table 3.3 Sensitivity test of the West Nile virus set A primers

Viral Strain	Quantity Value (cp/uL)	Quantity Mean Value (Ct)	Illumina Miseq Run Number	Total Number of Trimmate Reads	Number of WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
WNV L1 (reference used for the mapping on Genpat: WNV L1 FJ483548	10^6	18.68	230	1.457.278	503.152	99%	5043.56	10.959
	10^6		230	1.371.693	476.605	99%	4997.04	10.964
	10^6		230	938.406	375.653	99%	4372.01	10.959
		N of replicates with Coverage ≥ 95 %				3/3 (100 %)		
	10^5	23.45	230	1.342.407	426.914	99%	4587.33	10.960
	10^5		230	1.174.155	397.215	99%	4435.79	10.959
	10^5		230	825.135	315.219	99%	3776.67	10.956
		N of replicates with Coverage ≥ 95 %				3/3 (100 %)		
	10^4	27	230	906.690	292.718	96%	3456.26	10.964
	10^4		230	984.952	297.351	97%	3414.38	10.959
	10^4		230	1.247.127	327.095	96%	3570.22	10.959
		N of replicates with Coverage ≥ 95 %				3/3 (100 %)		
	10^3	30	230	1.096.401	249.798	94%	2735.41	10.962
	10^3		230	506.380	153.284	93%	1853.42	10.955
	10^3		230	569.039	170.272	91%	2099.54	10.955
		N of replicates with Coverage ≥ 95 %				0/3 (0 %)		
	10^2	33	230	57.229	20.115	82%	295.76	10.366

Viral Strain	Quantity Value (cp/uL)	Quantity Mean Value (Ct)	Illumina Miseq Run Number	Total Number of Trimmate Reads	Number of WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
	10^2		230	53.435	18.515	81%	281.027	10.958
	10^2		230	41426	15.149	80%	227.617	10.951
		N of replicates with Coverage ≥ 95 %				0/3 (0 %)		
WNV L2 (reference used for the mapping: WNV L2 MN652880)	10^6		230	1.262.167	12.484	35%	391.547	10.028
	10^6	18.68	230	1.315.568	11.576	33%	383.276	10.928
	10^6		230	1.103.838	16.652	26%	706.146	10.928
		N of replicates with Coverage ≥ 95 %				0/3 (0 %)		
	10^5		230	1.210.495	11.251	36%	347.197	10.928
	10^5	22.87	230	823.025	10.243	33%	345.101	10.928
	10^5		230	1.478.160	12.253	34%	394.675	10.928
		N of replicates with Coverage ≥ 95 %				0/3 (0 %)		
	10^4		230	1.228.945	9.715	32%	337.862	10.928
	10^4	26.41	230	1.090.849	9.547	32%	327.57	10.928
	10^4		230	947.964	4.405	30%	159.122	10.928
		N of replicates with Coverage ≥ 95 %				0/3 (0 %)		
	10^3		230	442.063	2.796	26%	121.744	10.928
	10^3	30	230	577.277	3.500	27%	143.439	10.926
	10^3		230	369.041	1.615	22%	79.118	10.245

Viral Strain	Quantity Value (cp/uL)	Quantity Mean Value (Ct)	Illumina Miseq Run Number	Total Number of Trimmate Reads	Number of WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
		N of replicates with Coverage ≥ 95 %				0/3 (0 %)		
	10^2		230	35.426	1.362	17%	789.157	10.924
	10^2	33.14	230	68.307	627	17%	360.495	10.926
	10^2		230	63.643	731	18%	571.297	10.926
		N of replicates with Coverage ≥ 95 %				0/3 (0 %)		
USUV (reference used for the mapping on Genpat: USUV HM569263)	10^6		230	1.189.449	376.979	100%	4607.17	10.837
	10^6	19	230	1.269.455	403.033	100%	4838.74	10.836
	10^6		230	1.198.499	392.297	100%	4745.8	10.837
		N of replicates with Coverage ≥ 95 %				3/3 (100 %)		
	10^5		230	1.240.823	379.133	99%	4581.63	10.837
	10^5	23	230	1.073.364	351.179	99%	4289.55	10.807
	10^5		230	1.235.377	386.080	99%	4624.55	10.837
		N of replicates with Coverage ≥ 95 %				3/3 (100 %)		
	10^4		230	1.392.113	381.234	99%	4339.31	10.838
	10^4	27	230	1.169.792	349.199	97%	4178.8	10.499
	10^4		230	1.094.369	334.163	96%	4135.57	10.499
		N of replicates with Coverage ≥ 95 %				3/3 (100 %)		
	10^3	30	230	641.826	223.790	96%	2848.31	10.499

Viral Strain	Quantity Value (cp/uL)	Quantity Mean Value (Ct)	Illumina Miseq Run Number	Total Number of Trimmate Reads	Number of WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
	10^3		230	695.820	238.992	95%	3069.17	10.351
	10^3		230	462.528	182.763	92%	2441.9	10.502
		N of replicates with Coverage ≥ 95 %				2/3 (66 %)		
	10^2		230	85.909	50.388	88%	709.483	10.348
	10^2	34	230	44.720	28.768	88%	406.636	10.346
	10^2		230	37.560	24.606	83%	366.618	10.340
		N of replicates with Coverage ≥ 95 %				0/3 (0 %)		

3.5.2.1.3 Specificity test

Amplicon-based whole-genome sequencing with set A primers was conducted on six flavivirus species (YFV, ZIKV, DENV-2, WSLV, KDGV, USUV), as well as RVFV and CHIKV, in order to assess the specificity of this WNV targeted approach. All the samples failed the bowtie2 1000 mapped-read threshold and no consensus genome could be assembled.

3.5.2.1.4 WNV Set A primers validation on real homogenates

Thirty-one (31) WNV-L1 and fifty-four (54) WNV-L2 homogenates with known Ct values by RT-qPCR were selected for targeted sequencing using the set A primers. Homogenates were obtained from mosquito pools and the internal organs of birds with low to high viral loads. Among WNV-L1 homogenates, horizontal coverage was between 34% and 100%. A total of 35% of the samples reached above 95% horizontal coverage and about 65% of samples for 90% horizontal coverage. Most complete genomes had Ct values between 16 and 28. However, we also noted that among the least well-covered samples, Ct values ranged from 25 to 35, highlighting that factors other than the viral load could be involved. Additionally, five samples were WNV-L1/WNV-L2 co-infections, and the amplicon-based approach yielded from 87% to 96% WNV-L1 horizontal coverage, even when WNV-L2 had a higher viral load. Relatively correct coverage (between 74% and 92%) was obtained from other four samples from mosquitoes trapped in Senegal, for which viral co-infections with either alphaviruses, mesoniviruses, or flaviviruses were reported. All these results are summarized in Table 3.4.

*Table 3.4 Test of the West Nile virus set A primers with WNV-L1 homogenates. (*Samples with multiple viral species/WNV lineage)*

Viral Homogenate WNV L1 – Sample Number	Host	RT-PCR Ct Value	Co-Infection Ct Value	# Total Trimmate Reads	# WNV L1 Reads	% HCoverage	VCoverage	Consensus Sequence Length
1	*Accipiter gentilis*	15	-	2,037,215	591,954	100%	6362.04	11,027
2	*Pica pica*	16	-	14,648,025	1,333,187	100%	6381.67	11,016
3	*Corvus cornix*	16	-	6,447,209	657,948	99%	5135.45	10,966
4	*Pica pica*	18	-	2,923,237	387,986	99%	4264.56	10,966
5	*Phalacrocorax carbo*	19	-	11,441,518	1,107,291	99%	5888.49	10,961
6	*Corvus cornix*	19	-	2,347,380	351,292	99%	4177.69	10,963
7	*Culex pipiens*	20	-	3,495,685	744,562	99%	5560.68	10,968
8	*Culex pipiens*	22	-	7,571,826	597,499	98%	3973.2	10,967
9	*Corvus cornix*	22	-	5,382,363	629,364	99%	4806.11	10,960
10	*Passer domesticus*	22	-	3,942,375	304,215	97%	3084.4	10,962

Viral Homogenate WNV L1 – Sample Number	Host	RT-PCR Ct Value	Co-Infection Ct Value	# Total Trimmate Reads	# WNV L1 Reads	% HCoverage	VCoverage	Consensus Sequence Length
11	*Culex pipiens*	23	-	4,560,711	283,771	93%	2899.47	10,960
12	*Corvus Cornix*	24	-	3,052,530	342,271	93%	3508.25	10,966
13*	*Culex pipiens*	25	Lin2 Ct 28	3,576,787	403,166	94%	3761.48	10,966
14*	*Culex pipiens*	25	Lin 2 Ct 28	1,557,373	263,149	90%	2471.3	10,954
15	*Larus michahellis*	25	-	1,813,567	129,834	88%	1805.95	10,952
16	*Streptopelia decaocto*	26	-	1,439,897	203,172	91%	2592.09	10,961
17	*Pica pica*	26	-	7,677,172	251,982	81%	2920.03	10,963
18	*Parus major*	26	-	755,951	387,80	69%	747.61	9,389
19*	*Culex pipiens*	27	Lin2 Ct 32	3,371,541	369,610	96%	3287.31	10,956
20	*Turdus merula*	27	-	2,710,572	66,229	82%	1039.08	10,954
21	*Culex pipiens*	28	-	6,865,408	269,727	94%	2618.84	10,962
22*	*Culex pipiens*	28	Lin 2 Ct 31	3,378,716	190,102	87%	2201.29	10,966
23	*Streptopelia decaocto*	28	-	1,842,010	22,006	68%	430.494	10,946

Viral Homogenate WNV L1 – Sample Number	Host	RT-PCR Ct Value	Co-Infection Ct Value	# Total Trimmate Reads	# WNV L1 Reads	% HCoverage	VCoverage	Consensus Sequence Length
24	*Equus caballus*	28	-	1,805,957	159,703	92%	2101.31	10,960
25	*Athene noctua*	29	-	3,394,989	15,767	34%	404	10,802
26	*Columba palumbus*	31	-	835,362	9,948	57%	211.706	10,960
27*	*Culex pipiens*	33	Lin2 Ct 25	4,662,340	220,508	91%	2381.21	10,963
28*(Alphavirus, Mesonivirus)	*Cx. neavei*	28,5	-	391,494	1,135,180	92,42%	2514.3	10194
29*(Barkedji, Mesonivirus)	*Cx. poicilipes*	35,59	-	64,716	616,500	82,35%	1789.36	9083
30*(Barkedji)	*Cx. neavei*	29,52	-	302,175	1,394,466	89%	2001.3	9819
31*(Alphavirus, Barkedji, Usutu)	*Cx. neavei*	25,03	-	180,791	484,221	73.89%	822.02	8150

Regarding WNV-L2 homogenates, experiments undertaken with the set A primers were consistent with the data from inclusivity and specificity tests. Indeed, less than 6% of the samples processed had above 95% of the genome covered (3 out 54), and 87% had ≤ 64% horizontal coverage whatever the viral load (Table 3.5).

*Table 3.5 Test of the West Nile virus set A primers with WNV-L2 homogenates. (*Samples with multiple viral species/WNV lineage)*

Viral Homogenate WNV L2 – Sample Number	**Host**	**RT-PCR Ct Value**	**Co-Infection Ct Value**	**# Total Trimmate Reads**	**# WNV Reads**	**% HCoverage**	**VCoverage**	**Consensus Sequence Length**
1	*Accipiter gentilis*	16	-	3.336.278	450.617	100%	3304.6	10.926
2	*Accipiter gentilis*	16	-	1.773.333	393.125	99%	2982.38	10.926
3	*Accipiter gentilis*	19	-	2.753.185	225.096	72%	1533.63	10.926
4	*Garrulus glandarius*	20	-	3.540.566	304.094	96%	1588.44	10.923
5	*Cx. pipiens*	22	-	1.092.527	517.44	58%	779.912	10.921
6	*Cx. pipiens*	23	-	568.562	54.658	59%	905.155	10.834
7	*Corvus cornix*	23	-	1.383.831	85.880	62%	1168.95	10.922
8	*Passer italiae*	24	-	476.120	45.644	47%	1089.79	10.923
9	*Columba palumbus*	26	-	1.313.900	76.773	64%	908.822	10.923
10	*Columba palumbus*	27	-	5.377	7.319	25%	257.256	10.868
11	*Columba palumbus*	28	-	545.944	5073	23%	281.009	8.425
12	*Turdus merula*	31	-	514.655	72	4%	26.336	375

Viral Homogenate WNV L2_–Sample Number	**Host**	**RT-PCR Ct Value**	**Co-Infection Ct Value**	**# Total Trimmate Reads**	**# WNV Reads**	**% HCoverage**	**VCoverage**	**Consensus Sequence Length**
13	*Pica pica*	31	-	330.374	280	5%	685.197	4.128
14	*Phasianus colchicus*	32	-	243.656	242	5%	543.463	7.510
15	*Pica pica*	33	-	341.153	613	9%	760.203	8.413
16	*Pica pica*	33	-	376.185	128	4%	307.715	3.779
17	*Egretta garzetta*	34	-	616.781	395	6%	715.396	7.923
18	*Culex pipiens*	29	-	1.537.448	24.517	33%	676.501	9.393
19	*Culex pipiens*	28	-	588.313	32.437	49%	749.224	10.924
20	*Culex pipiens*	27	-	606.886	28.181	52%	494.551	10.923
21	*Culex pipiens*	25	-	884.756	43.657	52%	864.517	10.891
22	*Culex pipiens*	30	-	259.173	2.572	3%	989.276	381
23	*Culex pipiens*	28	-	436.461	14.473	29%	630.097	10.923
24	*Culex pipiens*	24	-	2.172.952	45.568	45%	716.974	10.923
25*	*Culex pipiens*	31	L1 Ct 28	3.378.716	9.132	27%	447.986	10.928
26	*Culex pipiens*	25	-	339.617	33.681	42%	827.229	10.790
27	*Culex pipiens*	21	-	1.223.422	35.451	23%	1197.74	10.921
28	*Culex pipiens*	23	-	1.832.341	38.134	15%	1569.34	10.555
29	*Culex pipiens*	25	-	686.602	25.509	18%	1178.55	10.609

Viral Homogenate WNV L2 – Sample Number	**Host**	**RT-PCR Ct Value**	**Co-Infection Ct Value**	**# Total Trimmate Reads**	**# WNV Reads**	**% HCoverage**	**VCoverage**	**Consensus Sequence Length**
30	*Culex pipiens*	27	-	502.162	8.253	3%	3113.1	388
31	*Corvus cornix*	28	-	1.357.321	7.159	31%	312.307	10.475
32	*Pica pica*	20	-	6.417.489	198.613	94%	1616.11	10.927
33*	*Culex pipiens*	25	L1 Ct 33	4.662.340	33.608	60%	534.955	10.926
34*	*Culex pipiens*	24	USUV Ct 27	5.133.183	77.022	61%	888.532	10.925
35	*Culex pipiens*	24	-	153.601	10	47%	275.337	556
36	*Corvus cornix*	29	-	877.218	0	0%	0	0
37	*Culex pipiens*	23	-	199.550	99.490	88%	1126.14	10.890
38	*Culex pipiens*	24	-	323.115	5.048	23%	308.136	4.395
39*	*Culex pipiens*	27	L1 Ct 25	3576787	9.461	51%	239.312	10.928
40	*Culex pipiens*	22	-	78.378	42.637	63%	824.461	10.922
41	*Larus marinus*	23	-	546.215	142.178	81%	1460.97	10.923
42*	*Culex pipiens*	27	L1 Ct 32	3.371.541	5.603	47%	154.32	10.928
43	*Culex pipiens*	29	-	153.413	4.367	31%	187.09	9.364
44	*Culex pipiens*	28	-	66.202	7.786	36%	290.742	10.018
45*	*Culex pipiens*	28	L1 Ct 25	1.557.373	8.945	48%	243.941	10.926
46	*Culex pipiens*	28	-	118.396	2.420	15%	219.733	7.809
47*	*Culex pipiens*	27	USUV Ct 27	1.516.324	230	44%	985.194	7.469

Viral Homogenate WNV L2 – Sample Number	**Host**	**RT-PCR Ct Value**	**Co-Infection Ct Value**	**# Total Trimmate Reads**	**# WNV Reads**	**% HCoverage**	**VCoverage**	**Consensus Sequence Length**
48	*Corvus cornix*	28	-	470.113	6.194	40%	207.827	10.923
49*	*Culex pipiens*	23	USUV Ct 21	4.272.994	248	29%	941.936	10.806
50	*Culex pipiens*	15	-	327.238	88.896	61%	1398.22	10.922
51	*Ochlerotatus caspius*	25	-	361.194	16.436	32%	684.576	10.844
52	*Culex pipiens*	24	-	401.408	25.091	42%	804.597	9.592
53	*Pica pica*	23	-	899.597	2.771	24%	150.215	8.402
54*	*Culex pipiens*	29	USUV Ct 26	117.768	18.204	54%	451.546	10.907

3.5.2.2 Validation of set B

3.5.2.2.1 Inclusivity test

Five WNV-L2 isolates from Italy were selected to assess the set B primers. A total of 100% horizontal coverage was obtained for all the strains after sequencing on an Illumina MiSeq (Table 3.6).

Table 3.6 Inclusivity test of the West Nile virus set B primers

	RT-PCR Ct Value	# Total Trimmate Reads	# WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
Viral strain WNV L2 Italy	15	1.218.086	284.232	100%	3820.77	10.926
	15	1.792.478	402.082	100%	5234.36	10.926
	15	1.440.061	338.706	100%	4543.81	10.926
	17	1.711.005	328.182	100%	4374.41	10.926
	18	941.641	224.716	100%	3023.77	10.926
	N of replicates with Coverage ≥ 95 %			5/5 (100%)		

3.5.2.2.2 Sensitivity test

In order to identify the set B primers' detection limit under optimal conditions, serial dilutions from 10^6 to10^2 cp/μL of the strain WNV Thessaloniki_MC82m/2018 (accession number: MN652880) were processed in triplicate for sequencing (except the to10^2 cp/μL concentration, which was carried out in duplicate due to insufficient volume during the experiment). A total of 100% horizontal coverage was obtained between 10^6 to10^3 cp/μL, while the two replicates for to10^2 cp/μL covered 93% and 95% of the genome, as shown by Table 3.7.

Table 3.7 Sensitivity test of the West Nile virus set B primers

Viral Strain	Quantity Value (cp/5uL)	Quantity Mean Value (Ct)	Illumina Miseq Run Number	# Total Trimmate Reads	# WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
WNV L2 (reference used for the mapping: WNV L2 MN652880)	10^6	19	27	1.808.185	381.930	100%	5038.73	10.913
	10^6		27	4.928.502	673.900	100%	6845.55	10.926
	10^6		27	3.099.180	511.446	100%	6107.38	10.913
	N of replicates with Coverage ≥ 95 %					3/3 (100%)		
	10^5	22	27	2.665.260	409.989	100%	5157.74	10.914
	10^5		27	1.049.020	237.471	100%	3194.36	10.926
	10^5		27	2.820.387	429.662	100%	5335.99	10.912
	N of replicates with Coverage ≥ 95 %					3/3 (100%)		
	10^4	26	27	1.651.945	261.024	100%	3450.12	10.913
	10^4		27	2.483.786	337.982	100%	4234.91	10.914
	10^4		27	2.681.807	356.233	100%	4334.71	10.926
	N of replicates with Coverage ≥ 95 %					3/3 (100%)		
	10^3	30	27	1.570.036	236.060	100%	3029.53	10.894
	10^3		27	1.153.288	196.257	100%	2614.91	10.904
	10^3		27	782.424	285.136	99%	3070.47	10.912
	N of replicates with Coverage ≥ 95 %					3/3 (100%)		
	10^2	33	27	1.764.307	159.591	95%	2189.31	10.597

Viral Strain	Quantity Value (cp/5uL)	Quantity Mean Value (Ct)	Illumina Miseq Run Number	# Total Trimmate Reads	# WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
	10^2		27	2.082.360	177.212	93%	2389.54	10.800
	10^2		27	NA	NA	NA	NA	NA
	N of replicates with Coverage ≥ 95 %					1/2 (50%)		

3.5.2.2.4 Specificity test

Similar to the test conducted for set A, no amplification was observed using set B on the six flavivirus species mentioned above, as well as RVFV and CHIKV.

3.5.2.2.5 WNV Set B primers validation on real homogenates

Fifteen WNV-L2 homogenates from Italy with known CT values by RT-qPCR were selected for targeted sequencing using the set B primers. Homogenates were obtained from mosquito pools, as well as the internal organs of birds and horses with low to high viral loads. Overall, horizontal coverage between 97% and 100% was obtained on 14 out of 15 homogenates (93.3% with horizontal coverage > 95%). Only the horse sample exhibited 93% horizontal coverage. This sample was also the one with the lowest viral load (CT value: 35). All these results are summarized in Table 3.8.

Table 3.8 Test of the West Nile virus set B primers with WNV-L2 homogenates

Viral Homogenate WNV L2 – Sample Number	**RT-PCR Ct Value**	**Host**	**# Total Trimmate Reads**	**# WNV Reads**	**% HCoverage**	**VCoverag e**	**Consensus Sequence Length**
1	17	*Pica pica*	1.134.961	295.649	100%	3977.58	10.892
2	27	*Corvus cornix*	1.448.962	284.947	100%	3818.15	10.912
3	21	*Cx. pipiens*	1.733.553	385.088	100%	5042.35	10.913
4	23	*Cx. pipiens*	1.528.111	339.207	100%	4525.72	10.912
5	21	*Athene noctua*	1.801.390	376.213	100%	4946.53	10.878
6	22	*Cx. pipiens*	1.407.784	312.376	99%	4201.89	10.879
7	19	*Passer domesticus*	1.515.878	205.605	100%	2770.45	10.914
8	30	*Corvus cornix*	1.470.367	209.316	98%	2799.57	10.872
9	30	*Pica pica*	2.150.396	205.466	99%	2662.61	10.868
10	27	*Sylvia atricapilla*	3.649.208	281.102	99%	3426.07	10.880
11	25	*Cx. pipiens*	2.094.249	349.710	100%	4076.35	10.878
12	29	*Anopheles maculipennis*	3.435.959	563176	100%	5709.14	10.912
13	25	*Cx. pipiens*	3.120.601	281.102	100%	4601.81	10.912
14	27	*Cx. pipiens*	2.193.040	259.483	97%	3231.74	10.904
15	35	*Horse*	1.623.442	133.088	93%	1814.74	10.936

3.5.2.4 Validation of set A+B

In order to obtain a system able to efficiently sequence both WNV-L1 and WNV-L2 strains, the first set of primers (set A) was combined with the second one (set B) in equal volume. The new system, set A + B primers, was evaluated and compared in parallel with set A and set B after sequencing the WNV-L1 (n = 4) and WNV-L2 (n = 7) positive samples from internal organs of birds and horses, as well as mosquito homogenates, at different CT values (Table 3.9).

Table 3.9 Test of the West Nile virus set A+B primers with WNV-L1 and WNV-L2 homogenates

Viral Homogenate WNV L1	RT-PCR Ct Value	Host	Used Primers	# Total Trimmate Reads	# WNV Reads	% HCoverage	VCoverage	Consensus Sequence Length
1	L1 19	*Corvus cornix*	Set A	2.347.380	351.292	99%	4177.69	10.963
			Set A+B	1.118.615	287.672	99%	3987.12	10963
2	L1 25	*Larus michahellis*	Set A	1.813.567	129.834	88%	1805.95	10.952
			Set A+B	651.723	91.642	93%	1305.83	10.960
3	L1 18	*Pica pica*	Set A	2.923.237	387.986	99%	4264.56	10.966
			Set A+B	4.965.722	512.725	99%	4562.28	10.966
4	L1 28	*Horse*	Set A	1.805.957	159.703	92%	2101.31	10.960
			Set A+B	2.319.560	165.376	92%	2200.39	10.960
Viral Homogenate WNV L2	**RT-PCR Ct Value**	**Host**	**Used Primers**	**# Total Trimmate Reads**	**# WNV Reads**	**% HCoverage**	**VCoverage**	**Consensus Sequence Length**
1	L2 17	*Pica pica*	Set B	2.059.659	351.670	100%	4232.78	10.892
			Set A+B	1.134.961	295.649	100%	3977.58	10.892
2	L2 27	*Corvus cornix*	Set B	1.793.046	187.826	100%	2156.81	10.926
			Set A+B	1.448.962	284.947	100%	3818.15	10.912
3	L2 21	*Cx. pipiens*	Set B	2.219.785	312.851	100%	3515.05	10.926
			Set A+B	1.733.553	385.088	100%	5042.35	10.913
4	L2 23	*Cx. pipiens*	Set B	2.045.957	275.354	100%	2943.77	10.926

			Set A+B	1.528.111	339.207	100%	4525.72	10.912
5	L2 21	*Athene noctua*	Set B	3.413.467	359.916	100%	4106.39	10.892
			Set A+B	1.801.390	376.213	100%	4946.53	10.878
6	L2 22	*Cx. pipiens*	Set B	3.109.597	239.795	95%	2761.23	10.892
			Set A+B	1.407.784	312.376	99%	4201.89	10.879
7	L2 19	*Passer domesticus*	Set B	1.621.442	123.813	89%	1836.85	10.924
			Set A+B	1.515.878	205.605	100%	2770.45	10.914

In WNV-L1 samples, no loss of sensitivity was observed between set A and set A + B for all the samples tested. Notably, for one sample from a yellow-legged gull at CT value 25, a gain of sensitivity was observed at 88% horizontal coverage using set A to 93% using set A + B joined. In the same way, sequencing conducted on WNV-L2 samples worked just as well with set B as with set A + B, regardless of Ct values. Indeed, almost 72% of the samples had 100% full genome (n = 5 out of 7).

3.6 Discussion

NGS is now an essential tool in the study of infectious diseases, both at the fundamental level and in its application to public health. The COVID-19 pandemic has thus been a patent example of the importance of being able to obtain information on the genetic signature of pathogens in real time. However, it should be noted that sequencing technology, and in particular whole-genome sequencing, remains an expensive approach with significant experimental constraints (for instance, the host genome background with a relatively lower amount of genetic material of the pathogen of interest in clinical specimens) in order to have some quality of data generated. A multiplex PCR-based target enrichment or amplicon-based protocol [14] was mostly used to overcome these challenges during SARS-CoV-2 genomic surveillance, yielding more than 14 million genomes in the GISAID platform at the time of writing this manuscript [27]. WNV is becoming a major health problem in Europe and cases have also recently been detected in Africa [7,12]. WNV cases are mainly due to lineages 1 and 2. The mechanisms of diffusion of viral strains, in particular by the migratory movements of birds, are actively studied. The genetic characterization of the identified strains allows better control of the dissemination routes for effective sanitary measures. NGS showed the persistence of a WNV strain after winter in Andalusia in Spain, suggesting endemicity with potential future epidemics in the area [28]. Another recent genomic study evidenced continuous WNV-L2 circulation in Italy throughout the year [29], while a reintroduction event was identified from Europe to Senegal, highlighting a

potential threat [12]. Genomic characterization is even more important because it has been shown that West African lineages have higher virulence and replicative efficiency in vitro and in vivo compared to similar lineages circulating in the United States and Europe [6]. Genomic surveillance is thus essential as it allows a better understanding of the dissemination and dynamic of WNV strains. In order to ensure the sustainability of this type of surveillance, we describe here the development and evaluation of a whole-genome amplicon-based sequencing approach for WNV-L1 and WNV-L2 by Illumina technology in different types of vertebrate and mosquito species from Senegal and Italy. Three sets of primers were then designed and assessed with WNV-L1 and WNV-L2 strains. Set A and set B are specific to WNV-L1 and WNV-L2 strains, respectively, while the third one, a mixture of the two previous sets, is able to amplify both lineages. Thus, the use of one set or another depends on the context. Indeed, in the case where the lineage is already well defined, it is appropriate to use the specific sets, whereas set A + B fits more in a context where no lineage characterization could be made before sequencing. The evaluation in this study could only be carried out with the WNV-L1 and WNVL2 strains. Because set A was designed from at least one representative of all the WNV lineages, it would be appropriate to undertake a similar evaluation with at least set A and set A + B on other lineages than WNV-L1 and WNV-L2. Moreover, the repetition of these experiments by other groups allows the observed results to be refined, particularly in terms of correlation with Ct values. Indeed, even if this work was carried out with rigor and with two teams in Senegal and Italy, external factors such as the sample quality after long-term storage or the sample type may have impacted the outputs of the results. In any case, the approach presented in this manuscript could be a valuable tool for any WNV genomic investigation.

3.7 Funding statement

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3.8 Conflict of interest

The authors declare no conflict of interest. All authors have read and agreed to the published version of the manuscript.

3.9 Author contributions

Conceptualization, M.M.D., M.H.D.N., G.M., O.F., C.C., A.R., G.S., and O.F. (Oumar Faye); methodology, M.M.D., M.H.D.N., G.M., A.D., M.D.D., C.C., A.R., G.S., and O.F. (Oumar Faye); software, A.D., M.M.D., G.M., C.C., and C.L.; validation, M.M.D., M.H.D.N., G.M., M.D.D., V.C., M.M., C.L., O.F. (Ousmane Faye), and C.C.; formal analysis, M.M.D., M.H.D.N., G.M., A.D., M.K., N.M.T., and M.M. (Maïmouna Mbanne); investigation, M.M.D., M.H.D.N., G.M., E.h.N., D.D., M.K., N.M.T., M.M. (Maïmouna Mbanne), M.A., B.S., V.D.L., L.T., A.L., I.P., and A.G.; resources, M.M.D., A.A.S., C.L., M.D., O.F. (Ousmane Faye), C.C., G.S., and O.F. (Oumar Faye); data curation, M.M.D., M.H.D.N., G.M., A.D., E.h.N., D.D., N.M.T., L.T., A.L., and I.P.; writing—original draft preparation, M.M.D.; writing—review and editing, M.H.D.N., G.M., A.D., M.D.D., E.h.N., M.D., C.C., A.R., G.S., and O.F. (Oumar Faye); visualization, M.M.D., G.M., M.D.D., V.C., M.M., M.A., B.S., V.D.L., L.T., A.L., I.P., R.R., F.M., C.C., A.R., and G.S.; supervision, M.M.D., M.H.D.N., M.D.D., V.C., C.C., G.S., and O.F. (Oumar Faye); project administration, A.R., G.S., and O.F.

(Oumar Faye); funding acquisition, M.M.D., A.A.S., O.F. (Ousmane Faye), A.R., G.S., and O.F. (Oumar Faye). All authors have read and agreed to the published version of the manuscript.

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3.11 Supplementary materials

The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/v15061261/s1, Table S3.1: WNV Sequences aligned for set A primers design, Table S3.2: WNV-L2 Sequences aligned for set B primers design.

Table S3. 1 List of 15 WNV different lineage sequences used to perform a sequence comparison with primer set A

WNV Sequences Aligned for Set A Primers Design
KC243146.1 West Nile virus isolate ATH002316 partial genome
AY765264.1 West Nile virus strain Rabensburg isolate 97-103 complete genome
NC 001563.2 West Nile virus lineage 2 complete genome
KC496016.1 West Nile virus strain Novi Sad-2010 complete genome
KC496015.1 West Nile virus strain 578/10 complete genome
MN057643.1 Koutango virus isolate PM148 complete genome
EU082200.2 Koutango virus strain Dak Ar D 5443 polyprotein gene complete cds
MT863559.1 West Nile virus isolate Akela/France/2015 complete genome
MF797870.1 West Nile virus strain WNV Cy2016 complete genome
KT163243.1 West Nile virus isolate 68856-ICDC-4 complete genome
MH244513.1 West Nile virus strain 200.B/2013/Secovce/SVK complete genome
KM052152.1 West Nile virus isolate 349/77 polyprotein gene complete cds
DQ118127.1 West Nile virus isolate goose-Hungary/03 complete genome
DQ318019.1 West Nile virus strain ArD76104 complete genome
NC 009942.1 West Nile virus lineage 1 complete genome

Table S3. 2 List of 82 WNV L2 sequences used to perform a sequence comparison with primer set B

WNV-L2 Sequences Aligned for Set B Primers Design
AY532665.1 West Nile virus strain B956 polyprotein gene complete genome
EF429200.1 West Nile virus H442 complete genome
HM147824.1 West Nile virus from Democratic Republic of the Congo complete genome
JN393308.1 West Nile virus strain HS101 08 complete genome
KY523178.1 West Nile virus isolate UG2274/Uganda/2009 complete genome
EF429197.1 West Nile virus SPU116/89 complete genome
EF429198.1 West Nile virus SA93/01 complete genome
HQ537483.1 West Nile virus isolate Nea Santa-Greece-2010 complete genome
KJ883345.1 West Nile virus strain Greece/2013/Xanthi 3 complete genome
KU206781.1 West Nile virus strain Bulgaria/2015/Sofia complete genome
KP109691.1 West Nile virus strain blood donor/Vienna/2014 complete genome
MF984347.1 West Nile virus strain Blood donor 2/Austria/2016 (BD2/16) complete genome
MH244511.1 West Nile virus strain 291.B/2013/Velky Biel/SVK complete genome
MF984340.1 West Nile virus strain Blood donor 4/Austria/2015 (BD4/15) complete genome
MF984342.1 West Nile virus strain Patient 1/Austria/2015 (Pa1/15) complete genome
MF984344.1 West Nile virus strain Goshawk/Austria/2015 (Bi1/15) complete genome
MH244512.1 West Nile virus strain 286.B/2013/Velky Biel/SVK complete genome
KF179640.1 West Nile virus strain Austria/2008 gh complete genome
MF984343.1 West Nile virus strain Patient 2/Austria/2015 (Pa2/15) complete genome
KP780838.1 West Nile virus isolate Mismo complete genome

DQ116961.1 West Nile virus isolate goshawk-Hungary/04 complete genome
KF588365.1 West Nile virus strain Italy/2013/Rovigo/32.1 complete genome
KF647252.1 West Nile virus strain Italy/2013/Rovigo/35.1 complete genome
KP789960.1 West Nile virus strain Italy/2013/Mantova/36.1 complete genome
KF647251.1 West Nile virus strain Italy/2013/Padova/34.1 complete genome
KF647249.1 West Nile virus strain Italy/2013/Rovigo/33.2 complete genome
KP789956.1 West Nile virus strain Italy/2014/Verona/35.2 complete genome
MW862081 West Nile virus strain Italy/2015/Emilia-Romagna
MW862084 West Nile virus strain Italy/2015/Emilia-Romagna
MW862085 West Nile virus strain Italy/2015/Emilia-Romagna
MW862087 West Nile virus strain Italy/Emilia-Romagna 02/11/2017
MW862101 West Nile virus strain Italy/Emilia-Romagna Italy15/07/2019
MW862091 West Nile virus strain Italy/Emilia-Romagna 31/07/2018
MW862090 West Nile virus strain Italy/Emilia-Romagna /07/2018
MW862093 West Nile virus strain Italy/Emilia-Romagna 08/09/2018
MW862088 West Nile virus strain Italy/2017/Emilia-Romagna
MW862092 West Nile virus strain Italy/Emilia-Romagna 13/08/2018
KF823806.1 West Nile virus strain Italy/2013/Mantova/40.1 polyprotein gene complete cds
KP789953.1 West Nile virus strain Italy/2014/Pavia1 complete genome
KP789954.1 West Nile virus strain Italy/2014/Cremona2 complete genome
MW862075 West Nile virus strain Italy/2015/Emilia-Romagna
MW862076 West Nile virus strain Italy/2015/Emilia-Romagna

MW862077 West Nile virus strain Italy/2015/Emilia-Romagna
MW862080 West Nile virus strain Italy/2015/Emilia-Romagna
MW862079 West Nile virus strain Italy/2015/Piedmont
MW862078 West Nile virus strain Italy/2015/Piedmont
MW862082 West Nile virus strain Italy/2015/Lombardi
MW862089 West Nile virus strain Italy/2017/Lombardi
MW862096 West Nile virus strain Italy/Lombardi 31/08/2018
MW862103 West Nile virus strain Italy/Piedmont 23/10/2019
MW862104 West Nile virus strain Italy/Piedmont 26/09/2019
MW862106 West Nile virus strain Italy/Piedmont 26/09/2019
MW862107 West Nile virus strain Italy/Piedmont 26/09/2019
MW862102 West Nile virus strain Italy/Veneto 23/10/2019
MW862108 West Nile virus strain Italy/Piedmont 23/09/2020
KP789959.1 West Nile virus strain Italy/2014/Pavia4 complete genome
MW862073 West Nile virus strain Italy/2012/Sardinia
MW862074 West Nile virus strain Italy/2013/Sardinia
MW862083 West Nile virus strain Italy/2015/Sardinia
MW862086 West Nile virus strain Italy/2016/Sardinia
MW862094 Lineage2 West Nile virus strain Italy/Sardinia 15/07/2019
MW862099 West Nile virus strain Italy/Sardinia 06/10/2018
MW862095 West Nile virus strain Italy/Sardinia 19/09/2019
MW862097 West Nile virus strain Italy/Sardinia 05/09/2018

MW862100 West Nile virus strain Italy/Sardinia 2/10/2018
MW862105 West Nile virus strain Italy/Sardinia 18/11/2019
MW862098 West Nile virus strain Italy/Sardinia 13/10/2018
KF647250.1 West Nile virus strain Italy/2013/Rovigo/33.1 complete genome
MH021189.1 West Nile virus isolate WNV/Belgium/2017/Antwerpen complete genome
KC496016.1 West Nile virus strain Novi Sad-2010 complete genome
KP780839.1 West Nile virus isolate Berliner complete genome
KC407673.1 West Nile virus isolate Sad/12 complete genome
MH986055.1 West Nile virus strain 1382/2018/Berlin/Ger complete genome
MH986056.1 West Nile virus strain 1617/2018/Rostock/Ger complete genome
KP780840.1 West Nile virus isolate Tammy complete genome
FJ425721.1 West Nile virus isolate Reb VLG 07 H polyprotein gene complete cds
KJ934710.1 West Nile virus strain Hyalomma/Romania/2013 polyprotein precursor gene complete cds
KT207791.1 West Nile virus isolate 792/14 complete genome
EF429199.1 West Nile virus SA381/00 complete genome
DQ176636.2 West Nile virus strain Madagascar-AnMg798 complete genome
MF797870.1 West Nile virus strain WNV Cy2016 complete genome

CHAPTER 4. WEST NILE VIRUS: A VIRTUAL BRIDGE THAT LINKS AFRICA AND EUROPE

Manuscript

by

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4.1 Abstract

Phylogenetic and phylogeographic inferences are considered important tools to uncover the spatial and temporal viral dynamics of viruses. By integrating newly obtained genomic data, previously published NCBI sequences, and detailed geographical observations with phylogenetic and novel phylogeographic inference reconstructions it is nowadays possible to accurately uncover the pattern of viral circulation between countries and continents. Here we focus on West Nile virus (WNV) spread between Africa and Europe, two key continents in the study of this arthropod-borne pathogen that represents an emerging threat to public health worldwide. Particularly, we address our analysis towards lineages 1 (L1) and 2 (L2), considered the most diffused among the eight lineages described until now.

Our study, uncovering the origins and dispersal history of African and European WNV L1 and L2 strains between the two continents, shows two diverse geographical patterns of transmissibility. It highlights: i) a complex structure and unpredictable behavior for WNV L1 strains, with back-and-forth exchanges most between West Africa and Europe, particularly through an ideal corridor connecting Senegal, Morocco, and Southern-Western Mediterranean countries; and ii) WNV L2 homogeneity, with one main independent introduction from South Africa to Hungary, from where the virus spread and established in Europe. Finally, we suggest the two WNV L1 and L2 migratory routes between Africa and Europe to overlap two important Afro-Palearctic bird migratory flyways, making assumptions about the connection between the two lineage's diverse evolutionary history and the role of bird species on L1 and L2 trans-continental diffusion. Future innovative studies coupling bird species susceptibility, migratory connectivity patterns, and viral phylogeographic inferences are suggested to uncover the dynamics of emerging viruses, possibly preventing future outbreaks at a global scale.

4.2 Keywords

West Nile virus lineage; phylogeny; phylogeography; evolution; Europe; Africa

4.3 Introduction

Phylogenetic analyses and phylogeographic inference reconstructions integrating detailed geographical observations at high-resolution scale can provide valuable insight into the spatial dynamics of pathogens in a geographic and temporal context [1]. Starting from virus genome data and inferring continuous phylogeographic diffusion through space and time, Bayesian statistical approaches are nowadays precious for monitoring the viral spread between diverse geographic areas of the world and providing relevant information for epidemic prediction and preparedness [2]. This approach is particularly interesting for viruses known to be carried by migratory birds crossing vast distances across the globe, and playing a crucial role in pathogen ecology, circulation, and spread [3]. West Nile virus (WNV) is one of the most widely distributed viruses in the world, whose geographical expansion has been associated with migrating wild birds [3,4]. Considered one of the main One Health challenges, this arthropod-borne virus belonging to the genus *Flavivirus*, family *Flaviviridae*, and Japanese Encephalitis serocomplex, is maintained in nature through a transmission cycle involving wild birds and vector competent mosquitoes [5]. Although in most bird infection generally does not lead to severe clinical signs, in some susceptible species it might cause severe neurological signs [6,7]. Competent infected mosquitoes, mainly belonging to the *Culex* genus, can transmit the virus to humans and other animals, with an increasing rate and impact on human and animal health worldwide [8]. Infected humans are mostly asymptomatic, but in 20% of cases the infection can lead to febrile illness (West Nile Fever (WNF)), and in 1% of cases, mainly in elderly or immunocompromised people, to severe and sometimes fatal neurological disease (West Nile virus neuroinvasive disease (WNND)) [9]. Public health concerns also lie with the possible impact that WNV infection might have

on blood transfusion and organ transplantation (https://www.cdc.gov/westnile/transmission/blood-organ.html). WNV might as well represent a serious economic issue and source of emotional distress if horses are infected. In this species, infections can cause severe neurological disorders with a high mortality rate (33%) [10].

First identified in Africa in 1937, WNV is characterized by high genetic diversity. At least 8 lineages are described in the world, 4 of which are circulating in Africa [lineage 1 (L1 or L1a), lineage 2 (L2), lineage 7 (L7 - nowadays classified as the Koutango virus), and putative lineage 8 (L8)] [10]. In Europe, L1 and L2 strains are the most prevalent [11]. According to phylogenetic studies on WNV strains, connections between Africa and Europe appear evident. It seems that some European epidemics were the result of WNV African strain introductions. With regard to WNV L1, the first introduction probably occurred more than 25 years ago from Northern-Western African countries to Italy or France, where the strain was first detected in 1998 and 2000, respectively [10,12]. WNV L2, instead, was first introduced in Hungary in 2004 [11] but its origins are still uncertain [10,13]. Following these first introductions and probably after few other introductory events, both lineages spread and established in many European countries. In 2022, they were responsible for 965 human cases and 115 deaths (https://www.ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/disease-data-ecdc) [10].

Despite the existence of numerous studies focusing on WNV circulation [14–16,10], little has been revealed about the genetic relationships of WNV L1 and L2 European and African strains. This study aims to uncover the viral spread of the two lineages between Africa and Europe, which are two key continents to study the ecology and evolution of this pathogen. Specifically, we aim to (i) provide a new dataset of genomes, including sequences from Italy collected between 2021 and 2022 and Senegal from 2006 to 2016; and ii) describe the dispersal dynamics of the two lineages in Africa and Europe

through time and space. These data might be very useful for the scientific community and public health authorities to address future research studies and novel control strategies.

4.4 Materials and Methods

4.4.1 Sample collection in Senegal and Italy

In Italy, sample activities were carried out between 2001 and 2022 under the national surveillance plan coordinated by the Ministry of Health, the Istituto Superiore di Sanità (epidemiology and national reference laboratory, human), and the Istituto Zooprofilattico of Abruzzo and Molise (IZS-Teramo) (epidemiology and national reference laboratory, animal/entomology) (https://westnile.izs.it/j6_wnd/home, https://www.epicentro.iss.it/westnile/), as previously described [17]. In Senegal, sample activities were carried out by the Institut Pasteur de Dakar (IPD-Dakar) in collaboration with the Ministry of Health, within the mosquito-based arbovirus surveillance system and a sentinel syndromic surveillance network (4S). Both surveillance plans, in place since 1988 [18] and 2015 [19] respectively, aim at better understanding the transmission dynamics of arboviruses including WNV in the country [12].

4.4.2 Sample analysis

4.4.2.1 Tissue Homogenisation, Viral stock preparation, RNA extraction, Real-time RT-PCR

At IZS-Teramo, pools of bird organs and mosquito pools were homogenized in phosphate-buffered saline (PBS) with antibiotics. WNV strains were obtained from birds' internal organs or mosquito pool homogenates after one passage on C6/36 cell lines and one to two passages on Vero monolayer cell lines followed by an infection [20]. Viral RNA was extracted by using the MagMAX CORE Nucleic Acid Purification KIT (Applied Biosystem, Thermo Fisher Scientific, Life Technologies

Corporation, TX, USA) and amplified by multiplex real-time reverse transcription polymerase chain reactions (qRT-PCR) to detect WNV L1, WNV L2, and Usutu virus, by using the Superscript III Platinum OneStep qRT-PCR System (Invitrogen) as previously described [21].

At IPD-Dakar, arthropod and human samples derived from the WHO Collaborating Center for arboviruses and hemorrhagic fever viruses (CRORA) have been collected from the field. WNV strains were obtained after infection of C6/36 monolayer cells with homogenized mosquito pools, followed by indirect Immunofluorescence assay (using in-house hyper immune mouse ascitic fluids, as previously described [12]. To confirm WNV detection, the extraction of viral RNA was performed with the QIAamp viral RNA mini kit (Qiagen, Heiden, Germany) according to the manufacturer's instructions. Viral RNAs were amplified by qRT-PCR with the Quantitect reverse transcription kit (Qiagen, Heiden, Germany) according to the manufacturer's instructions and a consensus WNV RT-PCR assay [22].

4.4.3 Sequence retrieval and dataset preparation

In Italy and Senegal, 19 WNV L2 Italian samples, collected between 2021 and 2022, and 7 WNV L1 and 3 WNV L2 Senegalese samples, obtained between 2006 and 2016, were processed at IZS-Teramo and IPD-Dakar by Next Generation Sequencing (NGS) technology, as fully described in detail in [12,23]. Briefly, the complete WNV genomes were obtained from RT-PCR positive samples by using the high-throughput sequencing technique in the Miseq device (Illumina, San Diego, CA, USA). The total RNA was treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 20 min, and then purified by an RNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA, USA). The purified RNA was used for the assessment of sequencing independent single primer amplification protocol (SISPA) [12,23]. The PCR product was purified using the Molecular Biology Kit BioBasic (Biobasic inc., Markham, ON, Canada), and then quantified by using the Qubit® DNA

HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) [23]. The sample was diluted to obtain a concentration of 100–500 ng and used for library preparation by using the Illumina DNA Prep kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol [12,23]. Thus, the obtained libraries were each identified by indexes (Nextera XT index kit V2, Illumina) and then pooled at the same concentration. Whole-genome sequencing was performed with paired-end reads using the Illumina MiSeq reagent kit v2 (300 cycles) on an Illumina MiSeq instrument, as previously described [12,23]. The consensus sequences were obtained using iVar v 1.3.1 [24] after trimmed reads were mapped to the WNV L2 MN652880 (Greece, 2018) and WNV L1 FJ483548 (Italy, 2008) reference sequences, by using Snippy (https://github.com/tseemann/snippy).

Furthermore, 10 WNV L1 and 10 WNV L2 whole genome sequences, obtained from samples collected in Senegal between 1985 and 2018, have been shared by the Institut Pasteur Dakar of Senegal and added to the dataset. The final dataset also included 30 WNV L1 and 45 L2 whole genome sequences, obtained at IZS-Teramo between 2008 and 2022, which were downloaded from the Supplementary Materials of [17] and [Origin and evolution of West Nile virus lineage 1 in Italy].

By using a custom R script for automatic sequence retrieval, a new search has been conducted on the NCBI on 04/01/23, and 37 newly published worldwide WNV L1 and 39 WNV L2 sequences >= 200 nt have been downloaded.

4.4.4 Sequence quality-filtering and formatting

Quality-filtering was performed as previously described [17], but no sequences were removed because they did not contain a percentage of ambiguous bases above 10%. A total of 229 WNV L1 and 298 WNV L2 genomes, 188 of which came from Italy (81 L1 and 97 L2) and 31 from Senegal (18 L1 and 13 L2), were selected for further analysis.

A table of sequence curated metadata can be found in Supplementary Table S4.1.

4.4.5 Alignment, recombination detection and model selection

Sequence alignment was conducted using MAFFTv7 (https://mafft.cbrc.jp/alignment/server/) and aligned sequences were trimmed using trimAlv2 [25]. Suspected recombinant sequences (the L1 sequence OP846974.1 and L2 sequence OK239667.1) were identified by running the RDP4 program [26] and excluded from the final dataset. Modelfinder program [27] was used to carry out a model selection analysis, using parameters "-T AUTO -m TESTONLY". The best-fit model for both the worldwide and the Bayesian down-sampled datasets was GTR+F+I+G4, chosen according to both Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC).

4.4.6 Maximum-likelihood phylogenies

A maximum likelihood phylogeny of the dataset including the 228 WNV L1 and 297 L2 sequences was reconstructed by using RAxMLv8.2.12 [28], with commands "-p 1989 -m GTRGAMMAI -x 2483 -# 100 -f a -T 20". Clades were annotated using the resulting topology when having bootstrap supports ≥ 90%.

4.4.7 Molecular clock and phylogeographic analysis

Molecular clock analysis was conducted by sub-selecting 4 sequences of each subclade belonging to the highly supported group shown in the maximum likelihood tree of Supplementary Fig. S4.1. The selection was made based on genetic divergence (as many sequences were almost identical), year and location. Phylogeography was reconstructed by using continuous traits (latitudinal and longitudinal coordinates for each sequence) in BEASTv1.10.4 [29]. Both analyses are accurately described in [17], with the only difference of the tree prior, which was set as constant coalescent due to the large set of

parameters to be estimated. Convergence was assessed using Tracer V1.7.1 [30], making sure that all parameters were above a significance threshold of ESS (>200). Spatiotemporal patterns of WNV evolution were visualized using SpreaD3 [31].

A table of geographic coordinates used for the phylogeographic analysis can be found in Supplementary Table S4.2.

4.5 Results

4.5.1 Genome sequence analysis

Illumina sequencing produced an average total number of 2,330,817 trimmed reads. The numbers of mapped reads (151 nucleotides [nt] in length) ranged from 238,259 to 1,035,983, with coverage depth ranging from 2,770.45× to 6,862.12×. Consensus sequences were characterized by 19 WNV L2 Italian samples, collected between 2021 and 2022, and 7 WNV L1 and 3 WNV L2 Senegalese samples, obtained between 2006 and 2016, that were uploaded to BankIT NCBI (https://submit.ncbi.nlm.nih.gov/about/bankit/) the 20th of June 2022, the 27th of October 2022, and the 15th of November 2022, respectively.

4.5.2 Phylogenetic and phylogeographic inferences of WNV

Two phylogenetic and phylogeographic inferences of the WNV L1 and L2 genome sequences were obtained, separately.

4.5.2.1 Phylogenetic inference of WNV L1

Our maximum likelihood phylogeny and molecular clock analyses highlighted most of the WNV L1 American, European, Middle Eastern, Asian, and African strains to be included inside one major

clade, clade 1A (Supplementary Fig. S4.1). Fig. S4.1 identifies seven additional clusters within clade 1A.

Cluster 1 includes the first WNV L1 strain recovered in Africa in Egypt in 1951 (Genbank AF260968) and strains circulating in Israel (1953), Russia (1963), Azerbaijan (1967-70), India (1968, 2015), and Portugal (1971).

Cluster 2 contains more recent isolates. Particularly, three major sub-clusters are defined within this cluster: i) the Western-Mediterranean (W-Med), including strains (the chronological order of detection) of Morocco (1996 and 2003), Italy (1998 - 2022), Israel (2000), France (2000, 2004, and 2015), Portugal (2004), Spain (2007, 2008, 2010, and 2020), and Senegal (2012 - 2018); ii) the Eastern-European, with strains of Romania (1996) and Russia (1999 - 2006); and iii) a group of strains from Kenya (1998 and 2010), Senegal (1990), Zambia (2019), United Arab Emirates (2015), and Cyprus (2016). Our maximum-likelihood identifies two further main groups of sequences (WMed-1 and WMed-2) within the W-Med subtype. These groups are slightly different from the early and less evolved WMed WNV strains of Morocco (1996), France and Israel (2000), already proposed by [32], strains collected in Italy between 2011 and 2013 (Livenza, Ancona, Piave) and the more divergent strain from Senegal of 2012 (Genbank ON813216) (Fig. 4.1 and Supplementary Fig. S4.1).

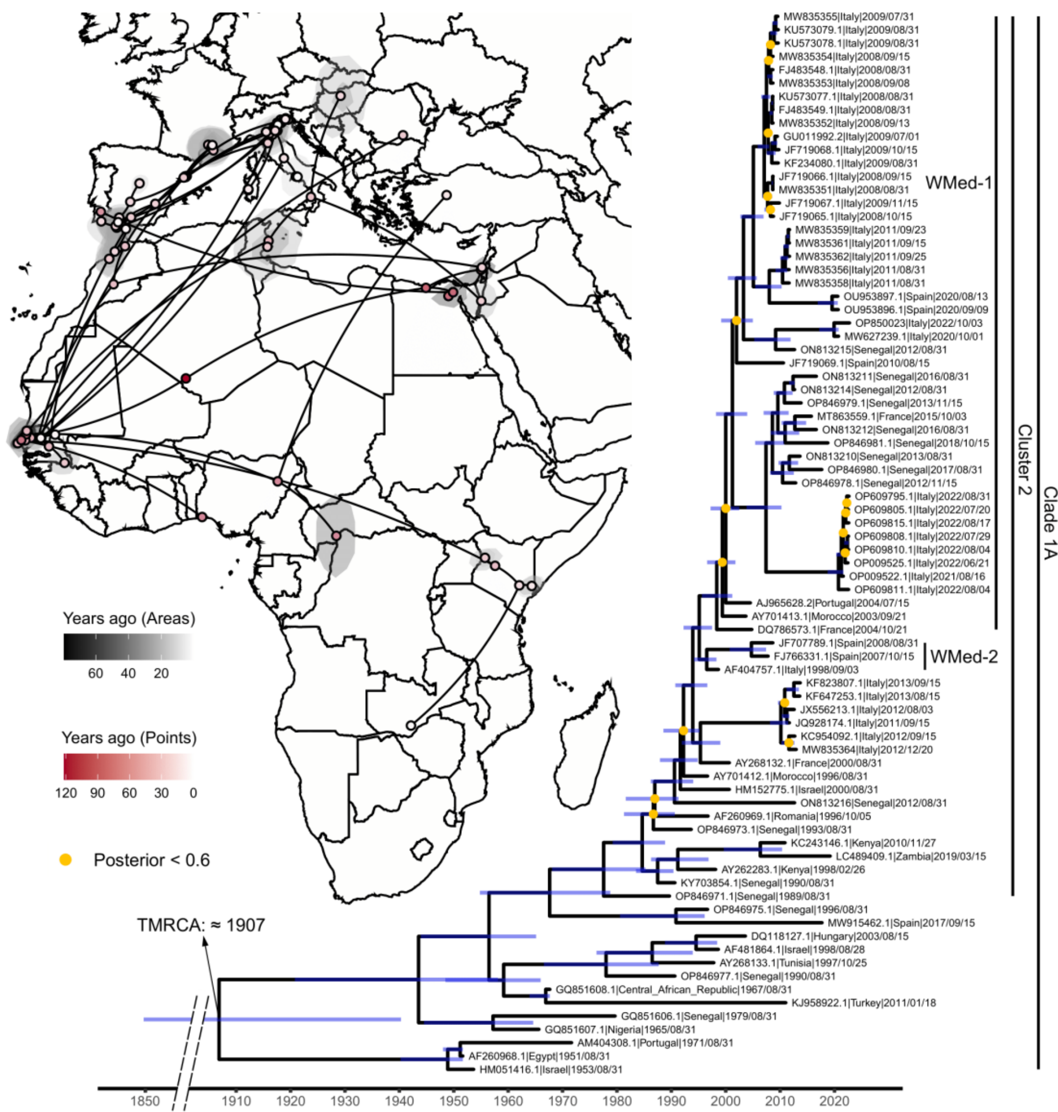

Figure 4.1 Phylogenetic inference of West Nile virus lineage 1 strains

A) Geographic diffusion pattern of 80 WNV L1 genomes and their ancestors was reconstructed. Black areas correspond to the 80% HPDs of the inferred location. The time of each area or sample is indicated with different shades (black for areas and red for dots), with the oldest samples corresponding to the most intense shade. B) A molecular clock shows phylogenetic relationships between the same 80 WNV L1 genomes. Yellow dots indicate nodes with posterior probability < 0.6, while 95% HPDs of the median ages are indicated with blue bars. The time of the most recent common ancestor (TMRCA) for clade 1A was estimated to be around 1907. On the right, bars highlight 4 groups of interest: cluster 2, Western Mediterranean clade 1 and Western Mediterranean clade 2.

The WMed-1 within the WMed sub-cluster includes most of the strains collected since 2003 from Morocco (2003), Portugal (2004), France (2004), Italy (2008 - 2022), Spain (2010 and 2020), and Senegal (2012 - 2018). The WMed-2 comprises one isolate from Italy (1998) and three from Spain (2007 and 2008) (Fig. 4.1). The WMed-1 and 2 appear to be all rooted in the strain of France of 2000 (Genbank AY268132).

Cluster 3 includes strains from Russia (1999), India (2011), and China (2011) while cluster 4 contains a large group of strains from Senegal (1990), Tunisia (1997), America (1999-2019), Israel (1998 and 2000), Hungary (2003), and Belgium (2014).

Cluster 5 includes strains from Nigeria and Senegal in 1965 and 1979, respectively.

Cluster 6 contains strains from the Central African Republic (CAR; 1967) and Turkey (2011).

Cluster 7 includes strains from Senegal (1996) and Spain (2017).

Our analysis shows clusters 2, 3, 4, 6, and 7 to be rooted in the two ancient sequences from Nigeria and Senegal included in cluster 5 (Fig 4.1 and Supplementary Fig. S4.1). It is also shown that all strains within cluster 2 are rooted by the 1989 Senegalese strain (Genbank OP846971), with bootstrap support = 100. Moreover, within cluster 2 WMed-1 subtype, a close genetic similarity is shown by a group of ten strains of Senegal (2012-18) and one strain of France of 2015, which cluster together, and appear to be closely related to a group of 2021-22 Italian sequences (bootstrap support = 1). Finally, the WNV strains collected in 2020 and 2022 from Campania region (Italy) (Genbank MW627239 and OP850023) are closely related to one strain of Senegal of 2012 (Genbank ON813215), which in turn are genetically similar to a group of strains of Italy (2008, 2009 and 2011) and Spain (2020).

4.5.2.2 Phylogeographic inference of WNV L1

Our phylogeographic inference evidences all strains included in Clade 1A to have originated from the African continent (Fig. 4.1), where the oldest strain recovered appears to be the one of Egypt of 1951 (Genbank AF260968). In particular, it is likely that the WNV L1 clade 1A African ancestor has originated in the 1900s in a North-Western area of Africa (median=1906.85; 95% HPD: 1849.68, 1940.28). From this area, WNV L1 of clade 1A probably spread around the 1940s into two opposite directions, towards Egypt (median=1948.89) and towards Western-Central Africa (median=1943.53), forming genetically related clusters and sub-clusters that are nowadays spread all over the World (Fig. 4.1 and Supplementary Fig. S4.1).

Within cluster 2, the common ancestor of the WNV L1 W-Med sub-cluster appears to be most likely located in North Africa, probably Morocco (median=1992.2; 95% HPD: 1988.55 - 1995.17). From this area, few independent introductions to Western-Mediterranean countries are highlighted around the 1990s, stressing the existence of a corridor between Senegal, Morocco, and Western-Mediterranean European countries, such as France and Italy. Moreover, sporadic back introductions between these countries are pointed out. Particularly, our analysis shows i) the Moroccan strain of 2003 to have originated from an area located in North-East of Spain, from where the sequences of Portugal and France of 2004 also probably arrived; ii) the existence of a genetic flow from an area stretching from the North-west of Italy to the South of France to Senegal.

4.5.2.3 Phylogenetic inference of WNV L2

In support of what was previously shown by the literature [33], our maximum-likelihood phylogeny and molecular clock analyses of the entire WNV L2 dataset stress the presence of four well-supported clades including most African and European strains. Clade 2a includes the most genetically divergent

strain of Madagascar of 1978. Clade 2b contains the oldest WNV L2 strain of South Africa of 1958 closely related to a Namibia strain of 2020, other than a strain from Cyprus of 1968. Clade 2c includes strains of Madagascar of 1988. Clade 2d is the largest and it is rooted in a strain of the Democratic Republic of Congo (DRC) of 1958 (Genbank HM147824) (bootstrap support = 100) (Fig. 4.2 and Supplementary Fig. S4.2).

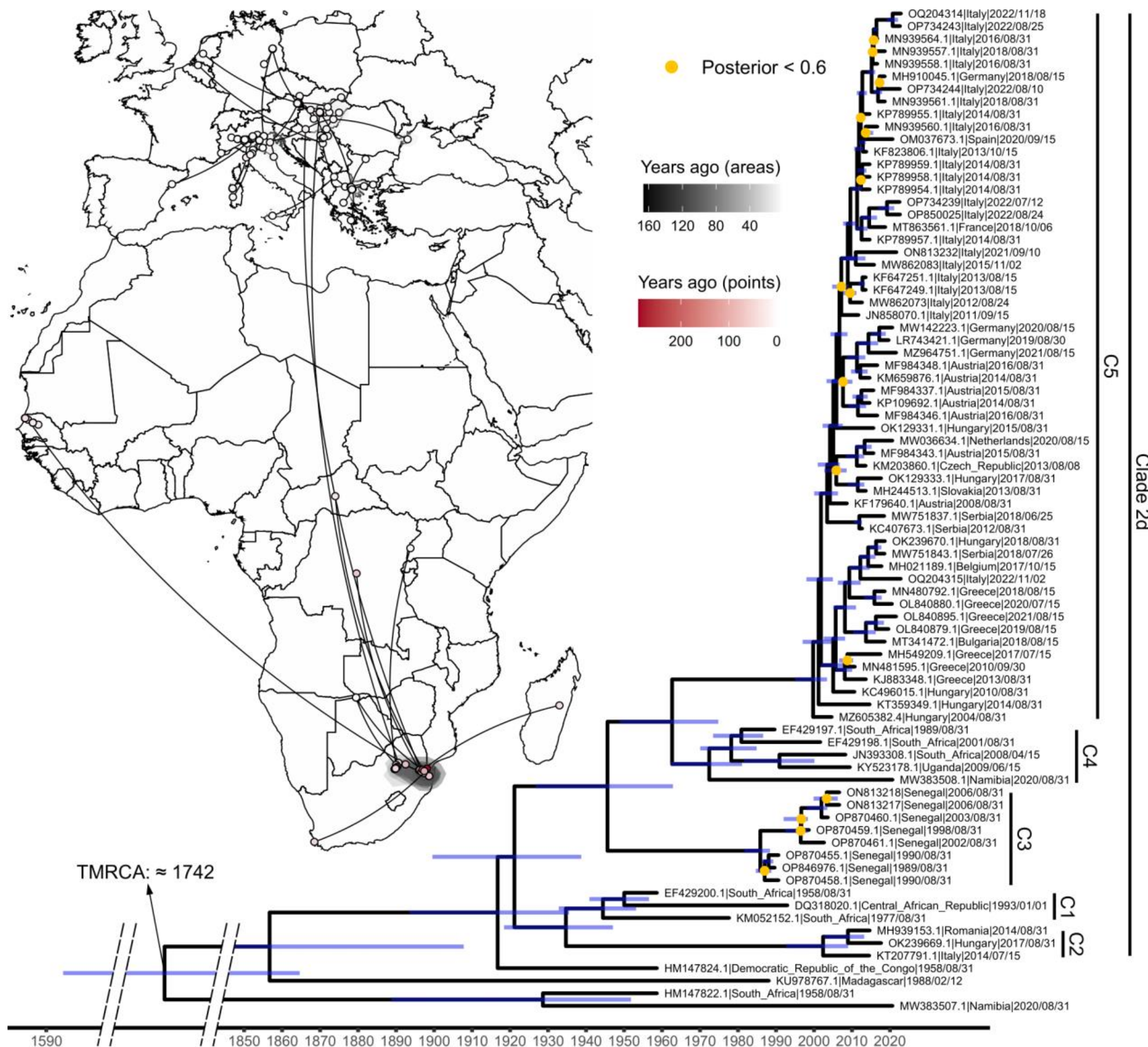

Figure 4.2 Phylogenetic inference of West Nile virus lineage 2 strains. Clusters 1-4 are indicated with C1-4 codes.

Phylogenetic inference of West Nile virus lineage 2 strains. A) Geographic diffusion pattern of 80 WNV L2 genomes and their ancestors was reconstructed. Black areas correspond to the 80%HPDs of the inferred location. The time of each area or sample is indicated with different shades (black for areas and red for dots), with the oldest samples corresponding to the most intense shade. B) A molecular clock shows phylogenetic relationships between the same 80 WNV L2 genomes. Yellow dots indicate nodes with posterior probability < 0.6, while 95%HPDs of the median ages are indicated with blue bars. The time of the most recent common ancestor (TMRCA) for all WNV L2 sequences included in the analysis was estimated to be around 1742. On the right, bars highlight 6 groups of interest which are considered relevant to our discussion: clusters 1-5 are indicated with C1-5 codes.

Clade 2d includes strains from (chronologically ordered) South Africa, Senegal, Uganda, CAR, Europe, and Russia (Fig. 4.2). Particularly, we define five well-supported clusters within this clade. Cluster 1 contains the two South Africa strains of 1958 and 1977 and a strain of CAR of 1993. It roots Cluster 2 including strains of Russia (2018), Iran (2017 and 2018), other than a little group of strains from Europe, such as Romania (2013 and 2014), Italy (2014), and Hungary (2017). Cluster 3 contains 13 Senegalese strains (1989 - 2006), other than a strain of Ukraine (1980), and is closely related to clusters 4 and 5. Cluster 4 includes South African strains (1989, 2001, and 2008) other than one strain from Uganda (2009) and one from Namibia (2020). Finally, cluster 5 includes all Central-Southern European strains, rooted by the Hungarian sequence of 2004 (Genbank MZ605382) and is rooted by cluster 4 (Fig. 4.2).

4.5.2.4 WNV L2 phylogeographic inference

Our analysis evidenced the WNV L2 common ancestor to be most likely located in South Africa (Fig. 4.2) and to have originated between the 18th and the 19th century (median=1741.6; 95% HPD: 1594.44 - 1864.61). The existence of corridors between African countries, and, in particular, between

South Africa, DRC, Senegal, CAR, and Uganda is also evident (Fig. 4.2). Two major European introductions from Southern Africa are also shown: i) one in Hungary in 2004, from where most of Central - Southern European strains have originated; and ii) a second introductory event which involved Italy and Bulgaria in 2014 (Fig. 4.2).

4.6 Discussion and conclusions

Numerous studies have drawn attention to the circulation of WNV in Africa and Europe, but little has been revealed about the genetic relationships and the introductory event dynamics of WNV L1 and L2 European and African strains [14–16,10]. In this study, we used phylogenetic and phylogeographic inferences to uncover the origins, genetic connections, dispersal history, and geographical patterns of most of the WNV L1 and L2 strains which have circulated and are circulating in both continents.

Amongst the two, WNV L1 is by far the lineage that appears to have the more complex history, with several clades, clusters and sub-clusters, and genetic flows among countries and continents around the world. Previous studies have described three WNV L1 major clades: 1A, which included strains from Africa, Europe, the Middle East, Asia, and America; 1B, which contained Kunjin virus, a virus circulating in Australia; and 1C, which comprised strains from India [11,34]. Within clade 1A, further WNV L1 sub-classifications have been made in the past [35–37], with seven clusters (clusters 1 to 7) recognised, providing evidence of the very complex structure of this lineage. Our phylogenetic analysis is consistent with the previous classification and contributes to giving novel insights into the hierarchical structure of WNV L1 clade 1A (Fig. 4.1 and Supplementary Fig S4.1).

According to our analyses, the WNV strains included in most of the Clade 1A clusters (2, 3, 4, 6, and 7) are likely to originate from Africa, more specifically from West Africa. Clade 1A clusters 2, 3, 4, 6, and 7 are in fact rooted by the two ancient sequences from Nigeria and Senegal included in cluster

5 (Fig 4.1 and Supplementary Fig. S4.1). Going deeper, it is probably of more interest to see cluster 2, which included most European strains to be rooted by the Senegal strain of 1989 (Genbank OP846971) (Fig. 4.1). This means that the first European introduction probably originated from Senegal around the 1990s, as previously reported by Ndione et al. [12]. This supposition is further validated by our phylogeographic inference which not only confirms the middle of North and West Africa as the location of the common ancestor of all WNV L1 Clade 1A strains but also traces its origin back to the 19th/20th century. From this ancestral location, the virus probably spread towards Egypt and Senegal, the latter being at the origin of all WNV strains within the WMed sub-cluster (20th - 21st centuries) (Fig. 4.1).

The 1996 Morocco strain (Genbank AY701412) at the root of the WMed 1 and 2 subtypes implies a Moroccan origin of most of the Western-Mediterranean European WNV L1 strains. It seems to occur around the 1990s and reinforces the previously hypothesized assumption of the existence of a genetic flow between North of Africa (Morocco) and Italy and France [Origin and evolution of West Nile virus lineage 1 in Italy, unpublished manuscript][38].

These findings confirm the presence of a corridor between Senegal, Morocco, and Western-Mediterranean European countries, such as Portugal, Spain, France, and Italy (Fig. 4.1). According to our phylogenetic inference, this is not a one-way corridor as incursions of WNV strains from Europe to Africa have also shown to occur. The WMed 1 and WMed 2 groups within the WMed sub-cluster which includes European, Moroccan (2003), and Senegalese (2012 - 2018) strains are rooted by the 2000 France strain (Fig. 4.1 and Supplementary Fig. S4.1). It appears evident, from our analyses, that the 2003 Morocco strain has originated from an area located in the South of Spain. This is the first time that such an evolutionary relationship has been disclosed. The genetic similarities observed between i) the 2012-2018 Senegalese and 2015 France strains [12], all closely related to the group of

2021-22 Italian sequences; and ii) the 2020-2022 Italian and 2012 Senegalese strains and those from Italy (2008 and 2011) and Spain (2020) (Fig. 4.1) further support this new reconstructive theory.

The evolutionary history of WNV L2 strains is much simpler to describe, with few introductory events from South Africa to Europe and no genetic flow in the opposite direction. Our maximum-likelihood phylogeny and molecular clock analysis stress the presence of several WNV L2 African clades with common origins (Fig. 4.2 and Supplementary Fig. S4.2). Historically, WNV L2 has circulated in Sub-Saharan Africa and Madagascar [39], before spreading to Europe [11,40]. Malagasy strains appear far apart from all the other strains included in this analysis, highlighting the presence of a local cycle probably sustained by resident birds and vector competent mosquitoes on the island, and independent of annual movements of migratory birds [41].

Evolving from permanent and stable local cycles established after the rare incursions into new areas appear to be the main characteristic of the WNV L2 strains. Often, the WNV L2 clade and cluster genetic similarity appear to be related to the specific geographical areas of circulation. This assumption is sustained by most of our WNV L2 strains grouping within the same geographical areas, as shown by i) clade 2a and c, including Madagascar strains only; ii) clade 2b, with most strains from South Africa and Namibia; and iii) clade 2d, including cluster 1, composed by Southern and Central African strains; clusters 3 and 4, constituted respectively by Senegal and Southern African strains (South Africa, Namibia, Uganda) only; and cluster 5, with most Central-Southern European strains grouping together (Fig. 4.2 and Supplementary Fig. S4.2).

Interestingly, our phylogenetic inference shows clades 2c and 2d to be a sister group of clade 2b, which includes the first strain obtained in South Africa in 1958 (Supplementary Fig. S4.2). Moreover, the clade 2d, including many African and European strains, appears to be rooted in the strain of the DRC of 1958, with high posterior support (Fig. 4.2). Particularly, cluster 5 within clade 2d including

the Central-Southern European strains, all rooted by the Hungarian strain of 2004, appears to be closely related to the South African cluster 4. These results stress the existence of a possible connection between South Africa and Europe.

Our phylogeographic inference also confirms this hypothesis, showing WNV L2 common ancestor to have probably originated in South Africa (Fig. 4.2). From there, the WNV L2 strains have probably started spreading among African countries, heading to Madagascar, DRC, CAR, Uganda, and Senegal in the 20th century, and to Europe in the 21st century. The virus, probably carried by long-distance migratory birds, was introduced in Hungary in 2004, where it was first detected, and then spread to many European countries, as shown by previous studies [11,42].

Overall, while WNV L1 evolution history is characterized by a complex behaviour and due to the constant connections between various continents, WNV L2 seems to be less complicated showing only one main independent introduction from Southern African countries (cluster 4) to Europe (cluster 5) and probably another of minor importance. Our maximum likelihood phylogeny shows the existence of a well-supported separate cluster 2 within clade 2b, which includes three European strains (Italy/2014, Romania/2014, Hungary/2017) closely related to the strains of CAR (1993) and South Africa (1958 and 1977) of cluster 1. Our phylogeographic analysis confirms this assumption, stressing the presence of a second flow between Southern African countries and Europe (Italy, Romania, and Hungary) through the CAR, which probably happened around the 21st century. The lineage, once in Europe, started spreading all over the European continent, founding favorable eco-climatic conditions which allowed it to become endemic and a very serious public health concern in numerous regions and countries [17,40].

According to these findings, it is clear that WNV L1 and L2 strains have had very different eco-epidemiological and genetic evolution features. The more complex genomic heterogeneity of WNV

L1 strains might be a consequence of the different time WNV L1 and L2 strains emerged from Africa [43], which in turn might derive by an apparent less inclination to spread from WNV L2 strains. For this reason, WNV L2 might appear to be more conserved than WNV L1 Clade 1A strains.

If we observe the two main Eastern and Western phylogeography and genomic epidemiology of WNV L1 and L2 strains between Africa and Europe, they perfectly overlap two most important Afro-Palearctic bird migratory flyways [44], strongly evidencing the essential role of migrating bird species in introducing WNV. This assumption is confirmed by several studies showing WNV to be carried around by migratory birds [4,10,45] that make magnificent movements across the globe (https://migrationatlas.org/) and, during this natural process, spread the virus from their original niches to new areas [9]. During their annual cycle, in fact, birds cross the borders of numerous countries having multiple stopovers while heading to their breeding and non-breeding grounds [46]. Each species appears to be characterized by a variability in the patterns of migration, creating a complex network of connectivity between Africa and Europe (https://migrationatlas.org/), which might be the basis of the tremendous and variable spread of WNV L1 and L2 strains worldwide. After saying that, it is still difficult to understand the reason why WNV L1 spreads more efficiently than L2, having both lineages the same chance to infect species of birds. A possible explanation could be that WNV L1 and L2 infection in these species might have a different outcome [7,47]. In other words, WNV L2 infection might result in a severe and fatal disease or in a very mild infection with short and low level of viraemia [48–51]. Either way, the host would reduce their capability of transmitting and spreading the infection.

Future coupled study of WNV L1 and L2 different species migratory bird susceptibility, connectivity patterns, and phylogeographic inferences might i) help uncover the dynamics of the two lineages, helping to understand how they spread and from which reservoirs they might be most carried around,

and ii) to prevent and predict future outbreaks, not only of this pathogen, but also of new emerging viruses.

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4.8 Supplementary Materials

All supplementary files including supplementary Figs. S4.1 and S4.2 (Maximum-likelihood phylogeny of WNV L1 and L2 strains) and Tables S4.1 and S4.2 (curated sequence metadata and geographic coordinates) can be found at 10.6084/m9.figshare.22182490.

Supplementary Figure S4.1. Maximum likelihood phylogeny of 228 WNV L1 genomes. Nodes with bootstrap supports (BS) lower than 50 are depicted with yellow dots. A scale bar at the bottom of the figure indicates the number of substitutions per site. All samples belong to clade 1, which is divided into different clusters (C1-C7) highlighted with colour bars on the right. Other relevant groups mentioned in the discussion are annotated in

the same way: Eastern-European clade (EEC), Western Mediterranean clade (WMed, which is divided into 2 subclades, WMed1 and WMed2) and the Italian clade sampled near Livenza in 2011-2013.

Supplementary Figure S4.2. Maximum likelihood phylogeny of 297 WNV L2 genomes. Nodes with bootstrap supports (BS) lower than 50 are depicted with yellow dots. A scale bar at the bottom of the figure indicates the number of substitutions per site. Clades (2a-d) are indicated by coloured bars on the right side of the figure. Clade 2d is further divided into 5 clusters, annotated with black bars on the right side of the figure.

4.9 Author contributions

Conceptualization, G.M., M.H.D.D., M.M.D., A.R., R.R., O.F., F.M.,and G.S.; methodology, G.M., A.S., M.H.D.D., and G.S.; validation, F.M., A.S., G.M., M.H.D.D., M.M.D., O.F., A.R., and G.S.; formal analysis, A.S., and G.M.; investigation, L.T., A.L., V.C., M.D.D., M.H.D.D., M.A., B.S., V.D.L., A.P., M.M.D., M.D., A.G., E.H.N., and G.M.; resources, G.S., A.R., and O.F. ; data curation, F.I., A.S., V.C., I.M., A.B., M.M.D., M.H.D.D., and G.M.; writing G.M.; original draft preparation, G.M., M.H.D.D., A.S., and G.S.; writing—review and editing, F.M., M.H.D.D., A.R., O.F., M.M.D., O.R.T., and G.S.; visualization, A.S., G.M., F.I., L.T., A.L., A.P., I.M., A.B., V.C., M.D.D., M.A., B.S., V.D.L., M.M., C.C., G.Mar., R.R., F.M., N.S., M.D., A.G., E.H.N, G.F., A.R., O.R.S., O.F., and G.S.; supervision, O.R.S., F.M., A.R., O.F., M.H.D.D., M.M.D., and G.S.; project administration, G.S.; funding acquisition, G.S. All authors have read and agreed to the published version of the manuscript.

4.10 Data availability statement

All scripts used to perform this analysis are available at the https://github.com/andrea-silverj/WNV-Afr_Eur GitHub repository. All scripts used to perform this analysis are available at the

https://figshare.com/account/home#/projects/160822. Particularly, alignments used for the phylogenetic analysis, model selection analysis, and tree files can be found at 10.6084/m9.figshare.22182418; phylogeographic inference tree files, geographic coordinates, and videos can be found at 10.6084/m9.figshare.22182409; supplementary files including supplementary Figs. S4.1 and S4.2 (Maximum-likelihood phylogeny of WNV L1 and L2 strains) and Tables S4.1 and S4.2 (curated sequence metadata and geographic coordinates) can be found at 10.6084/m9.figshare.22182490. Figures 4.1 and 4.2 can be found at 10.6084/m9.figshare.22182388.

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4.13 Conflict of interest

All the authors declare no conflict of interest.

CHAPTER 5. WEST NILE VIRUS LINEAGE 1 IN ITALY: NEWLY INTRODUCED OR A RE-OCCURRENCE OF A PREVIOUSLY CIRCULATING STRAIN?

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by

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5.1 Abstract

In Italy, West Nile virus (WNV) appeared for the first time in the Tuscany region in 1998. After 10 years of absence, it re-appeared in the areas surrounding the Po River delta, affecting eight provinces in three regions. Thereafter, WNV epidemics caused by genetically divergent isolates have been documented every year in the country. Since 2018, only WNV Lineage 2 has been reported in the Italian territory. In October 2020, WNV Lineage 1 (WNV-L1) re-emerged in Italy, in the Campania region. This is the first occurrence of WNV-L1 detection in the Italian territory since 2017. WNV was detected in the internal organs of a goshawk (*Accipiter gentilis*) and a kestrel (*Falco tinnunculus*). The RNA extracted in the goshawk tissue samples was sequenced, and a Bayesian phylogenetic analysis was performed by a maximum-likelihood tree. Genome analysis, conducted on the goshawk WNV complete genome sequence, indicates that the strain belongs to the WNV-L1 Western-Mediterranean (WMed) cluster. Moreover, a close phylogenetic similarity is observed between the goshawk strain, the 2008–2011 group of Italian sequences, and European strains belonging to the WMed cluster. Our results evidence the possibility of both a new re-introduction or unnoticed silent circulation in Italy, and the strong importance of keeping the WNV surveillance system in the Italian territory active.

5.2 Keywords

Arbovirus; WNV; WNV-L1; Italy; surveillance; whole genome sequencing; phylogenetic analysis

5.3 Introduction

West Nile virus (WNV) is a mosquito borne single-stranded RNA virus, a member of the Japanese encephalitis (JE) serocomplex belonging to the genus *Flavivirus* within the *Flaviviridae* family [1]. WNV is maintained in nature through an endemic cycle which involves mosquitoes (Diptera; Culicidae) as vectors, and birds as reservoir hosts [2]. Humans and horses are considered “dead-end”

hosts: they may develop disease; however, they are not able to infect vectors, and maintain the virus in the environment [3]. To date, eight different lineages of WNV have been described [4]. Lineages 1 and 2, often associated with cases of encephalitis in humans and horses, are by far those most widespread in Europe and the Mediterranean basin [5–8].

In Europe, WNV lineage 1 (WNV-L1) circulation was first evidenced in the 1960s in France, Portugal, and Cyprus [8–10]. Thirty years later, cases associated to WNV-L1 infection were reported in humans and horses in North African, Western, and Eastern European countries [8]. The WNV-L1 strains responsible for the Morocco (1996), Italy (1998), Israel (1998), and France (2000) human and horse cases [11–16] grouped into the Western-Mediterranean (WMed) clade, whereas those responsible for the Romanian (1996) and Russian (1999) human cases clustered in the Eastern-European clade [17–19]. A strong relationship has been observed between the WNV-L1 Israeli and Northern African strains which emerged in the Mediterranean region in the late 1990s, and the ones responsible for the 1999 New York epidemics, suggesting a viral flow between North Africa and North America via the Middle East [20]. In the early 2000s, WNV-L1 outbreaks were again recorded in Morocco, France, and Romania [16,21–23]. Starting from 2005, however, WNV lineage 2 (WNV-L2) strains belonging to the Hungarian and Volgograd clades started spreading to some South-Eastern and Eastern European countries [24–30]. In some regions, co-circulation of both WNV-L1 and L2 were observed [8,24,29,31–33]. Following the WNV-L2 incursions and spread, the circulation of WNV-L1 strains was less frequently observed. From 2010, it was reported in Morocco, Algeria, Tunisia, Spain, and Portugal [34–39]. Eastern WNV-L1 strains instead kept circulating in Romania, and spread in Bulgaria and Ukraine [27,40,41]. Human and horse infections with Eastern WNV-L1 strains were also reported in Turkey in 2010 and following years [42,43]. In recent years, WNV-L1 circulation appeared to be limited to Northern African countries, the Iberian Peninsula, Cyprus, Turkey, Israel, and Serbia [36–39,44–50].

In Italy, WNV-L1 emerged for the first time in the Tuscany region in 1998 [12,13]. Since then, the Italian Ministry of Health implemented a national veterinary surveillance plan for monitoring WNV in areas at risk of viral introduction and circulation [32]. The surveillance system did not detect any relevant WNV circulation until 2008, when WNV-L1 was identified in mosquitoes, birds, horses, and humans in the area surrounding the Po River delta [51]. Since 2008, WNV epidemics caused by genetically divergent isolates have been registered every year [29,52]. The phylogenetic analysis of the isolates confirmed the hypothesis of the virus overwintering, and the endemization in local host populations [53,54]. Between 2010 and 2011, WNV-L1 circulation further spread into Southern Italy, involving Sicily, Apulia, Calabria, Basilicata, and Sardinia regions [32,52,55]. Partial genome sequencing showed that strains isolated in the same area in 2011 were almost identical, but divergent from those responsible for the outbreak in Northern Italy in 2008–2009 [56,57] which, conversely, appeared strictly related to the WNV strains circulating in Europe and Israel from late 2004 to 2011 [55]. In 2011, WNV-L2 was first reported in Italy [31,58,59]. Since then, WNV-L1 circulation was reported only sporadically in birds and mosquito pools from North-Eastern regions (2012–2014, 2017) and Sardinia (2015–2016) [32]. In Italy, WNV-L1 was last detected in a mosquito pool collected in the Piacenza province in 2017 (https://westnile.izs.it/j6_wnd/wndItalia;jsessionid=D0C9EB639E7C322D0EFC34ECEB8E4D8E). According to a risk-based ranking of the Italian provinces, wild birds, mainly corvids (Eurasian jay, *Garrulus glandarius*; Carrion crow, *Corvus corone*; and Magpie, *Pica pica*), poultry, horses, and mosquitoes, are constantly sampled to obtain an early detection of WNV circulation and reduce the risk of human transmission. To date, 16 out of the 20 Italian regions are considered endemic (Italian epidemiological reports).

In October 2020, the WNV-L1 strain re-emerged in Italy, notably in the Campania region. Within the wildlife monitoring plan of the Campania region (PGMFS) and the National Plan for Prevention,

Surveillance, and Response to Arbovirus 2020–2025, WNV-L1, was detected in two wild birds, a kestrel (*Falco tinnunculus*) and a goshawk (*Accipiter gentilis*), found in Naples and Caserta provinces, respectively. This is the first occurrence of WNV-L1 detection in migratory and resident raptor birds since 2018.

In this paper, we describe the two cases, and characterize by whole genome sequencing (WGS) and phylogenetic analysis the WNV-L1 responsible for the goshawk infection.

5.4 Materials and Methods

5.4.1 Bird conditions and laboratory analyses

In October 2020, two wild birds, a kestrel and a goshawk, were found in critical conditions in Naples (Somma Vesuviana municipality 40.878958, 14.426694) and Caserta (Trentola Ducenta municipality 40.976368, 14.1664) provinces, respectively (Figure 5.1). The two birds, immediately transferred to the Regional Center for Wild Animals (CRAS) for rescue operations, died 48 h after the transfer. In detail, at the time of admission at the clinic, the kestrel was in a comatose state showing mydriasis. The radiography also evidenced the fracture of the tibia, and the presence of two bullet fragments. At the time of acceptance, the goshawk showed head injury, head tilt, and right lower limb ataxia. Necropsies were carried out, and selected tissues (heart, kidney, spleen, and brain) were collected, pooled, and homogenized in a sterile phosphate-buffered saline (PBS). Viral RNA was extracted from 200uL supernatants using Qiasymphony® DSP automatic instrumentation according to the manufacturer's instructions. Quantitative reverse transcription polymerase chain reactions (qRT PCR) to detect WNV-L1 and/or -L2 RNA was performed at the U.O.C. Virology of IZSM as described by Del Amo and colleagues [60]. The samples were sent to the National Reference Centre for Foreign

Animal Diseases (CESME) at the Istituto Zooprofilattico Sperimentale of Abruzzo and Molise in Teramo (IZSAM) for WNV confirmation and further analysis.

Figure 5.1 Geolocation of sites where the two wild birds were found

5.4.2 Virus strain and Laboratory Tests (IZSAM)

Virus RNA of the two samples' homogenates was extracted at IZSAM by using the MagMAX CORE Nucleic Acid Purification KIT (Applied Biosystem, Thermo Fisher Scientific, Life Technologies Corporation, TX, USA) according to the manufacturer's instructions. The virus RNA was tested by two qRT-PCR: (i) a 1-step RT-PCR assay for the simultaneous detection of WNV-L1 and 2 strains,

by using the QuantiTect Probe RT-PCR Kit (QIAGEN) [60]; and (ii) an RT-PCR assay for detection of all known lineages of West Nile virus [61], by using the Superscript III Platinum OneStep qRT-PCR System (Invitrogen). The RNA detected in the goshawk tissue samples was further fully sequenced by using next generation sequencing (NGS) technology. Briefly, total RNA was treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 20 min, and then purified by an RNA Clean & Concentrator™-5 Kit (Zymo Research, Irvine, CA, USA). The purified RNA was used for the assessment of sequencing independent single primer amplification protocol (SISPA) [62,63]. In detail, viral RNA underwent cDNA synthesis using 200 units of the SuperScript® IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), in the presence of 5X SSIV Buffer, 50 µM of the random hexamer FR26RV-N 5′-GCCGGAGCTCTGCAGATATCNNNNNN-3′, 10 mM of dNTPs mix, 100 mM of DTT, and 40U of RNAse OUT RNase inhibitor (Thermo Fisher Scientific, Waltham, MA). The reaction was incubated at 23 °C for 10 min, and 50 °C for 50 min. After an inactivation step at 80 °C for 10 min, 12.5 Units of 3′-5′ Klenow Polymerase (New England Biolabs, Ipswich, MA, USA) were directly added to the reaction to perform the second strand cDNA synthesis. The incubation was carried out at 37 °C for 1 h, and 75 °C for 10 min. Next, 5 µL of ds cDNA were added to 45uL of PCR master mix containing 5X Q5 Reaction Buffer, 10 mM of dNTPs, 40uM of the random primer FR20 Rv 5′-GCCGGAGCTCTGCAGATATC-3′, Q5® High Fidelity DNA polymerase (NEB, New England Biolabs, Ipswich, MA, USA), and Q5 High Enhancer [64]. The reaction was incubated at 98 °C for 10 s, 65 °C for 30 s, 72 °C for 3 min, and 72 °C for 2 min. The PCR product was purified using the Molecular Biology Kit BioBasic (Biobasic inc., Markham, ON, Canada), and then quantified by using the Qubit® DNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The sample was diluted to obtain a concentration of 100–500 ng, and used for library preparation by using the Illumina DNA Prep kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. Deep

sequencing was performed on the NextSeq 500 (Illumina Inc., San Diego, CA, USA) using the NextSeq 500/550 Mid Output Reagent Cartridge v2, 300 cycles, and standard 150 bp paired end reads. FASTQ files were generated using NextSeq Reporter (Illumina). The sequencing run delivered 300 Mb of sequence data. The reads obtained were trimmed using a Trimmomatic script (Trimmomatic v0.36) to remove low quality and short reads [65]. Furthermore, the reads were quality controlled by using FastQC v0.11.5 [63,66]. The resulting 2,149,990 reads were de novo assembled using SPADES v3.11.1 [67]. Based on genome assemblies, a de novo filtering for the scaffolds with a minimum length of 200 nucleotides, and a matching for the best reference for each assembly using ABRicate was carried out [63]. Finally, a mapping with the references found in the previous step using Bowtie2 (v.2.1.0) was performed [68]. The length of the final assembly (GenBank accession number MW627239) was of 10,990 bp, and it showed 98.33% nucleotide identity to the reference sequence FJ483548.

5.4.3 Phylogenetic analyses

A total of 64 sequences with information on country and year of isolation were downloaded from Genbank or from the IZSAM database for this study. In particular, the study was conducted using 34 complete genome sequences, 18 polyprotein gene complete coding DNA sequences (cds), and 12 partial cds (polyprotein and envelope glycoprotein gene). Sequences were obtained from mosquitoes (n = 7), birds (n = 24), horses (n = 6), humans (n = 16), and mice (n = 1) (10 sequences were from unknown hosts). They were selected from Italy (n = 27), Portugal (n = 2), France (n = 4), Spain (n = 4), Romania (n = 2), Israel (n = 2), Morocco (n = 2), Nigeria (n = 1), Russia (n = 5), Senegal (n = 3), Cyprus (n = 1), Turkey (n = 1), Japan (n = 1), Americas (n = 7), Kenya (n = 1), and Central African Republic (CAR) (n = 1). With the addition of 1 new sequence, a total of 65 sequences were aligned using the ClustalW algorithm implemented in Ugene v. 37.0 software (available at

http://ugene.net/download.html). Aligned sequences were manually curated using BioEdit v. 7.2 software (available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Metadata of all sequences referred to in this manuscript can be found in Table 5.1.

Table 5.1 Relevant data regarding the isolates used for the present study

Strain Number	Viral Species	Isolation Material	Host	Country	Year of Isolation	Accession Number
TE.362447.2020	WNV L1	Homogenate	Northern goshawk	Italy	2020	MW627239
TE.15803.2008	WNV L1	-	Magpie	Italy	2008	FJ483548
TE.15217.2008	WNV L1	-	Magpie	Italy	2008	FJ483549
TE.229892.2008	WNV L1	-	Magpie	Italy	2008	KU573077
Ita09	WNV L1	Blood	Human	Italy	2009	GU011992
Italy/2009/J-225677	WNV L1	C636 cells P1, Vero cells P3	Eurasian jay	Italy	2009	JF719068
Italy/2009/FIN	WNV L1	-	Human	Italy	2009	KF234080
Italy/2008/J-242853	WNV L1	C636 cells P1, Vero cells P3	Eurasian jay	Italy	2008	JF719065
Italy/2009/G-223184	WNV L1	C636 cells P1, Vero cells P3	Gull	Italy	2009	JF719067
Italy/2008/M-	WNV L1	C636 cells P1,	Magpie	Italy	2008	JF719066

Strain Number	Viral Species	Isolation Material	Host	Country	Year of Isolation	Accession Number
203204		Vero cells P3				
204913/2009	WNV L1	-	*Culex pipiens* mosquito	Italy	2009	KU573078
TE.14444.2011	WNV L1	Organ pool	Magpie	Italy	2011	MW835356
TE.17196.2011	WNV L1	Organ pool	Owl	Italy	2011	MW835357
TE.17208.2011	WNV L1	Organ pool	Crow	Italy	2011	MW835358
TE.20224/1.2011	WNV L1	Plasma	Chicken	Italy	2011	MW835359
TE.21370.2011	WNV L1	Plasma	Horse	Italy	2011	MW835361
TE.20875.2011	WNV L1	Organ pool	Eurasian jay	Italy	2011	MW835360
TE.23237.2011	WNV L1	Plasma	Chicken	Italy	2011	MW835363
TE.20224/8.2011	WNV L1	Plasma	Chicken	Italy	2011	Under publication
TE.21412.2011	WNV L1	Brain	Owl	Italy	2011	MW835362
04.05	WNV L1	Brain	Horse	Morocco	2003	AY701413

Strain Number	Viral Species	Isolation Material	Host	Country	Year of Isolation	Accession Number
PT5.2	WNV L1	-	-	Portugal	2004	AJ965628
PT6.16	WNV L1	-	-	Portugal	2009	AJ965626
Spain/2010/H-1b	WNV L1	Brain	Horse	Spain	2010	JF719069
WN Italy 1998-equine	WNV L1	-	Equine	Italy	1998	AF404757
96-111	WNV L1	Brain	Equine	Morocco	1996	AY701412
PaAn001	WNV L1	-	-	France	2003	AY268132
France 405/04	WNV L1	Brain	House sparrow	France	2004	DQ786572
WNV_0304h_ISR00	WNV L1	-	Human	Israel	2000	HM152775
GE-2o/V	WNV L1	Vero cells	Golden eagle	Spain	2007	FJ766332
GE-1b/B	WNV L1	BSR cells	Golden eagle	Israel	2007	FJ766331
HU6365/08	WNV L1	-	*Culex perexiguus*	Spain	2008	JF707789

Strain Number	Viral Species	Isolation Material	Host	Country	Year of Isolation	Accession Number
			mosquito			
RO97-50	WNV L1	-	*Culex pipiens* mosquito	Romania	1996	AF260969
KN3829	WNV L1	-	*Culex univittatus* mosquito	USA	2003	AY262283
VLG-4	WNV L1	Brain	Human	Russia	1999	AF317203
Tomsk/bird/2006/A 4	WNV L1	-	Blyth's reed warbler	Russia	2006	MN149538
LEIV-Vlg99-27889	WNV L1	Brain	Human	Russia	1999	AY277252
LEIV-Vlg00-27924	WNV L1	Blood	Human	Russia	2000	AY278442
Italy/2012/Livenza/ 37.1	WNV L1	Urine	Human	Italy	2012	KC954092
Italy/2012/Livenza/ 31.1	WNV L1	Culture viral isolate from	Human	Italy	2012	JX556213

Strain Number	Viral Species	Isolation Material	Host	Country	Year of Isolation	Accession Number
		blood				
Italy/2013/Livenza/35.1	WNV L1	Plasma	Human	Italy	2013	KF647253
Italy/2011/Livenza	WNV L1	Plasma	Human	Italy	2011	JQ928174
Italy/2013/Livenza/37.1	WNV L1	Urine	Human	Italy	2013	KF823807
Akela/France/2015	WNV L1	-	Human	France	2015	MT863559
Italy/2011/AN-1	WNV L1	Urine	Human	Italy	2011	JN858069
Italy/2011/Piave	WNV L1	Urine	Human	Italy	2011	JQ928175
WNV_Cy2016	WNV L1	Urine	Human	Cyprus	2016	MF797870
T2	WNV L1	Blood	Equine	Turkey	2011	KJ958922
ArB310/67	WNV L1	-	-	Central African Republic	1967	GQ851608
IBAN7019	WNV L1	-	-	Nigeria	1965	GQ851607

Strain Number	Viral Species	Isolation Material	Host	Country	Year of Isolation	Accession Number
ArD27875	WNV L1	-	Mosquito	Senegal	1979	GQ851606
PaH001	WNV L1	-	-	France	2003	AY268133
NY99-flamingo382-99	WNV L1	Chicken embryo	Flamingo	USA	1999	AF196835
ABB-B13	WNV L1	-	Mouse	Spain	2007	KC407667
NY99	WNV L1	Vero cell P2	-	USA	1999	NC009942
NY99iso-1	WNV L1	Vero cell E6	-	Japan	2007	FJ411043
NY99-crow-V76/1	WNV L1	-	American crow	USA	1999	FJ151394
3356K VP2	WNV L1	Kidney	American crow	USA	2000	EF657887
WNV-1/US/BID-V6527/2001	WNV L1	Kidney and Spleen	American crow	USA	2001	KJ501343
WNV-1/US/BID-V6506/2002	WNV L1	Kidney and Spleen	American crow	USA	2002	KJ501489
Ast99-901	WNV L1	Blood	Human	Russia	1999	AY278441

Strain Number	Viral Species	Isolation Material	Host	Country	Year of Isolation	Accession Number
KN3829	WNV L1	-	*Culex univittatus* mosquito	Kenya	1998	AF146082
RO97-50	WNV L1	-	-	Romania	1997	AF130362
SEN-ArD93548	WNV L1	-	Mosquito	Senegal	1993	AF001570
Dak Ar D 5443	KOUTANGO VIRUS	-	-	Senegal	2013	EU082200

The Bayesian phylogenetic analysis was performed through Bayesian Inference (BI) using a general time-reversible with gamma-distributed rate variation, a gamma category count of 4, and an invariant sites model (GTR + Γ + I), as selected by Akaike's information criterion (AICc) in jModelTest 0.1 [69]. A Bayesian MCMC approach using BEAST with JRE v2.6.3 was then employed. Ten independent MCMC runs with up to 100 million generations were performed to ensure the convergence of estimates. Tracer v.1.7.1 (available at http://beast.bio.ed.ac.uk/Tracer) was used to ensure convergence during MCMC by reaching effective sample sizes greater than 100. Trees were summarized in a maximum clade-credibility tree with common ancestor heights after a 10% burn-in [69] using TreeAnnotator v.2.6.3. A maximum likelihood tree was estimated using FigTree v1.4.4 [70] after identical alignment and curating methods. FigTree was run using the GTR + Γ + I nucleotide model with 2000 Γ-rate categories, exhaustive search settings, with 5000 bootstrap replications using the Shimodaira–Hasegawa (SH) test. All sequence alignments referred to in this manuscript can be found in supplementary materials.

5.5 Results

5.5.1 Strain Characterization and Phylogenetic Analysis

5.5.1.1 WNV-L1 Detection

WNV-L1 was detected and confirmed in both the kestrel and the goshawk organs (heart, kidney, spleen, and brain pooled together in PBS).

5.5.1.2 Phylogenetic Tree Inferred with Maximum-Likelihood Analysis

Phylogenetic inference of WNV using a maximum-likelihood tree is shown in Figures 5.2 and 5.3. All Shimodaira–Hasegawa values are displayed at respective nodes.

According to the analysis, WNV-L1 is represented by three main clusters: (1) the Mediterranean-Eastern European-Kenyan WNV cluster, that includes isolates from Senegal (1993), Kenya (1998), Romania (1996–97), Russia (1999, 2000, 2006), and Cyprus (2016); (2) the WMed cluster, including isolates from Europe (Italy 1998, 2008–09–11–20; Spain 2007–08; Portugal 2004–09; France 2003–04 and 2015), Israel 2000–07, and Morocco 1996–2003; and (3) the Israeli-American WNV cluster, including several American strains, isolated between 1999 and 2002 (all phylogenetically very similar among them), in addition to strains from Japan (2007), Europe (France 2003 and Spain 2007), and Africa (Nigeria 1965, CAR 1967, Senegal 1979, and Tunisia 2011), confirming a WNV clusterization already highlighted in the past [71,72] (Figures 5.2 and 5.3).

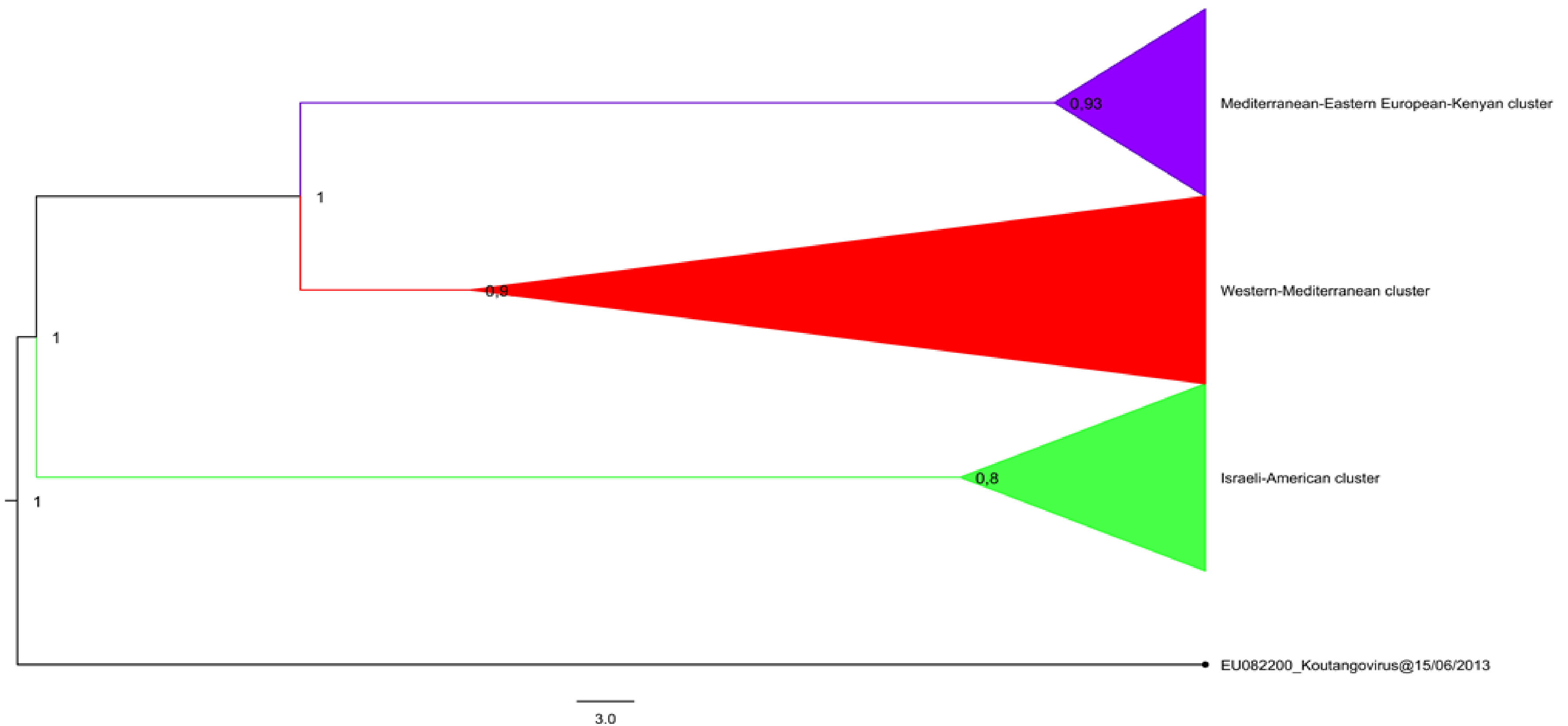

Figure 5.2 Maximum likelihood phylogenetic tree of the WNV complete and partial genome sequences analyzed in this study.

Violet, red, and green triangles represent the Mediterranean-Eastern European-Kenyan subtype, the Western Mediterranean subtype, and the Israeli-American subtype of WNV sequences, respectively. The Koutango virus strain EU082200 has been chosen as outgroup. The tree with the highest log-likelihood is shown. The Bayesian phylogenetic analysis was performed through Bayesian Inference (BI) using a general time-reversible with gamma-

distributed rate variation, a gamma category count of 4, and an invariant sites model (GTR + Γ + I). The evolutionary distances were computed using the optimal GTR + Γ + I model, with 2000 Γ-rate categories and 5000 bootstrap replications using the Shimodaira–Hasegawa (SH) test. The percentage of successful bootstrap replicate (n5000) is indicated at nodes. A Bayesian MCMC approach using BEAST with JRE v2.6.3 was employed. Ten independent MCMC runs with up to 100 million generations were performed to ensure the convergence of estimates. Tracer v.1.7.1 was used to ensure convergence during MCMC by reaching effective sample sizes greater than 100. Trees were summarized in a maximum clade-credibility tree with common ancestor heights after a 10% burn-in using TreeAnnotator v2.6.3.

The genome analysis includes the new strain TE_362447_2020 (accession number: MW627239 lineage 1, Italy, 2020) into the WMed single monophyletic group (Figure 5.3). A close similarity is observed between the MW627239 and the 2008–2011 groups of sequences clustered separately into the WMed subtype (Figure 5.3). Phylogenetically, a similarity is also observed between the Italian WNV-L1 (2008, 2011, 2020) and the viral strains circulating in Europe in the recent past (Spain 2010, France 2015), although they are less temporally close than the Italian strains [71].

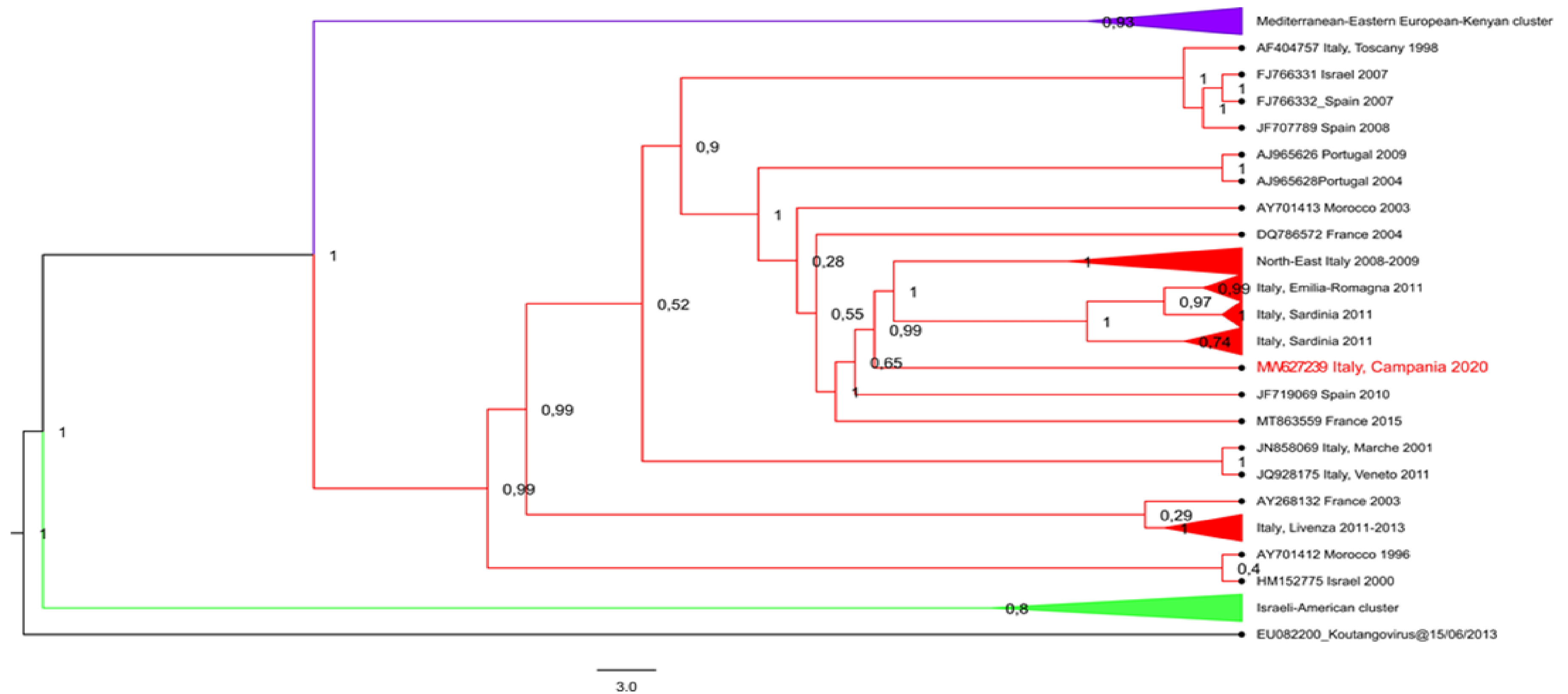

Figure 5.3 Maximum likelihood phylogenetic tree of the WNV complete and partial genome sequences analyzed in this study.

A detailed version of the phylogenetic tree is shown, showing all the Western Mediterranean WNV sequences. GenBank accession numbers are indicated for each strain, with country, lineage, and year of isolation. The WNV-L1 strain TE_362447_2020, obtained from the goshawk found in the Campania region in October 2020, is highlighted in red. The Koutango virus strain EU082200 has been chosen as outgroup. The tree with the highest log-likelihood is shown. The Bayesian phylogenetic analysis was performed through Bayesian Inference using a general time-reversible with gamma-distributed rate variation, a gamma category count of 4, and an invariant sites model (GTR + Γ + I). The evolutionary distances were computed using the optimal GTR

+ Γ + I model, with 2000 Γ-rate categories and 5000 bootstrap replications using the Shimodaira–Hasegawa (SH) test. The percentage of successful bootstrap replicate (n5000) is indicated at nodes. A Bayesian MCMC approach using BEAST with JRE v2.6.3 was employed. Ten independent MCMC runs with up to 100 million generations were performed to ensure the convergence of estimates. Tracer v.1.7.1 was used to ensure convergence during MCMC by reaching effective sample sizes greater than 100. Trees were summarized in a maximum clade-credibility tree with common ancestor heights after a 10% burn-in using TreeAnnotator v2.6.3.

5.5.1.3 Full-Length Polyprotein Sequencing

Using BioEdit v. 7.2 software (available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html), an amino acid sequence comparison between MW627239 and representative WNV-L1 Italian, European, and American isolates was performed. In particular, the strains (i) 115803 (accession number: FJ483548, Italy, 2008); (ii) WNV Italy 1998-equine (accession number: AF404757, Italy, 1998); (iii) Italy/2008/M-203204 (accession number: JF719066, Italy, 2008); (iv) Italy/2008/J-242853 (accession number: JF719065, Italy, 2008); (v) 21412 (accession number: MW835362, Italy, 2011); (vi) Italy/2013/Livenza/35.1 (accession number: KF647253, Italy, 2013); (vii) Spain/2010/H-1b (accession number: JF719069, Spain, 2010); (viii) GE-2o/V (accession number: FJ766332, Spain, 2007); (ix) Akela/France/2015 (accession number: MT863559, France, 2015); and (x) NY99 (accession number: NC009942, USA, 1999) were included in the analysis. Only representative amino acid residues were analyzed [7,52,73–75]. Results are shown in Table 5.2.

Table 5.2 Amino acid sequence comparison of MW627239 (goshawk, Italy, 2020) and representative WNV-L1 Italian, European, and American strains, conducted using BioEdit v. 7.2 software

Viral Protein	Amino Acid Position	MW627239 ITA 2020	AF404757 ITA 1998	JF719066 ITA 2008	FJ766332 SPA 2007	JF719065 ITA 2008	FJ483548 ITA 2008	MW835362 ITA 2011	KF647253 ITA 2013	NC_009942 USA 1999	JF719069 SPA 2010	MT863559 FRA 2015
C	34	M	M	M	V	M	M	M	M	M	M	M
	100	S	S	S	S	S	S	S	S	S	S	S
prM	72	S	S	S	S	S	S	S	S	S	S	S
M	36	I	I	I	I	I	I	I	I	I	I	I
E	35	S	S	S	S	S	S	S	S	S	S	S
	51	T	A	A	T	A	A	A	A	A	A	A
	76	T	T	T	T	T	T	T	T	T	T	T
	88	P	P	P	S	P	P	P	P	P	P	P
	126	T	T	T	T	T	T	T	T	I	T	T
	153	G	G	G	G	G	G	G	G	G	G	G

Viral Protein	Amino Acid Position	MW627239 ITA 2020	AF404757 ITA 1998	JF719066 ITA 2008	FJ766332 SPA 2007	JF719065 ITA 2008	FJ483548 ITA 2008	MW835362 ITA 2011	KF647253 ITA 2013	NC_009942 USA 1999	JF719069 SPA 2010	MT863559 FRA 2015
	159	I	I	I	I	I	I	I	I	V	I	I
	278	T	T	T	T	T	T	T	T	T	T	T
	312	L	L	L	L	L	L	L	L	L	L	L
	442	V	V	V	V	V	V	V	V	V	V	V
NS1	17	S	S	S	S	S	S	S	S	S	S	S
	35	Y	Y	Y	H	Y	Y	Y	Y	Y	Y	Y
	45	I	I	I	I	I	I	I	I	I	I	I
	70	S	S	S	S	S	S	S	S	A	S	S
	94	E	E	E	E	E	E	E	E	E	E	E
	138	P	P	P	P	P	P	P	P	P	P	P
	141	K	K	K	K	K	K	K	K	K	K	K

Viral Protein	Amino Acid Position	MW627239 ITA 2020	AF404757 ITA 1998	JF719066 ITA 2008	FJ766332 SPA 2007	JF719065 ITA 2008	FJ483548 ITA 2008	MW835362 ITA 2011	KF647253 ITA 2013	NC_009942 USA 1999	JF719069 SPA 2010	MT863559 FRA 2015
	188	V	V	V	V	V	V	V	V	V	V	V
	208	D	D	D	H	D	D	D	D	D	D	D
	288	S	S	S	S	S	S	S	S	S	S	S
	289	E	E	E	G	E	E	E	E	E	E	E
NS2A	85	I	I	V	I	V	V	I	I	I	I	I
	104	N	N	N	N	N	N	N	N	N	N	N
	119	H	H	H	H	H	H	H	H	H	H	H
	128	E	E	E	E	E	E	E	E	E	E	E
	138	V	V	V	V	V	V	V	V	V	V	V
	165	G	G	G	G	G	G	G	G	G	G	G
NS2B	82	D	D	D	D	D	D	D	D	D	D	D

Viral Protein	Amino Acid Position	MW627239 ITA 2020	AF404757 ITA 1998	JF719066 ITA 2008	FJ766332 SPA 2007	JF719065 ITA 2008	FJ483548 ITA 2008	MW835362 ITA 2011	KF647253 ITA 2013	NC_009942 USA 1999	JF719069 SPA 2010	MT863559 FRA 2015
	83	G	G	G	G	G	G	G	G	G	G	G
	103	A	A	A	A	A	A	A	V	V	A	A
	120	V	I	V	V	V	I	V	V	V	V	V
NS3	46	F	F	F	F	F	F	F	F	F	F	F
	244	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	H
	249	T	T	T	P	T	P	T	P	P	T	T
	356	I	I	I	I	I	I	I	I	T	I	I
	496	L	L	L	L	L	L	L	L	L	L	L
	503	N	N	N	N	N	N	N	N	N	N	N
	521	D	D	D	D	D	D	D	D	D	D	D
NS4A	85	V	V	I	V	I	I	V	V	A	V	V

Viral Protein	Amino Acid Position	MW627239 ITA 2020	AF404757 ITA 1998	JF719066 ITA 2008	FJ766332 SPA 2007	JF719065 ITA 2008	FJ483548 ITA 2008	MW835362 ITA 2011	KF647253 ITA 2013	NC_009942 USA 1999	JF719069 SPA 2010	MT863559 FRA 2015
	100	P	P	S	P	S	S	P	P	P	P	P
	122	P	P	P	P	P	P	P	P	P	P	P
NS5	53	H	H	H	H	H	H	H	H	H	H	H
	54	P	P	P	P	P	P	P	P	P	P	P
	257	D	D	D	D	D	D	D	D	D	D	D
	258	V	V	A	V	A	A	A	V	V	V	V
	280	K	K	K	K	K	K	K	K	K	K	K
	372	V	V	V	V	V	V	V	V	V	V	V
	374	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	422	R	R	K	R	K	K	K	R	R	R	R
	426	E	E	E	A	E	E	E	E	E	E	E

Viral Protein	Amino Acid Position	MW627239 ITA 2020	AF404757 ITA 1998	JF719066 ITA 2008	FJ766332 SPA 2007	JF719065 ITA 2008	FJ483548 ITA 2008	MW835362 ITA 2011	KF647253 ITA 2013	NC_009942 USA 1999	JF719069 SPA 2010	MT863559 FRA 2015
	436	M	M	M	I	M	M	M	M	M	M	M
	526	T	T	T	T	T	T	T	T	T	T	T
	653	F	F	F	F	F	F	F	F	F	F	F
	681	T	T	T	T	T	T	T	T	T	T	T

The strains (i) 115803 (accession number: FJ483548, Italy, 2008); (ii) WNV Italy 1998-equine (accession number: AF404757, Italy, 1998); (iii) Italy/2008/M-203204 (accession number: JF719066, Italy, 2008); (iv) Italy/2008/J-242853 (accession number: JF719065, Italy, 2008); (v) 21412 (accession number: MW835362, Italy, 2011); (vi) Italy/2013/Livenza/35.1 (accession number: KF647253, Italy, 2013); (vii) Spain/2010/H-1b (accession number: JF719069, Spain, 2010); (viii) GE-2o/V (accession number: FJ766332, Spain, 2007); (ix) Akela/France/2015 (accession number: MT863559, France, 2015); and (x) NY99 (accession number: NC009942, USA, 1999) were included in the analysis. Only representative amino acid residues were analyzed. C protein: amino acid (aa) 1–105; PreM protein: aa 124–290; M protein: aa 216–290; E protein: aa 291–791; NS1 protein: aa 792–1143; NS2A protein: 1114–1374; NS2B protein: 1375–1505; NS3 protein: 1506–2124; NS4A protein: 2125–2250; NS4B protein: 2274–2528; NS5 protein: 2529–3433. Significant amino acid residue substitutions are highlighted in red.

Using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi), a nucleotide and amino acid pairwise identity analysis was conducted among MW627239 and the Italian, European, and American sequences listed above, as shown in Table 5.3. Among the Italian representative sequences belonging to the WNV-L1 WMed single monophyletic group in the maximum-likelihood tree, the average nucleotide and amino acid pairwise identity was evidenced to be 98.13% (s.d. = 0.36) and 99.73 % (s.d = 0.07), respectively.

Pairwise identity analyses have been conducted among MW627239 and other WNV L-1 Italian, European, and American sequences, using Blast. Among the Italian representative sequences belonging to the WNV-L1 WMed single monophyletic group in the maximum-likelihood tree, the average nucleotide and amino acid pairwise identity was evidenced to be 98.13% (s.d. = 0.36) and 99.73% (s.d = 0.07), respectively.

Table 5.3 Nucleotide versus amino acid similarities of representative WNV Italian, European, and American strains

	MW627239 ITA 2020	**AF404757 ITA 1998**	**JF719066 ITA 2008**	**JF719065 ITA 2008**	**FJ483548 ITA 2008**	**MW835362 ITA 2011**	**KF647253 ITA 2013**	**NC_009942 USA 1999**	**FJ766332 SPA 2007**	**JF719069 SPA 2010**
MW627239 ITA 2020		98.5%	98.31%	98.33%	98.33%	98.02%	97.67%	95.80%	97.96%	98.27%
AF404757 ITA 1998	99.85%		98.50%	98.53%	98.55%	98.18%	98.54%	96.44%	98.93%	98.51%
JF719066 ITA 2008	99.68%	99.77%		99.95%	99.41%	98.93%	98.13%	96.04%	98.37%	98.75%
JF719065 ITA 2008	99.71%	99.80%	99.91%		99.94%	98.95%	98.15%	96.07%	98.41%	98.78%
FJ483548 ITA 2008	99.68%	99.83%	99.88%	99.91%		98.93%	98.15%	96.10%	98.39%	98.76%
MW835362 ITA 2011	99.68%	99.77%	99.71%	99.74%	99.71%		97.85%	95.76%	98.00%	98.43%

KF647253 ITA 2013	99.74%	99.83%	99.65%	99.68%	99.71%	99.65%		96.03%	98.34%	98.11%
NC_009942 USA 1999	99.62%	99.71%	99.56%	99.59%	99.62%	99.53%	99.71%		96.29%	96.16%
FJ766332 SPA 2007	99.65%	99.68%	99.50%	99.53%	99.56%	98.75%	99.62%	99.50%		98.35%
JF719069 SPA 2010	99.65%	99.74%	99.56%	99.59%	99.56%	99.39%	99.62%	99.50%	99.48%	

Results evidence a close genetic relatedness of the WNV-L1 strain that re-emerged in the Campania region in 2020, and the Italian and European strains belonging to the WNV-L1 WMed sub-cluster.

5.6 Discussion

This paper reports the first evidence of WNV-L1 strain circulation in the Campania region, Italy. The strain was detected in October 2020 in two wild birds found moribund in nearby areas, only a few days apart. It was the first detection of WNV-L1 after several years. The last WNV-L1 strain circulation evidenced in Italy dates back to 2017, when it was found in a pool of mosquitoes from Northern Italy (https://westnile.izs.it/j6_wnd/wndItalia;jsessionid=D0C9EB639E7C322D0EFC34ECEB8E4D8E).

The last evidence of WNV-L1 circulation in an area most nearby the Campania region dates back to 2016 when the circulation of a WNV-L1 strain was responsible for the death of several wild birds in Sardinia [31]. The first question which clearly came to mind when tackling this finding was: "was this strain the result of a new introduction or was it just a re-occurrence of a strain already circulating?".

The first assumption supposes the virus probably extinguished and reintroduced through migratory birds. The species where the strains were detected did not help in clearing the question. Goshawks and kestrels can in fact be considered either migrant or resident birds. Unfortunately, it was not possible to check the carcasses, and eventually make out the bird behavior from wing, claw, and feather characteristics [74–76]. In support of the re-introduction of WNV-L1 is the lack of detection for consecutive years by the national surveillance program, which has been in place since 2002, and has been drawn to detect virus circulation early. In the same way, a phylogenetic relationship (%), and nucleotide and amino acid similarities are observed between the new sequence, MW627239, and some European sequences obtained in the past (Spain 2010, France 2015) (Figure 5.3, Tables 5.2 and

5.3). This might suggest a possible viral circulation in the Mediterranean followed by a re-introduction in Italy in 2020.

Still on the phylogenic tree, however, the new strain MW627239 shows high nucleotide sequence identity (%) with the 2008–2011 Italian sub-clusters of the WMed single monophyletic group (Figure 5.3). In this respect, the second hypothesis, the WNV-L1 re-occurrence, seems to be the most credible scenario. Even if, in all these years, WNV has repeatedly proven its ability to overwinter and become endemic in many Italian regions, silence periods are factually not unusual for WNV-L1 [53,71]. After its first occurrence in the Tuscany region in 1998, WNV re-appeared in the areas surrounding the Po River delta after 10 years [55]. Similarly, in Sardinia, WNV-L1 was not detected for 3 consecutive years between 2011 and 2015 (https://westnile.izs.it/j6_wnd/wndItalia;jsessionid=D0C9EB639E7C322D0EFC34ECEB8E4D8E). In this particular case, the WNV-L1 presence was accidentally uncovered in two wild birds when they were in critical conditions. The presence of clinical signs in birds would indeed facilitate the discovery of virus circulation on many occasions, as observed in several WNV epidemics of the early 2000s [16,76,77]. Even though the majority of the WNV infections in birds are usually mild or asymptomatic [78], some species, such as birds of prey, jays, and crows, are highly susceptible, and can develop severe and even fatal encephalitis [78–80]. Clinical symptoms associated to WNV-L1 infection have been mainly reported in the orders of Passeriformes (corvids, blue jays, magpies) and Falconiformes (birds of prey) [81–83]. Fatal infections have been described in European eagles in Spain [84,85], and geese and poultry in Hungary [24]. The two WNV-L1 infected birds found in Campania were in critical conditions. Though the comatose state of the kestrel was likely the consequence of a gunshot wound, the origin of the clinical picture observed in the goshawks is not easy to assess. Head injury, head tilt, and right lower limb ataxia are definitely signs of neurologic pathology. However, whether these symptoms were a consequence of the head injury or vice versa couldn't be determined. In nature,

goshawks have been shown to be highly susceptible to WNV infection, probably because of their predatory habits [81]. Oral transmission of WNV by feeding on infected prey has been described and is believed to be an important route of transmission in birds of prey [79,83,86]. Both WNV-L1 and L2 experimental infections conducted in American kestrels (*Falco sparverius*), golden eagles (*Aquila chrysaetos*), red-tailed hawks (*Buteo jamaicensis*), barn owls (*Tyto alba*), and great horned owls (*Bubo virginianus*) showed high level of viraemia, and important clinical symptoms, such as lethargy, inappetence, body weight loss, and muscle tremor [82,87,88]. However, looking back over the past 13 years of circulation in Italy, WNV-L1 seems to be unable to cause important clinical manifestations and deaths in birds [52].

The virulence of WNV depends on several factors related to the pathogen, hosts, and their interaction [89,90]. Concerning the pathogen, changes in the amino acid positions may significantly influence the WNV strain virulence [7]. Multiple genetic variations correlated with increased or decreased pathogenicity have been highlighted in genetic and phenotypic studies of WNV mutants [74]. Among the WNV genes, the NS3 helicase domain is considered a virulence determinant [52,91]. In particular, increased avian virulence due to the point mutation NS3-T249P has been reported in American crows (*Corvus brachyrhynchos*) and in site-directed mutagenesis experiments [73,92–94]. In support of our analysis, the Italian strain MW627239 is not characterized by the NS3-T249P point mutation. A threonine residue was observed at the 249 position in the strain MW627239, as well as in JF719065 and MW835362, circulating in Italy in 2008 (jay) and 2011 (owl), and in JF719069 and MT863559, circulating in Spain (horse) and France (human) in 2010 and 2015, respectively. Furthermore, the amino acid valine at the residue 159 of the E protein is considered a determinant of WNV neurovirulence, influencing viral replication and pathogenesis, and being involved in WNV infection and T-cell infiltration in the brain [75]. This amino acid is observed in the isolate NC009942, circulating in the USA in 1999, but not in the Italian and European strains, all characterized by

isoleucine at this residue position. This point mutation might also help explain the low pathogenesis observed among birds in Italy and, more generally, in Europe. It is likely that the WNV pathogenicity is the result of a complex series of events which involve the virus, the vectors, and the hosts. Further studies correlated with the WNV genotype and phenotype may help in understanding the mechanisms underlying WNV clinical signs in birds, and the emergence of new pathogenic phenotypes.

5.7 Conclusions

In recent years, WNV-L2 has been, by far, the most frequent lineage detected in Italy, whereas WNV-L1 was detected only occasionally. Since 2008, about 1500 wild birds and 5000 resident birds belonging to target species (carrion crow, magpie, Eurasian jay) have been annually tested by real time RT-PCR to monitor the circulation of WNV L1 and L2 strains in Italy. Among them, 326 wild birds and 804 target species were found positive to WNV (https://westnile.izs.it/j6_wnd/home). Concerning WNV-L1, it was last detected in a sparrow hawk (*Accipiter nisus*) and in two carrion crows in the Sardinia region during the 2016 vector season.

Serological analysis conducted in Italy on humans and horses between 2008 and 2020 identified anti-WNV-IgM in 1189 persons and 1196 horses. No evidence of WNV circulation was detected in the Campania region [95] (https://westnile.izs.it/j6_wnd/home). The detection of the WNV-L1 strain in two wild birds described in this study emphasizes the importance of having in place an efficient surveillance system, and, in particular, the early warning function played by some avian species in detecting WNV circulation.

5.8 Supplementary Materials

The following are available online at https://www.mdpi.com/article/10 .3390/v14010064/s1, File S5.1: WNV Lineage 1 Sequence Alignment.

5.9 Author Contributions

Conceptualization, G.M., F.I., G.F., C.d.M. and G.S.; methodology, G.M., F.I. and G.S.; validation, F.M., A.P., V.C. and G.S.; formal analysis, V.C., A.P. and G.M.; investigation, A.D.G., O.P., V.C., A.P., S.B. and G.M.; resources, G.S.; data curation, F.I., V.C., A.P. and G.M.; writing G.S., G.F., C.d.M., F.I. and G.M., original draft preparation, G.F., C.d.M., F.I., G.M. and G.S.; writing— review and editing, F.I., F.M., G.F., C.d.M., V.C., A.P., E.D.F., R.R., A.R., G.M. and G.S.; visualization, G.M., F.I., F.M., G.F., C.d.M., O.P., A.D.G., V.C., A.P., S.B., E.D.F., R.R., A.R. and G.S.; supervision, G.S.; project administration, G.S.; funding acquisition, G.S. All authors have read and agreed to the published version of the manuscript.

5.10 Data Availability Statement

Sequence data are available via NCBI. The accession numbers for the sequences used can be found in Table 5.1.

5.11 Acknowledgments

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5.13 Conflicts of Interest

All the authors declare no conflict of interest.

5.14 References

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CHAPTER 6. ORIGIN AND EVOLUTION OF WEST NILE VIRUS LINEAGE 1 IN ITALY

Manuscript

by

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6.1 Abstract

5.1.1 Background

West Nile virus (WNV) is a zoonotic mosquito-borne pathogen capable of causing serious disease and death in infected individuals, equids, and some species of birds. Eight lineages are reported, with Lineage 1 (L1) and Lineage 2 (L2) being the most widespread and pathogenic. In 2021 and 2022, after several years of low circulation and sporadic cases, WNV L1 re-emerged in Italy, co-circulating with WNV L2 and causing 330 neurological cases and 37 deaths in humans as well as severe diseases in equids and birds.

5.1.2 Methodology and principal findings

We assembled 31 new WNV L1 genomes from samples collected in Italy between 2008 and 2022 through the national surveillance plan, and we reconstructed their evolutionary history using molecular clocks and phylogeographic methods. Our analysis indicates that the WNV L1 first introduction in Italy might have originated from Northern African countries around 1985. It also evidences in the Italian territory an interregional circulation, possible local endemization, and the presence of two different strains currently circulating in the country. It stresses the existence of a constant connection between Western-Mediterranean countries other than the presence of an ideal line connecting Italy, France, Spain, Portugal, and Morocco. Finally, it shows new genetic mutations possibly associated with increased virulence in humans and horses.

5.1.3 Conclusions

Our work tracks back the geographical spread of WNV L1 strains in Italy uncovering its viral dynamics, makes available a new consistent dataset of complete genome sequences that might be a

reference for future investigations, highlights the strong need for coordinated surveillance activities among Europe and Africa, and underline the need of *ad hoc* trials to confirm the association between point mutations and increased pathogenicity in infected hosts.

6.2 Keywords

West Nile virus lineage 1; neurological disease; phylogeny; phylogeography; evolution; Italy

6.3 Introduction

West Nile virus (WNV) is an arthropod-borne *Flavivirus* member of the Japanese encephalitis virus complex within the *Flaviviridae* family. In nature, it is maintained by several species of birds and competent mosquitoes mainly belonging to the *Culex* species. The former generally act as amplifying hosts while the latter as vectors [1,2]. Humans and other animals are considered incidental dead-end hosts. In humans, most of the infections are mild (flu-like symptoms) or asymptomatic (West Nile fever, WNF) [3]. In some cases (1%), however, especially in old or immunocompromised people, they can cause severe and sometimes fatal neurological disease (WNND) [3].

Since its first identification in Uganda in 1937 [4], WNV has progressively spread to Europe, America, Asia, and Oceania, becoming the most widespread arbovirus in the world [5]. Eight phylogenetic lineages are currently known - Lineage 1 (L1) to Lineage 8 (L8). Of them, WNV L1 and L2 are by far the most widespread and pathogenic. They are often associated with severe outbreaks in humans, horses and birds [6]. Phylogenetic analyses suggest that WNV L1 was introduced in Europe in the middle 1950s [7], and in North America in 1999 [8], from Morocco and the Middle East (Israel), respectively [9,10]. These and many of the subsequent introductions have been probably due to long distance migratory birds [11,12]. As a result, WNV L1 circulation has been observed in several European countries. Two major clades have been characterised: i) the Western Mediterranean clade,

with strains first identified in Italy (1998) and France (2000), and ii) the Eastern European clade which includes strains responsible for the outbreaks in Romania (1996) and Russia (1999) [10,13,14]. The 1998 WNV Italian strain was reported in the Tuscany region. It was able to cause severe neurological disease and deaths in horses, but no cases were recorded in humans [15]. A re-emergence of WNV L1 strains belonging to the Western Mediterranean clade were reported 10 years later in North-Eastern Italian regions (Emilia-Romagna, Veneto, and Lombardy). They were responsible for severe illness in humans and horses. Numerous birds and mosquitoes were also found infected with WNV L1 [16], (https://westnile.izs.it/j6_wnd/wndItalia). In 2010, circulation of WNV L1 was also observed in Southern Italy and outbreaks were reported in Sardinia in 2011 [17,18]. In the same year, WNV L2 circulation was detected for the first time in the Italian territory. It soon became the most prevalent lineage circulating in the country [19], meanwhile WNV L1 has been more and more sporadically reported. Evidence of its circulation was, however, observed in North-Eastern regions (2012–2014, 2017), Sardinia (2015–2016), and Campania (2020) [14].

In 2021, unexpectedly, a new WNV L1 incursion re-occurred in the Northern part of Italy (https://westnile.izs.it/j6_wnd/docBolletItaPeriodico?annoDocumento=2021). The virus re-emerged in the same area in 2022, co-circulating with WNV L2 and causing nearly 300 WNND cases and 37 deaths in humans. WNV positivities were also reported in 45 horses and 349 wild and target birds. Similarly, numerous mosquito pools were found infected (https://westnile.izs.it/j6_wnd/docBolletItaPeriodico?annoDocumento=2022). The Veneto region was the major hotspot area for viral circulation, with 142 human WNND and 17 deaths [20], (https://westnile.izs.it/j6_wnd/docBolletItaPeriodico?annoDocumento=2022).

The reappearance of WNV L1 in Italy, the pathogenic differences of the various circulating strains, the increasing incidence of WNND cases in humans, together with the gap of knowledge of several

aspects of WNV biology, ecology and genetics and the lack of safe vaccines and healing therapies, provide the motivation to improve research and deep the understanding of the genetic features of the WNV L1 strains in Italy. To this end, in this study we have deployed a novel phylogenetic and phylogeographic analysis of WNV L1 strains circulating in the country since 1998, displaying the dynamics of the viral circulation in the Italian territory.

6.4 Materials and Methods

6.4.1 Sample collection

WNV L1 positive samples were collected in Italy between 2008 and 2022 within the national surveillance plan, coordinated by the Ministry of Health, the Istituto Superiore di Sanità (epidemiology and national reference laboratory, human) and the Istituto Zooprofilattico of Abruzzo and Molise (IZS-Teramo) (epidemiology and national reference laboratory, animal/entomology) (https://westnile.izs.it/j6_wnd/home, https://www.epicentro.iss.it/westnile/).

6.4.2 Sample analysis

6.4.2.1 Tissue homogenization, RNA extraction, Real-time RT-PCR

At IZS-Teramo, pools of mosquitoes and bird organs (heart, kidney, spleen, and brain), collected from either residential or wild birds, were homogenised in phosphate-buffered saline (PBS) with antibiotics. Viral RNA was extracted by using the MagMAX CORE Nucleic Acid Purification KIT (Applied Biosystem, Thermo Fisher Scientific, Life Technologies Corporation, TX, USA) and amplified by multiplex real-time reverse transcription polymerase chain reactions (qRT-PCR) to detect WNV L1, WNV L2, and Usutu virus, by using the Superscript III Platinum OneStep qRT-PCR System (Invitrogen) as previously described [21].

6.4.3 Sequence data preparation and retrieval

Purified nucleic acids were sequenced by Next Generation Sequencing, as described in detail in [22]. Thirty WNV L1 consensus sequences were obtained at IZS-Teramo using iVar v 1.3.1 [23] after trimmed reads were mapped to the WNV L1 reference sequence FJ483548 (Italy, 2008) by using Snippy (https://github.com/tseemann/snippy). A map of WNV L1 sequence geo-localization sites is shown in Fig 6.1.

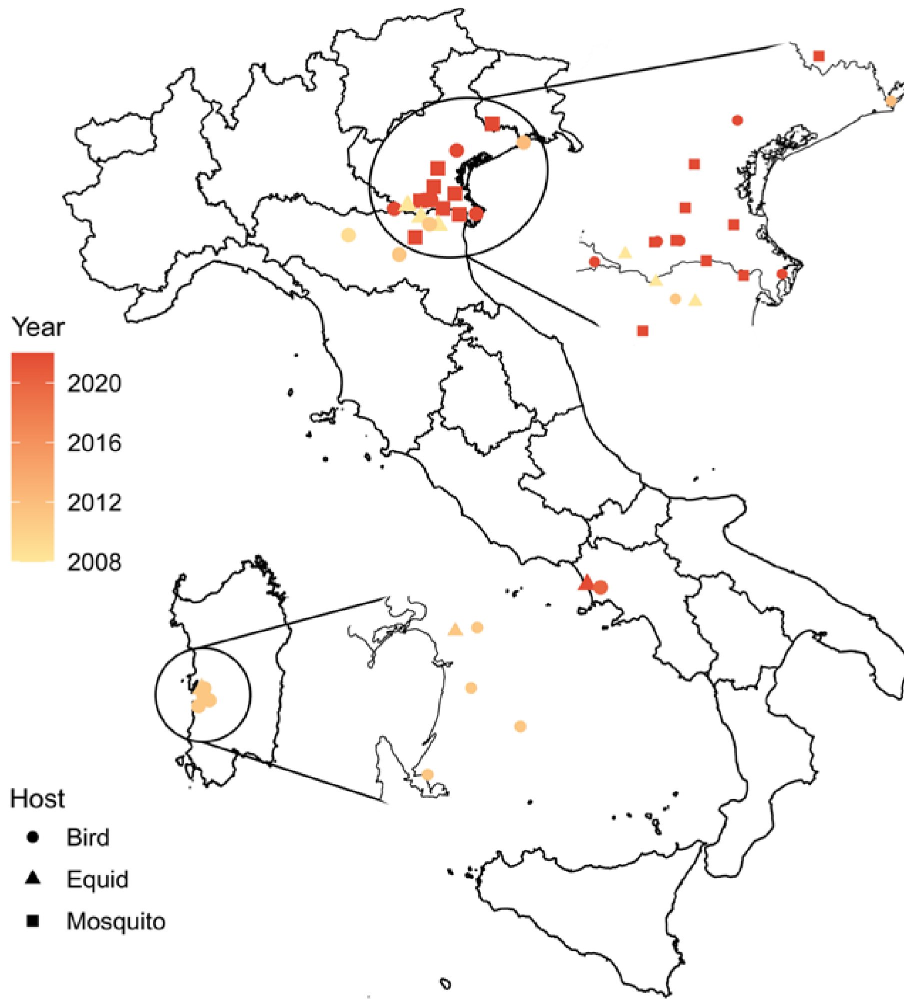


Figure 6.1 Geo-localization of West Nile virus Lineage 1 sequenced sample collection sites.

The sampling date is indicated by a colour scale. The shape indicates the host from which the sample was extracted.

Metadata for the IZS-Teramo newly generated sequences were obtained from the Laboratory Information Managements Systems (SILAB) at IZS-Teramo (https://www.izs.it/IZS/Engine/RAServePG.php/P/257610010719/L/1). A world-wide dataset of 130 WNV L1 sequences have been downloaded from the Supplementary Materials of [24], including

sequences obtained from NCBI on 01/03/2022. By using a custom R script for automatic sequence retrieval, a new search has been conducted on NCBI the 06/11/2022 and 28 newly published WNV L1 sequences >= 200 nt have been downloaded.

6.4.4 Sequence data cleaning and formatting

All sequences were quality-filtered, as described in [24]. After this step, 2 sequences (OP734273, OU953898.1) were removed, as they contained a percentage of ambiguous bases above 10%. A total of 186 WNV L1 genomes, 55 of which coming from Italy, were selected for further analysis. A table of sequence curated metadata can be found in S6.1 Table.

6.4.5 Alignment, recombination detection and model selection

All the datasets were aligned individually using MAFFTv7 (https://mafft.cbrc.jp/alignment/server/) with the "--auto" option and trimmed using trimAlv2 [25]. The presence of recombinant sequences in the final dataset was checked by running the RDP4 program [26], with default options. The sequence AJ965626.2 from Portugal was indicated as suspect recombinant and was therefore excluded from the alignment. Model selection was carried out on all datasets using Modelfinder [27], implemented in IQTREE2 [28], using parameters "-T AUTO -m TESTONLY". The best-fit model for both the worldwide and the Bayesian down-sampled datasets was GTR+F+I+G4, chosen according to both Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC).

6.4.6 Maximum-likelihood phylogenies

A maximum likelihood phylogeny of the dataset including the 185 WNV L1 sequences was reconstructed by using RAxMLv8.2.12 [29], with commands "-p 1989 -m GTRGAMMAI -x 2483 -

100 -f a -T 20”. Clades were annotated using the resulting topology when having bootstrap supports ≥ 90.

6.4.7 *Molecular clock and phylogeographic analysis*

We used the results obtained in the maximum-likelihood analysis to subset our dataset for molecular clock analysis. In particular, we selected the highly supported group which includes all Italian genomes, highlighted in Fig 6.2 and part of the C2 cluster, and downsampled it by selecting sequences according to the observed genetic divergence (as many sequences were almost identical), the year and the location, choosing 4 sequences for each subclade. When the exact position was not available, we approximated the location using the coordinates of the municipality from which the sample was collected (S6.2 Table). Phylogeography was reconstructed by using continuous traits (latitudinal and longitudinal coordinates for each sequence) in BEASTv1.10.4 [30]. We divided the analysis into two different partitions (one for sequence data and the other for continuous coordinates) and used the Cauchy RRW substitution model for our location partition, with bivariate traits representing latitude and longitude, adding random jitter to the tips (jitter window size: 0.01). For the location partition, we selected the option to reconstruct states at all ancestors. We employed an uncorrelated relaxed clock with a log-normal distribution and a Coalescent Bayesian Skyline tree prior. We set an MCMC length of $500*10^6$ generations, sampling every 50000 steps. Convergence was assessed using Tracer v1.7.1 [31], making sure that all parameters were above a significance threshold of ESS (>200). A table of sequence metadata used for the phylogeographic analysis can be found in S6.2 Table. Phylogenetic trees and phylogeographic diffusion patterns were represented using custom R scripts, employing the ggtree [32] and SERAPHIM [33] packages, respectively.

6.4.8 *Full-Length Polyprotein Sequencing*

Using Ugene software v44.0 (https://ugene.net/download.html), a sequence comparison between the amino acid residues of the group of strains included in the phylogeographic analysis was performed. In particular, we compared the 2021-22 group of Italian sequences and the strains: i) OP850023, Italy, Campania region, 2022; ii) MW627239, Italy, Campania region, 2020; iii) Italy, 2008 group; iv) Italy, 2011 group; v) AF404757, Italy, Tuscany region, 1998; vi) MT863559, France, 2015; vii) OU953897, Spain, 2020; viii) JF719069, Spain, 2010; vi) AY701413, Morocco, 2003; x) AJ965628, Portugal, 2004; and xi) DQ786573, France, 2004. We also included in the sequence comparison the strain NC_009942/1999 because, although it is not part of the major Western Mediterranean cluster here analysed, it is representative of all virulent L1 strains from the USA.

6.5 Results

6.5.1 Genome sequence analysis

Illumina sequencing produced an average total number of 5,096,291 trimmed reads. The numbers of mapped reads (151 nucleotides [nt] in length) ranged from 159,703 to 1,333,187, with coverage depth ranging from 2,101.31× to 6,381.67×. The mean consensus sequence length was 10,966bp. Consensus sequences were characterised by 31 WNV L1 genomes. They were obtained from samples collected in Italy between 2008 and 2022, and were uploaded to BankIT NCBI (https://submit.ncbi.nlm.nih.gov/about/bankit/) on the 18th of May 2021, the 26th of October 2022, and the 15th of November 2022.

6.5.2 Phylogenetic Tree Inferred with Maximum-Likelihood Analysis

Maximum-likelihood phylogenetic tree of the WNV L1 genomes analysed in this study showed the presence of 7 well supported clusters. All Italian genomes were included in cluster 2, together with a

sequence of a strain from Israel (Genbank HM152775) and one from Morocco (Genbank AY701412), as shown in Fig 6.2.

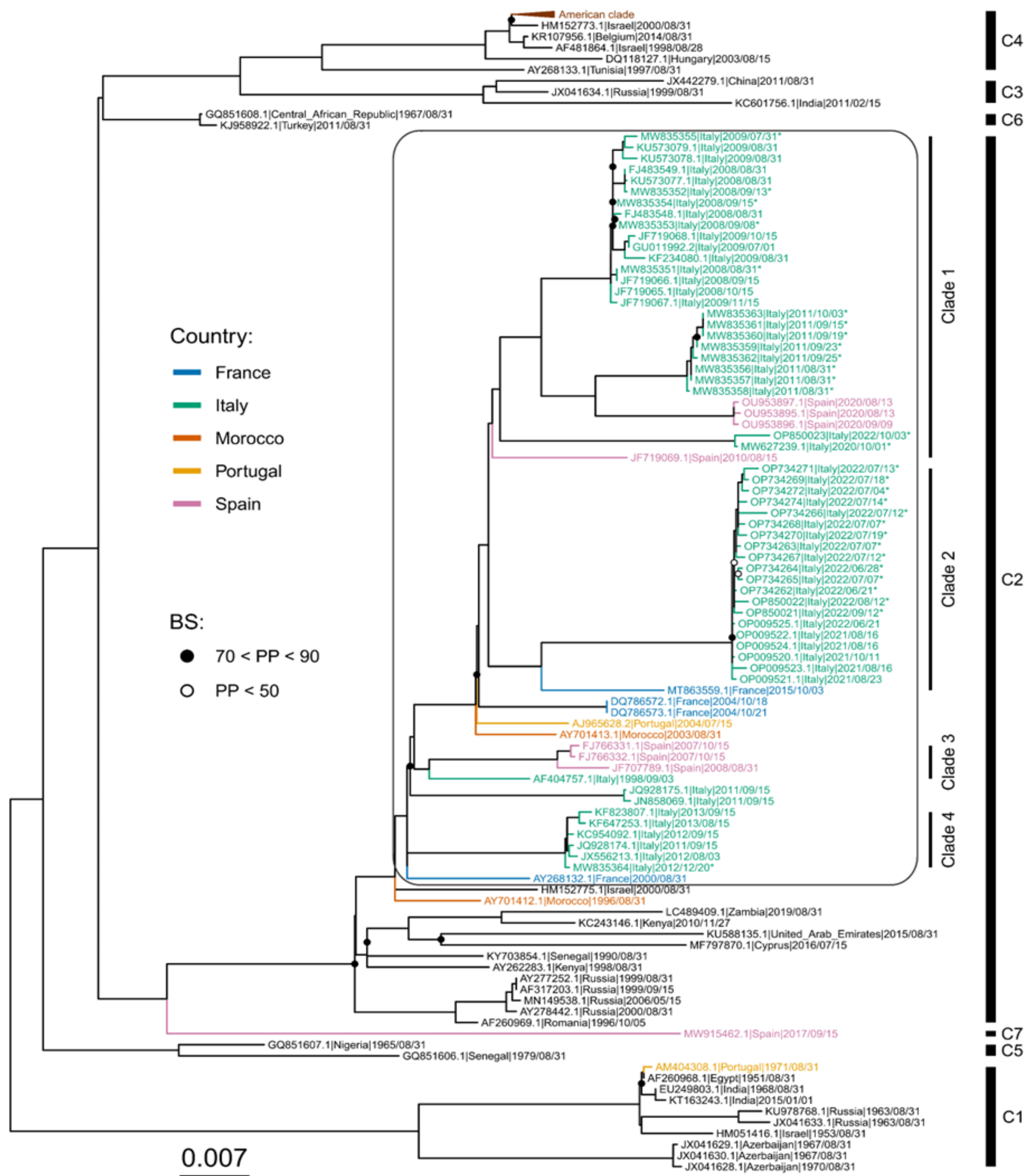


Figure 6.2 Maximum-likelihood tree of West Nile virus Lineage 1 sequences.

Seven major clusters (C1-C7, bar on the right) can be identified. Mediterranean countries that are part of a major cluster which includes all Italian genomes (highlighted in a black square with rounded corners) are indicated by different colours. Inside this group, 4 Italian clades (Clade 1-4) can be defined based on the

topology of the tree. Bootstrap support values (BS) > 90 are not shown; bootstrap support values between 70 and 90 are represented by a full black circle; low bootstrap supports (BS < 50) are represented with a white circle. Sequences obtained in this study are indicated by the "*" symbol. A scale bar representing substitutions per site is shown at the bottom-left part of the figure.

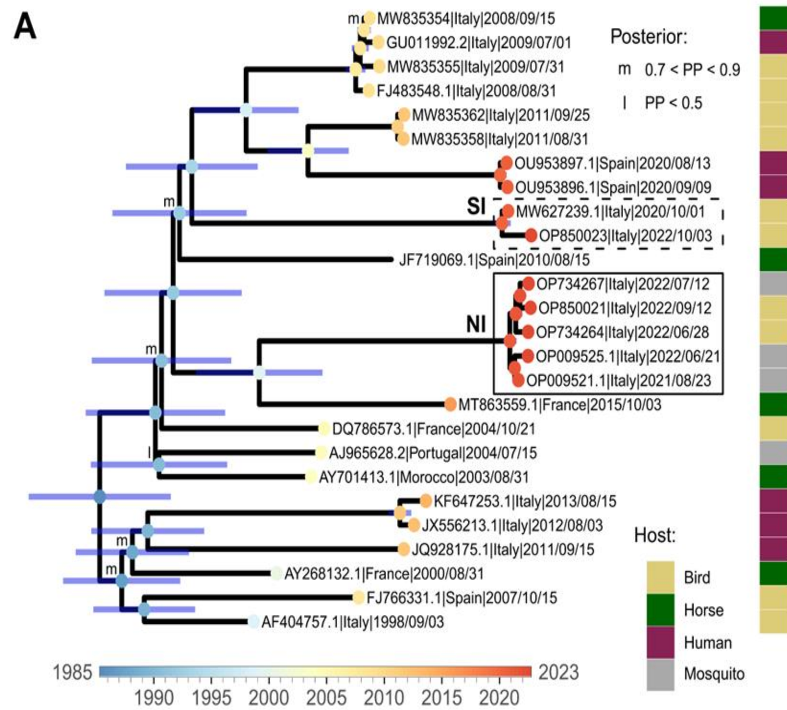
Sequences from Italy can be divided in 4 highly supported groups (clades 1-4, Fig 6.2). Clade 1 includes the 2008-09 and the 2011 groups of Italian WNV L1 sequences. They are genetically similar to the Spanish sequences of 2020 (bootstrap support = 100), and all are closely related to the 2020 (GenBank MW627239) and 2022 (GenBank OP850023) Campanian sequences (bootstrap support > 80). Clade 2 includes the 2022 WNV L1 Italian strains, originating from Veneto, Emilia-Romagna, Friuli-Venezia Giulia, and Lombardy regions (S6.1 Table). They appear to be in the same monophyletic group of the sequences obtained in the Veneto region in 2021, and closely related to the WNV L1 sequence (GenBank MT863559) obtained from a horse with neurological signs in Southern France in 2015 (bootstrap support = 100). Clade 3 includes Spanish sequences and the first WNV L1 genome ever sampled in Italy (Genbank AF404757), while clade 4 comprises sequences sampled between 2011 and 2013 near Livenza. The 2 remaining Italian genomes (JN858069/Italy/2011/AN-1 and JQ928175/Italy/2011/Piave), which group together, can't be placed confidently in the tree (the signal is too low to place them with sufficient precision).

The strains DQ786572/France/2004 and DQ786573/France/2004 appear to be at the root of clade 1 and 2 (bootstrap support = 68).

The two strains collected in the Campania region in 2020 and 2022 appear to be very closely related (bootstrap support = 100), and distant from the 2021-22 North-Eastern Italian strains.

6.5.3 Phylogeographic analysis of the WNV L1 Western Mediterranean clade

We reconstructed a phylogeographic diffusion pattern for a subsample of the cluster 2 (highlighted in Fig 6.2), including all Italian clades. The common ancestor of the sequences was most likely located in the area stretching from the North-west of Italy to the South of France, with a possible first introduction occurring around 1985 on the border between Italy and France (Fig 6.3).



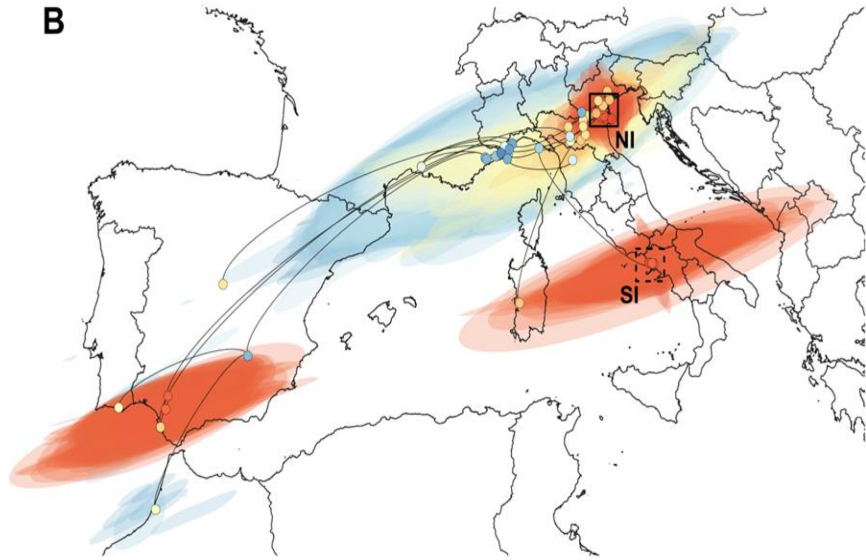


Figure 6.3 Phylogeographic analysis of the WNV L1 Western Mediterranean clade.

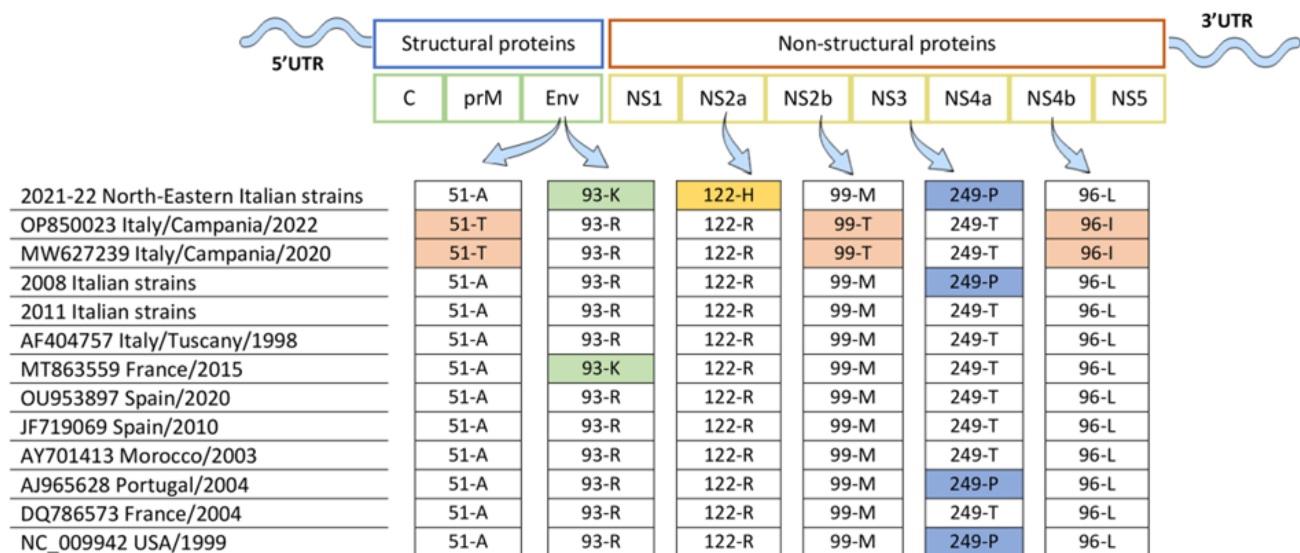
A) Molecular clock of the clade including all Italian sequences. Nodes with posterior probability (PP) below 0.9 are indicated with the “m” letter (medium) close to the node when between 0.7 and 0.9, while the “l” letter (low) represents nodes with a PP < 0.5. The bar on the right indicates the host from which the genome was isolated (mosquito, human, horse or bird). The time scale on the bottom indicates, with a colour scale, the sampling time (blue: old; red: recent) of the genomes (see tips) or the median estimated age of their ancestors

(see nodes). Genomes from the recent outbreaks in Southern Italy (SI) and Northern Italy (NI) are highlighted by a dotted square and a full square, respectively. **B)** Phylogeographic diffusion pattern in continuous scale of the virus in the Mediterranean basin. Genome sampling sites and reconstructed locations are plotted together on a map, showing the connections among all genomes and their inferred ancestors. Again, the same colour scale used in A) indicates the sampling time of the genomes or the median estimated age of their ancestors. The coloured areas in the figure represent the 95% HPDs of the locations and times inferred by the model.

In the following years, the virus moved towards east (Italy) and south-west (Spain). Our phylogeographic analysis also indicates a clear genetic flow connecting Morocco, Spain, France and Italy (Fig 6.3).

6.5.4 Amino acid sequence analysis

Pairwise alignment shows the presence of a new conservative amino acid point mutation (R122H) in the NS2A region of all 2021-22 Italian WNV L1 genomes. This mutation is not present on the strain OP850023 isolated in 2022 in the Campania region (Fig 6.4).



2021-22 North-Eastern Italian strains	51-A	93-K	122-H	99-M	249-P	96-L
OP850023 Italy/Campania/2022	51-T	93-R	122-R	99-T	249-T	96-I
MW627239 Italy/Campania/2020	51-T	93-R	122-R	99-T	249-T	96-I
2008 Italian strains	51-A	93-R	122-R	99-M	249-P	96-L
2011 Italian strains	51-A	93-R	122-R	99-M	249-T	96-L
AF404757 Italy/Tuscany/1998	51-A	93-R	122-R	99-M	249-T	96-L
MT863559 France/2015	51-A	93-K	122-R	99-M	249-T	96-L
OU953897 Spain/2020	51-A	93-R	122-R	99-M	249-T	96-L
JF719069 Spain/2010	51-A	93-R	122-R	99-M	249-T	96-L
AY701413 Morocco/2003	51-A	93-R	122-R	99-M	249-T	96-L
AJ965628 Portugal/2004	51-A	93-R	122-R	99-M	249-P	96-L
DQ786573 France/2004	51-A	93-R	122-R	99-M	249-T	96-L
NC_009942 USA/1999	51-A	93-R	122-R	99-M	249-P	96-L

Figure 6.4 Amino acid sequence analysis.

Amino acid sequence comparison between West Nile virus lineage 1 Italian strains and other Western-Mediterranean strains included in Cluster 2, other than the 1999 NC_009942 strain representative for the USA genome sequence group included in Cluster 4, is displayed. C = Capsid protein; prM = pre-membrane protein; M = Membrane protein; NS = non-structural proteins. Common point mutations (51-T, 99-T, 96-I) detected in the two Campanian strains OP850023/2022 and MW627239/2022 are displayed in pink; the point mutation 93-K of the Envelope protein detected in the 2021-22 Northern-Eastern Italian strains and in the 2015 French strain MT863559 is displayed in light green; the 122H amino acid substitution of the NS2a protein detected in the 2021-22 Northern-Eastern Italian strains is displayed in yellow; the 249-P point mutation is displayed in light blue.

Three conservative amino acid changes are observed in the E (A51T), NS2B (M99T), and NS4B (L96I) proteins of the OP850023/Campania/2022 and MW627239/Campania/2020 strains, but not in the other strains. The NS3T249P point mutation is also found in the recent 2021-22 Italian genomes,

in the group of 2008 Italian sequences, in the 2004 strain from Portugal, and in the 1999 American sequence. More details about the amino acid sequence comparison are shown in S6.3 Table.

6.6 Discussion

WNV is the most widespread mosquito-borne pathogen in the world [34]. Currently, it is seriously affecting many European countries [35].

In Italy, WNV L2 has been the most prevalent circulating lineage in the last decade. WNV L1 strains instead have been circulated sporadically, with only few evidences reported in North-Eastern regions (2012–2014, 2017), Sardinia (2015–2016), and Campania (2020) [14], (IZS-Teramo Annual Epidemiological Bulletins, https://westnile.izs.it/j6_wnd/wndItalia, https://westnile.izs.it/j6_wnd/wndItaliaPeriodici). This was the reason why health authorities and scientists involved in the surveillance and control were greatly surprised when it reappeared in the Italian territory in the 2021-22 epidemic seasons, even more as its emergence coincided with an increased incidence of severe WNND in humans [20,36]. This study investigated the possible origin and evolution of the WNV L1 strains circulating in Italy providing insights into the viral circulation dynamics of WNV L1 in the country since 1998. Two different and complementary approaches have been used: the phylogenetic and phylogeographic approaches.

The Bayesian and maximum-likelihood trees reflect the results obtained by other authors [37] which also observed the presence of seven well supported clusters (Fig 6.2). Of the seven identified clusters, cluster 2 contains most of the European strains included in this study. Since the 1996 Moroccan (Genbank AY701412) and the 2000 Israeli (Genbank HM152775) sequences are also in this cluster, it is likely that European and the Israeli WNV L1 strains have had a Northern African (Morocco) origin, as previously suggested [38]. Inside cluster 2, it is also worth noting the close relationship

between the 1998 Italian strain and the 2007-08 group of Spanish sequences (bootstrap support = 100) (clade 3). This evolutionary line if, on the one hand, suggests the existence of a corridor between Italy and Spain permeable to the circulation of WNV L1, on the other hand, considering the 9 year silence period recorded between the 2 epidemics, it supports the hypothesis that in the Western Mediterranean area WNV can circulate unnoticed for long periods [22,39,40]. This latter assumption questions the belief that old strains of clade 3 are not anymore circulating despite the date of their last detection.

The existence of a corridor connecting Italy and Spain is also evident when examining the sequences grouped in clade 1, in which the close genetic relationship existing between the 2008-09 and the 2011 Italian and 2020 Southern Spain strains is very clear [41].

Concerning the 2008-09 Italian group of strains, they re-emerged in Italy after 10 years of silence [40,42]. Unlike the 1998, the 2008 incursion involved a larger area (3 regions and 8 provinces) and caused disease not only in horses, but also in humans. Birds and mosquitoes were found infected too [16,40,43]. The virus later spread to Southern Italy (2010) and Sardinia in 2011 [17,18]. Considering the possibility of unnoticed circulation of WNV in the area, it is not easy to figure out whether this group of Italian sequences derives from a new re-introductory event or is just the re-emergence of a strain already circulating in the past. Previous studies seem to lean towards the latter hypothesis [42]. Nevertheless, our phylogenetic tree shows a close genetic relationship between the 2003 Moroccan strain (AY701413/2003) and other European strains (clade 2, clade 3), suggesting a possible new re-introductory event from Morocco to Italy, through Portugal, Spain, and France (Figs 6.2 and 6.3) [44]. The Campanian strains isolated in 2020 (GenBank MW627239) and 2022 (GenBank OP850023) are also in the clade 1. They are genetically similar to the 2008-11 group of Italian sequences, as well as to the sequence from Spain (Genbank JF719069). It is therefore difficult to determine their origin.

Much easier, instead, is establishing the evolutionary line of clade 2, including the 2021-22 North-Eastern Italian strains and the WNV L1 sequence MT863559, a strain isolated from the Natural reserve of Camargue, in Southern France in 2015 [10]. Their close relatedness sustains the hypothesis of a possible new re-introduction to Northern Italy from Southern France. The existence of a corridor between France and Italy is also suggested by the two 2004 French strains DQ786573 and DQ786573, closely related and sister group (even if with low bootstrap support = 68) of the 2 major Italian clades 1 and 2 described in our phylogenetic tree.

More and much clearer information on the shared ancestry assumed using phylogenetic methods has been obtained when using the phylogeographic reconstructions of WNV L1 diffusion, which provided valuable insight on the WNV L1 dynamics in a geographic and temporal context. According to our analysis, the first European WNV introduction dates back around 1985 (median= 1985.31; HPD 95%: 1978.66, 1990.99) in an area on the border between North-west of Italy and South of France, where an initial and undetected WNV L1 circulation seems to have occurred (Fig 6.3). All strains included in this analysis seem to have originated from this area. The virus has then probably spread eastwards (Italy) and South-westwards (Spain), touching Italy, France, Spain, Portugal, and Morocco (see Results and Fig 6.3). Our analysis supports the existence of a constant circulation in Europe, with possible new re-introductory events from Europe to Africa, although this scenario has to be taken with care, considering the low sample size of Moroccan sequences, which could affect the inferred directionality of the introductory events among countries.

In the European continent, the great abundance of susceptible hosts and vectors already existing in the Western Mediterranean area have indeed facilitated the rapid spread of WNV to the continent despite the diverse landscapes that it had to pass through [45,46]. While the first introduction of WNV L1 in Europe can be attributed to long distance migratory birds along their Western African migration routes

through Northern-western African countries, as Senegal and Morocco [39,47,48], the virus likely moved around the Western Mediterranean countries through short distance migratory birds in their shift between breeding grounds and overwintering quarters [22,39,44,49,50]. This could explain why clades 1-3 group together (Fig 6.2).

As demonstrated by Pesko and Ebel [51], changes to the WNV genome could also affect its pathogenesis. In many epidemics, including those occurring in Italy, there were some WNV L1 strains capable of causing severe neurological cases in humans, horses and birds, some others only in humans and still others only in horses [52],(https://westnile.izs.it/j6_wnd/home). Our pairwise alignment evidenced several point mutations, which might be referable to increased virulence (Fig 6.4 and S6.3 Table) [20]. This could be the case of the mutation observed in the NS2AR122H region of the 2021-22 strains from North-Eastern Italy, which were responsible for numerous severe WNDD cases in humans and horses (https://westnile.izs.it/j6_wnd/home). The ecological and climatic Italian conditions of the 2021-22 seasons might have created a favourable environment for a strong viral circulation and many replication events, and, in turn, the appearance of new genetic variants among the group of Italian strains [20]. *Ad hoc* trials with the NS2AR122H motif in mice and/or birds are however required to confirm this hypothesis. These studies might help in the understanding of the mechanisms underlying WNV clinical signs in humans, giving a possible explanation on the new WNV L1 North-Eastern Italian region's pathogenic phenotype.

Interestingly, the genome sequence OP850023, obtained from a horse in the Campania region in October 2022, is not included in the group of North-Eastern Italian sequences, although coming from the same epidemic season. It is closely related (posterior probability = 1) to the sequence MW627239, obtained also in the Campania region from a Northern goshawk 2 years before (October 2020) [44]. They do not carry the NS2AR122H point mutation reported in all 2021-22 North-Eastern strains but

show three common new amino acid substitutions (EA51T, NS2BM99T, and NS4BL96I) (Fig 6.4 and S6.3 Table). As previously evidenced, it is not simple to assess the exact origin of the Campanian introduction. If our Maximum Likelihood tree placed both strains close to the 2008-11 group of Italian sequences and to the 2010 Spanish sequence (Genbank JF719069) (clade 1), making a re-introductory event from Italy (Northern Italy/2008, Sardinia/2011) or Spain possible, our phylogeographic analysis seems to support the Italian introduction hypothesis (Fig 6.3). Anyway, it is likely that once introduced in Campania, these strains might have invaded an available ecological niche, establishing an intra-endemic circulation [44] and accumulating mutations in the newly colonised environment.

Our study provided information on the WNV L1 spatial and temporal dynamics in Italy in the last 24 years and identifies the presence of 2 diverse strains currently circulating in the country (one in the North-East, and one circulating intra-regionally in the Campania region). It evidenced how the different viral circulation observed in the various regions following introduction is strictly dependent on the presence of susceptible vectors and hosts and on the favourable climatic conditions [22,39,40]. Finally, it evoked the existence of a constant connection between Mediterranean countries, and, in particular, between Italy, Southern France, Southern Spain, Southern Portugal, and Morocco, with high risk for WNV to spread into new regions. These results also stress the importance to further intensify surveillance on WNV in the Mediterranean countries and, in particular, in areas where the virus might find favourable conditions for its endemic circulation, either in wetlands inhabited by birds and mosquitoes (Southern France and Spain) [41,53], or in areas where the circulation might seem reduced or absent. This will contribute to build a more consistent and homogeneous European dataset that could help clarify uncertainties related to the WNV dynamics.

6.7 Conclusion

In this study, the 31 new Italian genome sequences obtained by the IZS-Teramo from 2008 to 2022 are used to uncover the phylogenetic and phylogeographic relationships of WNV L1 Italian strains with other strains circulating worldwide. We provide evidence that WNV L1 spreads through an ideal line which connects Morocco, Spain, Southern France and Northern Italy, with further diffusion between diverse regions of the Italian territory, and possible events of endemisation. These results help clarify the WNV L1 dynamics in Europe, provide a new consistent dataset that can be used as reference data for future WNV investigations, and highlight the strong need of constant and coordinated surveillance activities among European and African countries.

6.8 Supporting information

Supporting information can be found at https://doi.org/10.6084/m9.figshare.21947681.

S6.1 Table. WNV Lineage 1 genome sequence curated metadata. Metadata of worldwide genome sequences used in this study to perform the Maximum-Likelihood Analysis are shown. Sequences obtained by IZS-Teramo are indicated by the "*" symbol, next to the Accession number; NA = Not available

S6.2 Table. Sequence metadata used for the phylogeographic analysis. Curated metadata of genome sequences used to perform phylogeographic analysis; NA = not available

S6.3 Table. Amino acid pairwise alignment. Amino acid sequence comparison of most strains included in Clade 1, 2, 3 of Cluster 2 and of the strain NC_009942/1999 coming from the USA, included in cluster 4. C = Capsid protein; prM = pre-Membrane protein; M = Membrane protein; NS = non-structural proteins. Protein amino-acid coordinates: C: 1-105; PreM: 124 – 215; M: 216 – 290;

E: 291-791; NS1: 792-1143; NS2A: 1114-1374; NS2B: 1375-1505; NS3: 1506-2124; NS4A: 2125-2250; NS4B: 2274-2528; NS5: 2529-3433. Common point mutations (51-T, 99-T, 96-I) detected in the two Campanian strains OP850023/2022 and MW627239/2022 are displayed in pink; the point mutation 93-K of the Envelope protein detected in the 2021-22 Northern-Eastern Italian strains and in the 2015 French strain MT863559 is displayed in light green; the 122H amino acid substitution of the NS2a protein detected in the 2021-22 Northern-Eastern Italian strains is displayed in yellow; the 249-P point mutation is displayed in light blue.

6.9 Author contribution

Conceptualization, G.M., F.M., and G.S.; methodology, G.M., A.S., and G.S.; validation, F.M., A.S., G.M., O.R.S., A.R., and G.S.; formal analysis, A.S., and G.M.; investigation, L.T., A.L., A.D.G., A.P., V.C., M.A., B.S., V.D.L., M.D.D., and G.M.; resources, G.S.; data curation, F.I., A.S., V.C., A.P., and G.M.; writing – original draft G.M., A.S., and G.S.; writing—review and editing, G.M., A.S., G.Mar., F.M., A.R., and G.S.; visualization, A.S., G.M., F.I., L.T., A.L., A.D.G., A.P., V.C., M.D.D., B.S., V.D.L., M.A., D.M., MG.P., G.Mar., R.R., F.M., N.S., A.R., O.R.S., and G.S.; supervision, O.R.S., F.M., A.R., and G.S.; project administration, G.S.; funding acquisition, G.S. All authors have read and agreed to the published version of the manuscript.

6.10 Data availability statement

All scripts used to perform this analysis are available at the https://github.com/andrea-silverj/WNV-L1_IT GitHub repository. Alignments and tree files can be found at https://doi.org/10.6084/m9.figshare.21940931.v1 and https://doi.org/10.6084/m9.figshare.21940898.v2.

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6.13 Conflict of interest

All the authors declare no conflict of interest. The contents of this publication are the sole responsibility of the authors and don't necessarily reflect the views of the European Commission.

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CHAPTER 7. WEST NILE VIRUS LINEAGE 2 OVERWINTERING IN ITALY

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(2022)

by

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7.1 Abstract

In January 2022, West Nile virus (WNV) lineage 2 (L2) was detected in an adult female goshawk rescued near Perugia in the region of Umbria (Italy). The animal showed neurological symptoms and died 15 days after its recovery in a wildlife rescue center. This was the second case of WNV infection recorded in birds in the Umbria region during the cold season, when mosquitoes, the main WNV vectors, are usually not active. According to the National Surveillance Plan, the Umbria region is included amongst the WNV low-risk areas. The necropsy evidenced generalized pallor of the mucous membranes, mild splenomegaly, and cerebral edema. WNV L2 was detected in the brain, heart, kidney, and spleen homogenate using specific RT-PCR. Subsequently, the extracted viral RNA was sequenced. A Bayesian phylogenetic analysis performed through a maximum-likelihood tree showed that the genome sequence clustered with the Italian strains within the European WNV strains among the central-southern European WNV L2 clade. These results, on the one hand, confirmed that the WNV L2 strains circulating in Italy are genetically stable and, on the other hand, evidenced a continuous WNV circulation in Italy throughout the year. In this report case, a bird-to bird WNV transmission was suggested to support the virus overwintering. The potential transmission through the oral route in a predatory bird may explain the relatively rapid spread of WNV, as well as other flaviviruses characterized by similar transmission patterns. However, rodent-to bird transmission or mosquito-to bird transmission cannot be excluded, and further research is needed to better understand WNV transmission routes during the winter season in Italy.

7.2 Keywords

West Nile virus; flavivirus; birds of prey; overwintering; bird-to bird transmission; rodent-to bird transmission; hybrid mosquitoes; surveillance

7.3 Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus, belonging to the family *Flaviviridae*, genus *Flavivirus* [1]. It is part of the Japanese encephalitis serocomplex, which includes other related viruses such as Usutu, Murray Valley encephalitis, Stratford, Alfui, Kunjin, and Saint Louis encephalitis [2]. WNV is transmitted in nature by vector-competent mosquitoes mainly belonging to the *Culex* genus [3]. Its transmission cycle involves several bird species and orders as main amplifier hosts and humans, equids, and other animals as incidental, dead-end hosts [3,4]. In humans, 80% of cases are generally asymptomatic, while in about 20% of cases, infection causes mild, flu-like symptoms known as West Nile fever (WNF). West Nile neuroinvasive disease (WNND) occurs in less than 1% of cases, reporting WNV meningitis, encephalitis, or poliomyelitis [2,5]. Among birds, several avian species can be infected by WNV, but corvids and raptors appear to be the most susceptible ones, showing neurological symptoms and deaths [5]. Once infected, horses usually do not have symptoms. In 20% of cases, they can develop clinical and neurological forms [6], which can lead to 30–50% deaths in unvaccinated animals [5]. The severity of symptoms has been often correlated to WNV genetic diversity [7]. Up to now, 8 lineages have been recognized. Among them, WNV lineage 1 (WNV L1) and lineage 2 (WNV L2) are by far the most widely spread and the most virulent, capable of causing numerous cases worldwide [2,8,9].

Nowadays, WNV represents a serious public health concern in Europe. In the last decades, WNV has expanded its geographical range. The number of West Nile disease (WND) cases have been increasing in animals and humans, especially in southern, central, and eastern Europe, where many countries have become endemic [10,11].

Italy is one of the European countries most affected by WNV circulation [12]. Since 2002, four years after the first incursion in the Tuscany region [13,14], the Italian Ministry of Health has implemented

a veterinary surveillance plan to monitor the viral introduction and circulation of WNV in the whole country. In Italy, the WNV circulation is currently monitored through an annually updated preparedness and response plan, aiming at limiting the risk of WNV transmission to humans either by mosquitoes or by substances of human origin. The current program, modulated on the basis of seasonality and local epidemiology, includes national integrated human, animal (equids and birds), and entomological surveillance (One Health Surveillance). Viral circulation is monitored from April to November by testing vector-competent mosquitoes, resident birds belonging to target species (*Pica pica*, *Corvus corone* cornix, and *Garrulus glandarius*) or sentinel chickens, wild birds found dead, horses showing nervous symptoms, and humans presenting neuroinvasive disease signs. On the basis of WNV occurrence and the eco-climatic characteristics of the territory, Italy is divided in three areas: (1) high-risk areas, where WNV is circulating or has circulated in at least one of the previous five years and where, therefore, episodes of infection have been repeatedly observed; (2) low-risk areas, where WNV has circulated sporadically in the past or has never circulated but whose eco-climatic characteristics are favorable for viral circulation; and (3) minimum-risk areas, where WNV has never circulated and where, given the eco-climatic characteristics of the territory, the probability of its circulation is considered as minimal (National Plan for Prevention, Surveillance, and Response to Arbovirus 2020–2025).

The Umbria region is classified as a low-risk area. WNV circulation has never been reported, at least up to 2019, when the death of a little grebe (Tachybaptus ruficollis subsp. ruficollis) was associated with WNV L2 infection [15].

In this report, a second clinical case associated with WNV L2 infection observed in a northern goshawk (*Accipiter gentilis*) in Umbria during the winter season is described.

7.4 Materials and Methods

7.4.1 Case Report

On 4 January 2022, a female adult northern goshawk was rescued in Torgiano, a municipality in the province of Perugia, Italy (Figure 7.1).

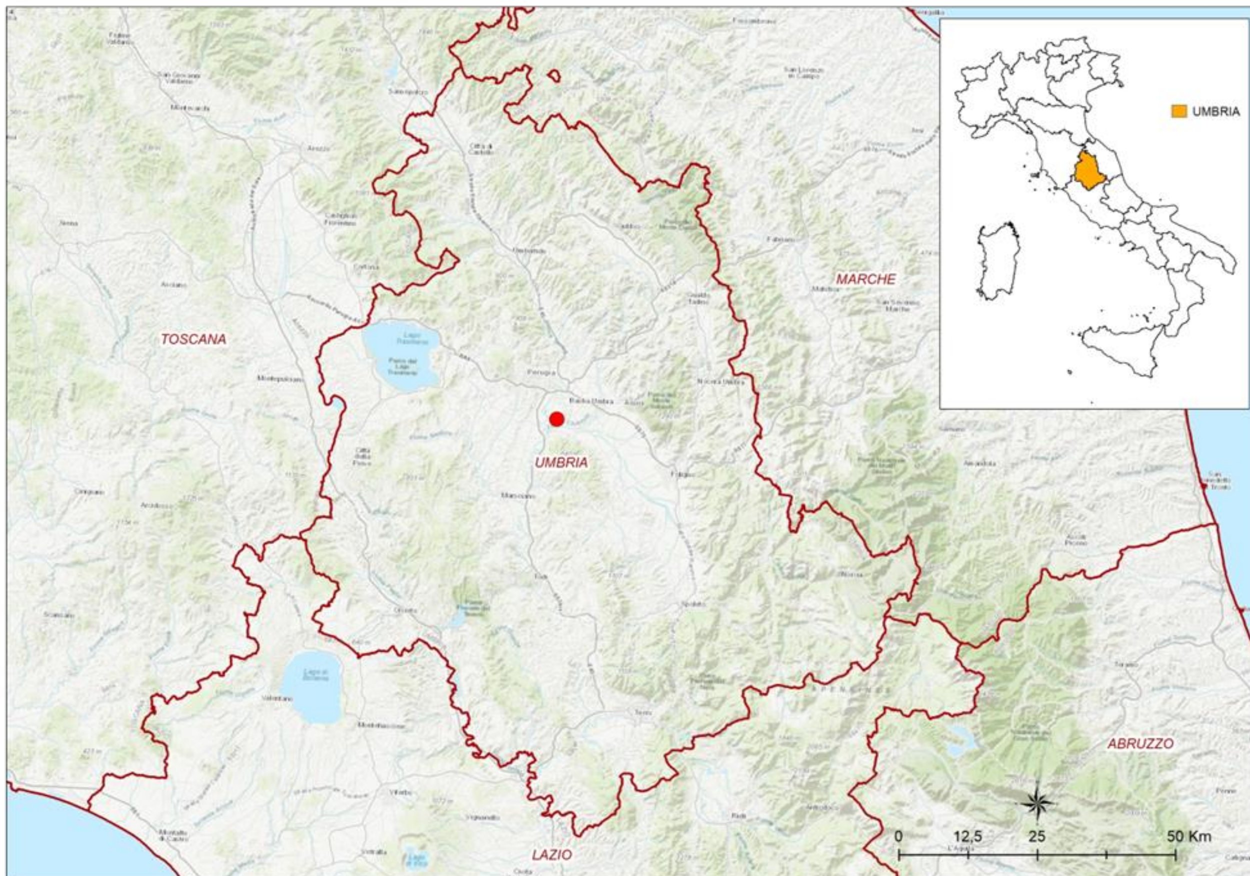

Figure 7.1 Map of geo-localization site.

The Northern goshawk was found in the Torgiano municipality (43.0893° N, 12.4410° E) in the Umbria region.

The bird was found on the roof of a private house while trying to defend itself from a mobbing attack by crows. The noise caused by the crows attacking the stunned goshawk attracted the homeowner, who recovered the bird and brought it to a wildlife rescue center (WRC). The northern goshawk

showed classical neurological symptoms, including stupor, blepharospasm, and an inability to maintain a normal posture. After an initial improvement of symptoms in which the bird restarted feeding on its own, the clinical signs suddenly worsened, and the bird died on 19 January 2022. The necropsy evidenced a good state of nutrition, generalized pallor of the mucous membranes, and mild splenomegaly. Cerebral edema was also observed. The picture and video of the WNV-infected northern goshawk are reported in Figure S7.1 and Video S7.1 (Supplementary Materials).

7.4.2 Laboratory Analyses

7.4.2.1 Real-Time PCR for WNV and USUV

The brain, heart, kidney, and spleen were collected, pooled, and homogenized in sterile phosphate-buffered saline (PBS). The viral RNA was extracted from 200 μL supernatant using Qiasymphony® DSP automatic instrumentation (Germantown, MD, USA) according to the manufacturer instructions. The extracted RNA was then tested using one-step quantitative reverse transcription polymerase chain reactions (qRT-PCRs) specific for USUV, all known lineages of West Nile virus, and West Nile virus L1 and L2 [16–18].

7.4.2.2 Illumina and Sanger Sequencing

A WNV-positive sample was selected for Illumina and Sanger sequencing. The total RNA was subjected to Turbo DNase treatment (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 20 min and then purified with an RNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA, USA). The purified RNA was used for the assessment of the sequence independent single primer amplification (SISPA) protocol [19,20]. In detail, a single-strand cDNA was obtained using reverse transcription (RT) in 20 μL reaction mixture with 5X SSIV buffer, 50 μM random hexamer FR26RV-N 50-GCCGGAGCTCTGCAGATATCNNNNNN-30, 10 mM dNTPs mix, 100 mM DTT, 200 units

SuperScript® IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA), and 40 U RNAse OUT RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer instructions. The reaction was incubated at 23 °C for 10 min, 50 °C for 50 min, and 80 °C for 10 min. To convert the single-stranded cDNA into double-stranded (ds) cDNA, 1 μL (2.5 U) 3′-5′ Klenow Polymerase (New England Biolabs, Ipswich, MA, USA) was directly added to the reaction. The incubation was carried out at 37 °C for 1 h and 75 °C for 10 min. Next, 5 μL of ds cDNA was amplified with a PCR master mix containing 5X Q5 reaction buffer, 10 mM dNTPs, 40 μM random primer FR20 Rv 50-GCCGGAGCTCTGCAGATATC-30, 0.01 U/μL Q5® High Fidelity DNA polymerase (NEB, New England Biolabs, Ipswich, MA, USA), and 5X Q5 High Enhancer. The reaction was incubated at 98 °C for 10 s, 65 °C for 30 s, 72 °C for 3 min, and 72 °C for 2 min. The PCR product was purified using Expin TM PCR SV (GeneAll Biotechnology CO., Seoul, Korea) and then quantified using a Qubit® DNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The sample was diluted to obtain a concentration of 100–500 ng, then used for library preparation with an Illumina DNA prep kit, and sequenced with a NextSeq 500 (Illumina Inc., San Diego, CA, USA) using a NextSeq 500/550 Mid Output Reagent Cartridge v2, 300 cycles, and standard 150 bp paired-end reads. After quality control and trimming with Trimmomatic v0.36 (Usadellab, Düsseldorf, Germany) [21] and FastQC tool v0.11.5 (Bioinformatics Group, Babraham Institute, Cambridge, UK) [22,23], reads were de novo assembled using SPADES v3.11.1 (Algorithmic Biology Lab, St Petersburg, Russia) [24]. The contigs obtained were analyzed with BLASTn to identify the best match reference. Mapping of the trimmed reads was then performed using the iVar computational tool [25] to obtain a consensus sequence. In order to close some large gaps, the WNV-positive sample was further sequenced using the Sanger method [26]. Briefly, the total RNA was extracted from the collected sample using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany) according to the manufacturer instructions and collected in 45

μL elution buffer prewarmed at 72 °C. The complete WNV-coding DNA sequences (cds) of the polyprotein precursor gene was amplified using 13 WNV primer pairs able to amplify 13 overlapping regions of the genome (the primer sequences are available upon request). Gel-based RT-PCR was performed using a Transcriptor One-Step RT-PCR kit (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) as described by the manufacturer instructions. The RT-PCR cycling conditions for the amplification were 50 °C for 15 min and 94 °C for 7 min, followed by 35 cycles of denaturation at 94 °C for 10 s, annealing at 57.5 °C for 30 s, and extension at 68 °C for 4 min and 30 s, followed by 1 extension cycle performed at 68 °C for 7 min. The gel-based RT-PCR amplicons were purified with a Qiaquick PCR Purification kit (Qiagen, Leipzig, Germany). The purified amplicons and the 13 WNV sequencing primers were sent to an external service, Eurofins Genomics (Eurofins Genomics, Germany GmbH, Anzinger Str. 7a, 85560 Ebersberg, Germany), to perform sequencing in both directions. The obtained sequences were analyzed with SeqScape v3.0 (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

7.4.2.3 Phylogenetic Analysis

A phylogenetic analysis was conducted including 62 WNV L2 genome sequences publicly available. Specifically, 57 complete and 5 partial genome sequences representative of different geographic regions and identified in different hosts were downloaded from Genbank. In addition, three sequences were added as outgroups: WNV L1 Italy 2020 (MW627239), WNV L1 France 2015 (MT863559), and Koutango virus (KOUTV) Senegal 2013 (EU082200). All 66 sequences were aligned using the MAFFT online alignment program (https://mafft.cbrc.jp/alignment/server/, accessed on: 15 April 2022) and curated using BioEdit v. 7.2.5.0 software (https://bioedit.software.informer.com/7.2/, Bioedit Company, Manchester, UK, accessed on: 15 April 2022). The WNV sequence alignment and the metadata of the WNV strains used for the present study are reported in File S7.1 and Table S7.1,

respectively (Supplementary Materials). Bayesian phylogenetic inference (BI) was performed using a Bayesian Evolutionary Analysis by Sampling Tree (BEAST) software package version 2.6.3 (http://www.beast2.org/, University of Auckland, Auckland 1142

New Zealand) [27,28]. In detail, using the interface program called Bayesian Evolutionary Analysis Utility (BEAUti) included in the BEAST package, the amino acid sequence alignment was uploaded by choosing a gamma-site model with a gamma category count of 4, as well as invariant sites model (GTR + Γ + I) [28]. A family of Bayesian Markov chain Monte Carlo (MCMC) algorithms with 10 independent MCMC runs with up to 100,000,000 generations was used to perform the inference. Using TreeAnnotator v.2.6.3 (https://www.beast2.org/treeannotator/), trees were summarized in a maximum-clade-credibility tree with common ancestor heights after a 10% burnin percentage [28]. Tracer v 1.7.1 (available at http://beast.bio.ed.ac.uk/Tracer, accessed on: 16 April 2022) was used to ensure convergence during the MCMC runs. Finally, the FigTree v2.6.3 program (http://tree.bio.ed.ac.uk/software/) allowed the estimation of a maximum-likelihood tree [29].

7.5 Results

The northern goshawk rescued in the Umbria region in January 2022 was positive for WNV L2 (Ct 25) and turned out negative for WNV L1 and USUV.

The Illumina sequencing run produced a total of 15,763,124 reads. BLASTn analysis was performed to identify the closest publicly available sequence in the GenBank database. The best match (99.49%) was with the West Nile virus isolate of Nea Santa-Greece-2010 (accession no. HQ537483), and this sequence was used to perform mapping with the iVar tool [25]. This analysis produced a consensus sequence with a horizontal coverage (HCov) of 57% and a mean vertical coverage of 575,535, probably due to the low quality of the RNA sample.

The large gaps were partially filled with Sanger sequencing data, and a consensus sequence of 11.056 nt in length was obtained (HCov 91%) and published in the NCBI database under acc. no ON032498 and the NCBI sequence name of 15935/22.

Phylogenetic analysis placed 15935/22 NCBI in the same cluster as the other central-southern European WNV L2 sequences [30] (highlighted in pink in Figure 7.2) with a posterior probability of 100%.

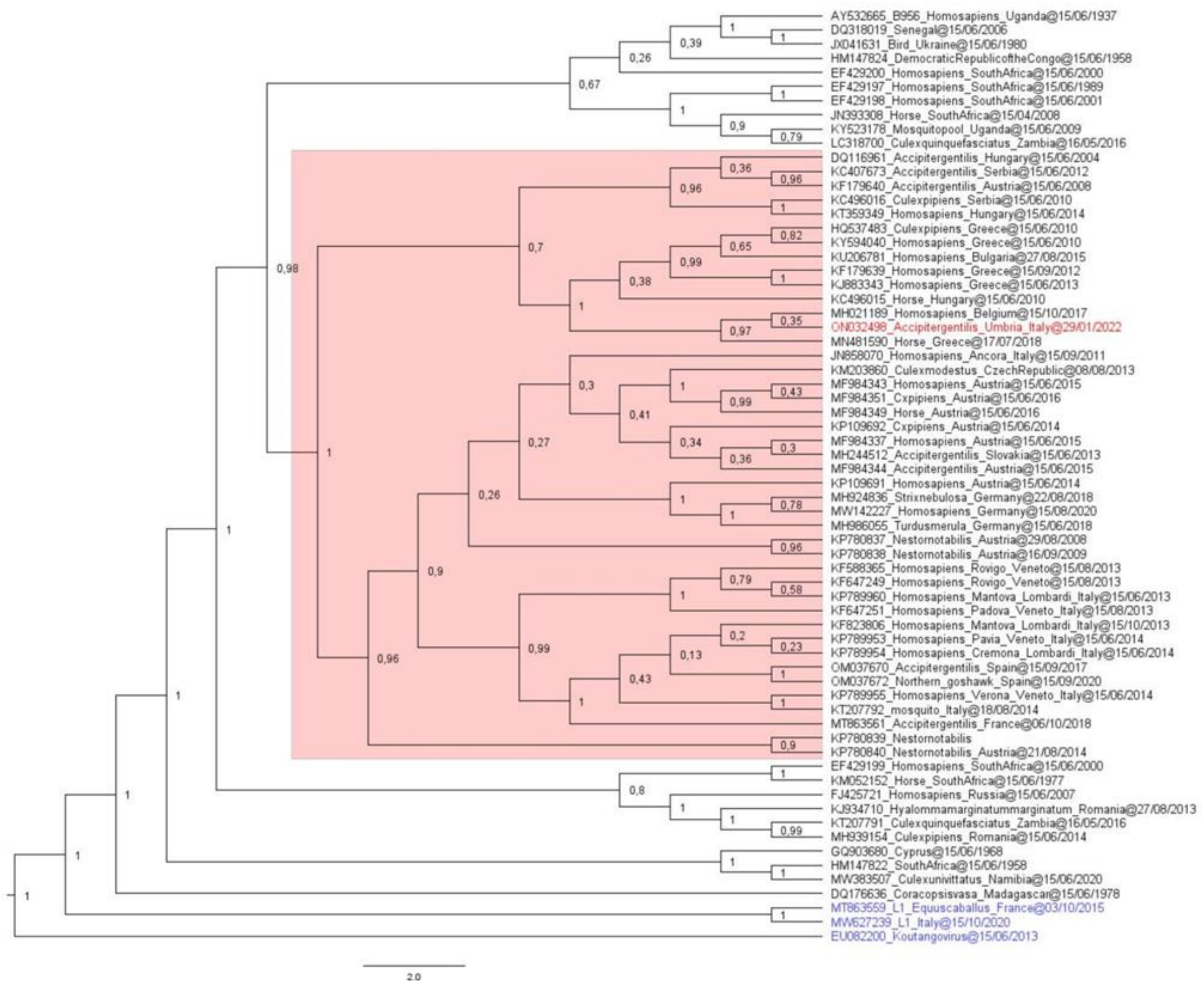

Figure 7.2 Evolutionary analysis inferred by using the maximum-likelihood method and the general time reversible model.

The tree with the highest log-likelihood is shown. The percentage of trees in which the associated taxa clustered together is displayed next to the branches. Initial trees for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among the sites (4 categories). The evolutionary distances were computed using the optimal GTR + Γ + I model, with 2000 Γ-rate categories and 5000 bootstrap replications using the Shimodaira–Hasegawa (SH) test. Ten independent MCMC runs with up to 100 million generations were performed to ensure the convergence of the estimates. GenBank accession numbers are indicated for each strain, with country, lineage, and year of isolation. The genome sequence ON032498 WNV L2 (HCov 91%), obtained from the goshawk organ homogenate, is highlighted in red. The WNV L1 MW627239 (Italy 2020) and MT863559 (France 2015), and Koutango virus (KOUTV) EU082200 (Senegal 2013), chosen as outgroups, are highlighted in blue. The new strain (ON032498) showed high genetic similarity with the central-southern European WNV L2 clade, highlighted in pink, with a posterior probability of 100%.

7.6 Discussion

This study reported the second evidence of WNV L2 circulation in the Umbria region. The current National Surveillance Plan includes Umbria amongst those areas whose eco-climatic characteristics are favorable for viral circulation but where WNV has never or sporadically circulated in the past (National Plan for Prevention, Surveillance, and Response to Arbovirus 2020–2025). In fact, no WNV circulation has ever been reported in the territory, neither in humans [31] nor in animals, before 2019, when the virus was detected for the first time in a little grebe [15]. Interestingly, in both reported Umbrian cases, WNV was detected in the winter months (December and January), a period when, normally, the most common vector, *Culex pipiens*, is less active.

The Northern goshawk (*Accipiter gentilis*) is a medium-sized bird of prey belonging to the family of *Accipitridae*. The family also includes other diurnal raptors, such as eagles, buzzards, and harriers [32]. With regard to WNV infection, Northern goshawks have been demonstrated to be highly susceptible. In fact, following WNV infection, severe clinical symptoms have often been described in this species and, in general, in raptors [19,30,33,34]. The clinical signs described in this report were compatible with WNV infection. Moreover, the absence of fractures in the bird skull excluded a possible traumatic origin of the observed neurological disorders.

The first aspect that needs to be clarified is the time of infection: did it really occur in winter? In this regard, the occurrence of clinical signs and the direct detection of WNV in the goshawk organs indicates that this case was related to a recent infection. WNV infections in periods of mosquito inactivity have been described, particularly in raptors [33,35,36]. This group of birds is, in fact, characterized by predatory habits [32] and the transmission of WNV by the predation of infected birds has been frequently observed [33,37]. In this reported case, it is then highly probable that the Umbrian goshawk became infected by eating an infected bird. If, on the one hand this explanation indeed clarifies the way the goshawk obtained the infection, on the other hand, it does not explain how the supposed goshawk prey was still infectious in a period of mosquito inactivity. The persistence of infectious WNV for prolonged periods in the organs of birds and, in particular, of Passeriformes has been evidenced by many authors [35,38–42]. Persistent infection has been defined as the detection of a virus in host tissues after viremia has subsided [43]. The persistent, high viral loads in organs of birds and, in particular, in those belonging to prey species might sustain WNV transmission to predators also months after mosquito season. The recent finding of WNV L2 in a little grebe collected in the winter months in Umbria [15] indicates that finding birds with WNV-infected organs in the winter months is not uncommon [33,34,38,41,43]. Thus, the symptomatic goshawk found in Umbria last January can be regarded as a case of bird-to bird WNV oral transmission.

This transmission route has been considered as one of the possible ways of overwintering for WNV [33,34,38,41,43]. Bearing in mind that common preys of goshawks also include rodents and that, also, in these animals infectious WNV has been detected months after infection [44,45], a possible rodent-to bird transmission cannot be excluded.

Further attention should be given to the *Culex pipiens* complex because of its vector role in WNV transmission [46,47]. Among this complex, the *Culex pipiens* (rural, mainly ornithophilic) and Culex molestus (urban, mainly mammophilic) biotypes can interbreed, giving birth to a hybrid form with intermediate ecological features found in a wide set of environments and acting as a WNV bridge-vector from birds to humans [46,47]. Interestingly, while *Cx. pipiens* are well-known to enter diapause during winter, *Cx. molestus* and *Cx. Pipiens–Cx. molestus* hybrids actively feed all year round [46] and might, for this reason, have been responsible for the goshawk WNV infection.

Irrespective of whether the goshawk was infected by hybrid mosquitoes or by the predation of infected animals, WNV has indeed been circulating in the winter months. Therefore, the second important issue to be clarified in this report is where the goshawk was infected. According to the National Surveillance Plan, Umbria is classified as a WNV low-risk area, while the neighboring regions (parts of Tuscany, Marche, and Lazio) are high-risk areas (National Plan for Prevention, Surveillance, and Response to Arbovirus 2020–2025). In most parts of Europe, the northern goshawk is a sedentary species [32,48]. In Italy, it is a rather scarce, localized breeder, mainly present in mature forests of the Apennines and the Alps that are especially rich in large trees and are particularly suitable for nest-building, where its common prey are abundant (e.g., squirrels, wood pigeons, woodpeckers, corvids, and rabbits) [32]. In January, even if it further reduces its movements, it can still keep moving for hundreds of kilometers [32]. Because of that and in view of the fact that Umbria has a very small surface (8.456 Km2), it is very difficult to determine whether the goshawk became infected in Umbria

or in the neighboring regions. Interestingly, during the WNV season 2021, there was no evidence for the circulation of WNV, not only in Umbria but also in the nearby area of central Italy [49]. The lack of WNV infection cases in high-risk areas (Latium and Tuscany) also in wintertime [15,50], when mosquitoes are less active, might suggest that birds have an important role in WNV overwintering. The potential transmission through the oral route in a predatory bird might explain the relatively rapid spread of WNV and of other similar flaviviruses, such as Tick-borne encephalitis virus and Usutu virus (USUV) [50–52]. It has to be said that Umbria is endemic for USUV [53] (National Plan for Prevention, Surveillance, and Response to Arbovirus 2020–2025), which is a mosquito-borne virus that shares the same life cycle and patterns of transmissibility with WNV [4]. If the eco-climatic conditions of Umbria are suitable for maintaining the USUV life cycle, they should also be favorable for WNV.

Based on these features and due to recent WNV positivities detected in the territory, the Umbria region was included among the high-risk areas in 2022 by the WNV National Surveillance Plan (National Plan for Prevention, Surveillance, and Response to Arbovirus 2020–2025).

7.7 Conclusions

In conclusion, our study highlighted the circulation of WNV L2 during the winter in Italy. Even though the reported cases remain rare during the cold season, this report is of fundamental importance because it evidences the potential for human transmission when veterinary active surveillance is suspended. It means that we cannot rely on the early warning system for WNV circulation mainly provided by mosquito and bird surveillance to prevent human infection. Further research is needed to better understand the transmission routes and the role of overwintering birds, rodents, and mosquitoes, as well as WNV infection per *os*. in WNV transmission and epidemiology in Italy. Considering the strong WNV circulation in the Italian territory and the observed changes in the seasonal and regional

patterns of WNV, with the virus observed lately in winter times, as well as in WNV low-risk areas, this work also highlighted the strong importance of the passive surveillance of wildlife. This should be coupled with the implementation of a harmonized protocol for necroscopies and biological sample collection, including gastro-intestinal contents, in order to obtain additional data needed to clarify the potential cause of death, especially in winter, when the National Surveillance Plan does not include an active search for the virus.

7.8 Supplementary Materials

The following are available online at https://www.mdpi.com/article/10.3390/tropicalmed7080160/s1, Figure S7.1: Picture of WNV-infected Northern goshawk; Video S7.1: Video of WNV-infected Northern goshawk; File S7.1: WNV List of Sequence dataset; Table S7.1: Metadata of WNV strains used for the present study.

7.9 Author Contributions

Conceptualization, G.M., F.I., F.M., and G.S.; methodology, G.M., F.M., A.P., M.M., and G.S.; validation, F.M., A.P., M.M., V.C., and G.S.; formal analysis, G.M., A.P., F.M., and M.M.; investigation, A.D.G., L.T., A.P., M.M., M.G., E.M., S.S., B.S., V.D.L., and G.M.; resources, F.M. and G.S.; data curation, M.G., E.M., A.P., and G.M.; writing—original draft, G.M., F.I., F.M., and G.S.; writing—review and editing, G.M., F.M., M.M., R.R., A.R., and G.S.; visual-ization, G.M., F.I., F.M., A.D.G., L.T., V.C., M.M., A.P., M.G., E.M., V.D.L., S.S., B.S., C.C., R.R., A.R., and G.S.; supervision, F.M. and G.S.; project administration, G.S.; funding acquisition, G.S. All authors have read and agreed to the published version of the manuscript.

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7.11 Data Availability Statement

Sequence data are available via NCBI. The accession numbers for the sequences used can be found in Table S7.1, supplementary materials.

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7.13 Conflicts of Interest

All the authors declare no conflict of interest.

7.14 References

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CHAPTER 8. EPIDEMIOLOGICAL AND EVOLUTIONARY ANALYSIS OF WEST NILE VIRUS LINEAGE 2 IN ITALY

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by

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8.1 Abstract

West Nile virus (WNV) is a mosquito-borne virus potentially causing serious illness in humans and other animals. Since 2004, several studies have highlighted the progressive spread of WNV Lineage 2 (L2) in Europe, with Italy being one of the countries with the highest number of cases of West Nile disease reported. In this paper, we give an overview of the epidemiological and genetic features characterising the spread and evolution of WNV L2 in Italy, leveraging data obtained from national surveillance activities between 2011 and 2021, including 46 newly assembled genomes that were analysed under both phylogeographic and phylodynamic frameworks. In addition, to better understand the seasonal patterns of the virus, we used a machine learning model predicting areas at high-risk of WNV spread. Our results show a progressive increase in WNV L2 in Italy, clarifying the dynamics of interregional circulation, with no significant introductions from other countries in recent years. Moreover, the predicting model identified the presence of suitable conditions for the 2022 earlier and wider spread of WNV in Italy, underlining the importance of using quantitative models for early warning detection of WNV outbreaks. Taken together, these findings can be used as a reference to develop new strategies to mitigate the impact of the pathogen on human and other animal health in endemic areas and new regions.

8.2 Keywords

West Nile virus lineage 2; epidemiology; evolution; early-warning; Italy

8.3 Introduction

West Nile virus (WNV) is a positive strand RNA virus, belonging to the Flaviviridae family, genus Flavivirus, within the Japanese encephalitis virus serocomplex [1,2]. It is maintained in nature through a transmission cycle involving competent mosquitoes and birds acting as main vectors and major

amplifiers, respectively [3,4]. Amongst the group of birds, corvids and raptors are highly susceptible. In these groups, WNV infection can result in severe, sometimes fatal neurological disorders [2,5,6]. In other groups, symptoms are less frequently observed, and in some cases chronic infection may develop [5,7,8,9]. Migratory birds can play a key role in introducing the virus to new regions, while resident competent birds are mostly involved in local transmission, which might eventually lead to the endemic circulation of the virus [5,10,11]. Humans, horses, and other vertebrates, such as reptiles, amphibians, and several mammals, are regarded as WNV incidental dead-end hosts [2,4]. In equids, only the minority of cases have severe clinical signs, associated to neurological symptoms, resulting in substantial economic costs and serious emotional distress [12,13]. In humans, WNV infection is generally asymptomatic (80%) [14]. In 20% of cases, however, it can lead to West Nile fever (WNF), characterised by flu-like symptoms. In a lower percentage of cases, mainly in old or immunocompromised people, infections can also cause severe and even fatal diseases characterised by neurological manifestations [2,15].

WNV was first detected in 1937 in Uganda. It has been circulating outside East Africa since the 1950s [2,3,4,15] and nowadays is reported in many countries worldwide [2,4,16]. Phylogenetic studies have revealed that WNV comprises at least eight lineages. WNV lineage 1 (L1) and lineage 2 (L2) are the most pathogenic and widespread, causing several outbreaks in birds, humans, and horses around the world [17]. From the 1960s to 2004, L1 was the main lineage circulating in Europe [18]. Since its first appearance in Hungary in 2004, L2 quickly spread throughout Europe, becoming endemic in many European countries [19]. Nowadays, it is the most prevalent WNV lineage circulating in the continent [20,21].

In Italy, the first West Nile disease (WND) outbreak was reported in the Tuscany region in 1998. The strain belonged to the WNV L1 genotype and was responsible for severe neurological cases and deaths among horses [22].

The virus has been monitored in the country since 2001. The surveillance plan did not detect any relevant circulation until 2005, when WNV antibodies were detected in sentinel chickens in northern Italy (see Material and Methods, as well as sample and data collection sections, for the details of the surveillance plan) [23]. Following the first years in which the surveillance plan was tackled almost separately by the human and veterinary health systems, a One-Health integrated surveillance program has been implemented since 2016. It integrates human, animal, and entomological surveillance to detect seasonal WNV circulation early. The detection of WNV in any host/vector is confirmed by a network of reference laboratories coordinated by the Ministry of Health, the Istituto Superiore di Sanità (National Reference Laboratory for WND in humans), and the Istituto Zooprofilattico of Abruzzo and Molise (IZS-Teramo) (National Reference Laboratory for WND in animals) (https://westnile.izs.it/j6_wnd/home, https://www.epicentro.iss.it/westnile/, accessed on 15 November 2022). The surveillance activities are refined every year according to the new WNV epidemiological findings in the country.

In 2008, several WNV L1 cases were reported in Emilia-Romagna, Veneto, and Lombardy [24]. In the following years, the virus became endemic in the northern part of the country and between 2010 and 2011 spread to the south [15]. Evidence of WNV L2 circulation in Italy was first reported in 2011 [25,26]. After its first appearance, it rapidly spread all over the country replacing WNV L1, which was only sporadically detected until October 2020, when it re-emerged in Italy in the Campania region [18]. The current 2022 vector season is characterised by an intense co-circulation of WNV L1 and L2 virulent strains, detected at the same time in mosquitoes, birds, horses, and humans [27], as reported

in the Annual Epidemiological bulletins produced for Italy by IZS-Teramo (https://westnile.izs.it/j6_wnd/periodicalItaly, accessed on 10 November 2022).

An exceptional number of WNV L2 cases were observed in Italy between June and November 2018 [21], with 577 human cases confirmed, including 230 neuroinvasive diseases (39.86%), 279 fevers (48.35%), and 42 deaths (7.27%). They occurred in Veneto, Emilia-Romagna, Lombardy, Piedmont, Sardinia, Friuli-Venezia Giulia, and Molise (only 1 case imported from Greece) regions. An abnormal number of WND cases were also observed in horses, birds, and mosquitoes, with 238 cases reported in equids (IgM or molecular test), while WNV L2 RNA was detected in 239 resident target birds (Pica, Corvus corone cornix, and Garrulus glandarius), 109 wild birds, and 433 mosquito pools in the Emilia-Romagna, Lombardy, Piedmont, Friuli-Venezia Giulia, Veneto, Sardinia, Lazio, Basilicata, and Puglia regions (https://storymaps.arcgis.com/collections/b50666024702441dac792d0cb3aee32c, year 2018, accessed on 10 November 2022). Currently, Italy is the European country with the highest number of WNV cases, and most of them are associated with WNV L2 infections (see Italian Epidemiological reports https://westnile.izs.it/j6_wnd/home, accessed on 10 November 2022).

In this paper, we reconstructed the phylogeny, phylogeography, and phylodynamics of WNV L2 in Italy to better characterise its spread and evolution. The epidemiological trend of WNV L2, since its first appearance in the country in 2011, is also described by analysing the seasonal patterns and the viral prevalence in mosquitoes, birds, horses, and humans over the years. These analyses gave us the opportunity to assess the accuracy of the recent early warning system based on the environmental and climate model [28] in predicting WND epidemic behaviour during the 2021 and 2022 vector seasons.

8.4 Materials and Methods

8.4.1 Sample and Data Collection

In 2001, following the first outbreaks of WNV L1 reported in Tuscany in the late '90s, a WNV and Usutu virus integrated surveillance plan was implemented in Italy. At that time, the plan aimed to detect the introduction and local spread of these viruses. Fifteen WNV-at-risk areas were selected according to their suitable eco-climatic conditions and monitored by using an approach based on serological screening in sentinel animals (horses and poultry), wild bird mortality, and mosquito surveillance. In 2002, hospitalised human cases of fever with rash or encephalitis and meningitis recorded in regions where virus circulation was evidenced by the animal surveillance, started being reported. No WNV L2 cases were detected until 2011, when the lineage first appeared in northern regions, soon becoming endemic. Since this year, WNV L2 samples were collected under an integrated surveillance on birds, mosquitoes, and humans, activated on a regional scale, in northern regions [29], while WNV monitoring in the rest of Italy was based on serological screening in sentinel horses and chickens. Since this year, samples were collected.

In 2016, the first national plan integrating human, animal (equids, resident, and wild birds), and mosquito surveillance was issued. Its evolution in the following years led to the 2020–2025 Integrated Surveillance and Response Plan for Arboviruses (PNA), which is currently still in place (https://westnile.izs.it/j6_wnd/ministeriale, accessed on 10 November 2022). Seasonal surveillance activities are defined on the basis of the previous year's virus circulation (risk areas), while any WNV detection in birds, mosquitoes, equids, and humans triggers the activation of prevention measures (blood and transplant measures including nucleic acid testing, vector control, and risk communication campaigns aimed at citizens). Therefore, the start date of these measures can vary each year and, in each province, depending on the viral circulation.

Any positive results from local surveillance activities are confirmed by the National Reference Centre for Foreign Animal Diseases (CESME) at IZS-Teramo. Notifications of outbreaks are registered by the official veterinary authorities in the national information system for the notification of outbreaks in animals (SIMAN) [30]. The notification system is designed to register and document the occurrence and evolution of important infectious animal diseases according to Council Directive 82/894/EC [29].

8.4.2 Sample Analysis

8.4.2.1 Tissue Homogenisation, RNA Extraction, and Real-Time RT-PCR

At IZS-Teramo, bird tissue samples (heart, kidney, spleen, and brain) and pools of mosquitoes were homogenised in phosphate-buffered saline (PBS) with antibiotics. WNV RNA was extracted by using the MagMAX CORE Nucleic Acid Purification KIT (Applied Biosystem, Thermo Fisher Scientific, Life Technologies Corporation, Waltham, MA, USA), according to the manufacturer's instructions. Every extracted RNA was subjected to one-step quantitative reverse transcription polymerase chain reactions (qRT-PCR) to detect WNV-L1 and/or -L2, and all known lineages of WNV by using the Superscript III Platinum OneStep qRT-PCR System (Invitrogen, Thermo Fisher Scientific, Life Technologies Corporation, Waltham, MA, USA) [31].

8.4.3 Epidemiological Data Analysis

All data collected within the Italian WNV surveillance plan between 2011 and 2021 (see Table S8.1) have been processed using LibreOffice Calc v 365 [32] and Microsoft Excel v 4.2.2 [33]. Data collected between 2011 and 2021 were analysed with LibreOffice Calc, Microsoft Excel, and R version 4.1.2 [34].

8.4.4 *Sequence Data Preparation and Retrieval*

8.4.4.1 Whole Genome Sequencing, Genome Assembly, and Sequence Processing at NCBI

Purified nucleic acids were sequenced by Next Generation Sequencing, as described in detail in [35]. WNV L2 consensus sequences were obtained using iVar v 1.3.1 [36] after reads were mapped to various WNV L2 reference sequences (KU573082_Italy_2013, MN652880_Greece_2018, KT207792_Italy_2014, KP789954_Italy_2014, and HQ537483_Greece_2010) by using Snippy (https://github.com/tseemann/snippy, accessed on 1 November 2022). A map showing the geo-localisation of the collection sites of the sequenced samples can be found in Figure 8.1.

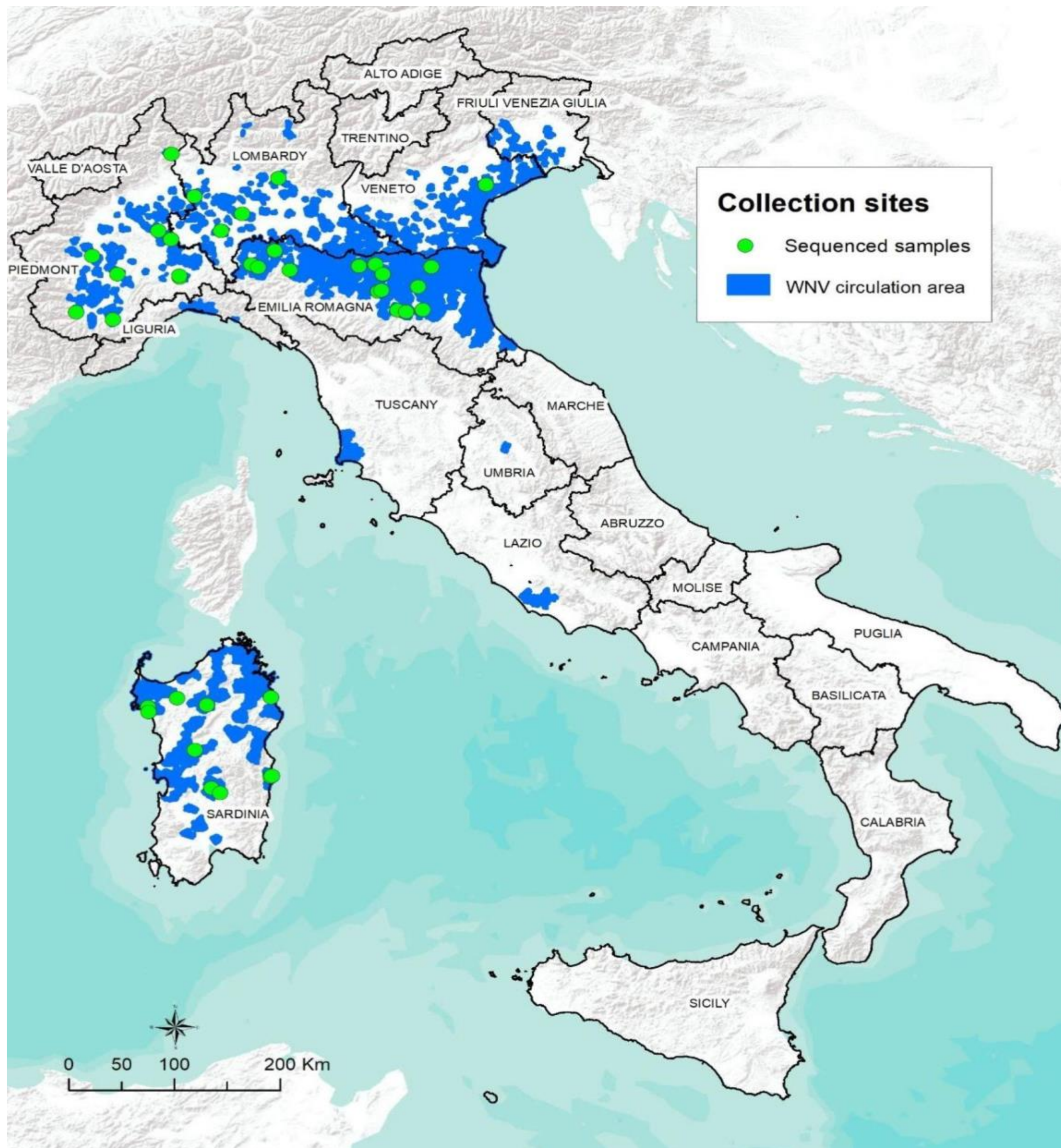

Figure 8.1 Geo-localization of WNV L2 sequenced sample collection sites

In blue, the WNV L2 circulation municipalities obtained from veterinary sample collection performed between 2011 and 2021 are displayed. The collection sites of samples from which sequences were obtained are shown in green.

8.4.4.2 Download of Reference Genomes

All available WNV sequences ≥ 200 nt have been downloaded from NCBI by using a custom R script for automatic sequence retrieval, which allows one to select sequences of the desired length by leveraging the "rentrez" R package (https://cran.r-project.org/web/packages/rentrez/index.html, accessed on 25 October 2022).

8.4.4.3 Sequence Metadata Collection and Curation

Metadata for the newly generated sequences were obtained from the Laboratory Information Managements Systems (SILAB) at IZS-Teramo. SILAB is a web application designed by IZS-Teramo IT staff that uses an automated electronic recording function to support all diagnostic activities performed on the incoming samples, from their registration to the report release (https://www.izs.it/IZS/Engine/RAServePG.php/P/257610010719/L/1, accessed on 20 October 2022). All the information recorded in SILAB is originally collected and presented in the Vetinfo portal (https://www.vetinfo.it/, accessed on 20 October 2022) and subsequently reported in the PNA (https://www.vetinfo.it/, https://www.salute.gov.it/imgs/C_17_pubblicazioni_2947_allegato.pdf, accessed on 20 October 2022).

Metadata of all WNV sequences present in NCBI were downloaded from the NCBI virus platform (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/, accessed on 20 October 2022) as a single csv file, including all available fields.

8.4.4.4 Sequence Data Cleaning and Formatting

Only data for sequences longer than 10 kb (i.e., sequences covering almost the entire WNV genome) were retained for subsequent steps. All sequences were filtered for quality, converting all unusual

nucleotides into “N” letters, and then sequences which contained a percentage of ambiguous bases superior to 10% were removed. After this step, a total of 2478 genomes, 95 of which came from Italy, were selected for further analysis. For these sequences, we checked the cases lacking in information regarding the collection date and host, attempting to retrieve it from the corresponding paper, if available. When unable to identify the collection date, we put a putative date, if at least the year was known, choosing 15/06 (i.e., approximately the half of the year). We filled in all fields lacking in data with “NA” values, obtaining a resulting table of curated metadata (Table S8.2). All sequences were annotated using custom Python scripts and the newly generated tables with curated metadata, formatting the headers in a “>id|country|yyyy/mm/dd” format.

8.4.5 Phylogenetic Analysis

8.4.5.1 Subsampling for Molecular Clock Analysis

In order to reduce redundancy and make data suitable for molecular clock analysis, we carried out a subsampling of our main dataset. First, a maximum-likelihood phylogeny using FastTreev2.1.10 [37] (as specified in the next sections of this study) was reconstructed. As all American genomes (representing 83.9% of our dataset, 2079 sequences out of 2478) belonged to the same clade and were part of the L1 lineage, a subsampling of these sequences was carried out as well. Genome-sampler [38] was used to perform our selection. A total of 49 selected sequences resulted by sampling according to collection date (with a 1095-day sampling frequency) and viral diversity (50%). These sequences were then merged with the remaining quality-checked genomes from all possible world locations, and the sampling step was repeated with the same tool by using them as context sequences (defined as genomes obtained from a global community resource), while using our selection of Italian genomes as focal sequences (i.e., genomes obtained locally). We sampled every 90 days, with a diversity threshold of 0.95 and sampling near neighbours of the focal sequences at 0.99 percent

genome diversity, picking up 3 sequences per cluster. A final dataset of 370 sequences (95 genomes from Italy and 275 from 43 other countries) was generated and used for further analysis.

8.4.5.2 Alignment and Recombination Detection

All the datasets were aligned individually using MAFFTv7.490 [39], with the "--auto" option. All the datasets, excluding the one comprising all WNV L2 Italian sequences (very similar to each other), were trimmed using trimAlv1.2 [40] with the option "-automated1". The presence of recombinant sequences in the final dataset was checked by running the RDP4 program [41], with default options. This program uses phylogenetic-based (BootScan, RDP, Siscan) and substitution-based (GenConv, Maxchi, Chimaera, 3SEQ) methods to infer recombination events. A recombination event was regarded as true when detected by at least 5 methods out of 7. The sequences EF429199.1 from South Africa and GQ851604.1 from India were excluded from our final set, as they were indicated as suspected recombinants. The dataset without recombinant sequences (n = 368) was realigned and trimmed using the same methods.

8.4.5.3 Model Selection

Model selection was carried out on all datasets using Modelfinder [42], implemented in IQTREEv2.1.2 [43], using parameters "-T AUTO -m TESTONLY". The best-fit model for both the worldwide and the Bayesian down-sampled datasets was GTR + F + I + G4, chosen according to both Akaike information criterion (AIC) and Bayesian information criterion (BIC).

8.4.5.4 Maximum-Likelihood Phylogenies

To have an initial estimation of the phylogenetic signal in our dataset, FastTree 2 [35] was used to reconstruct a worldwide phylogeny of all WNV sequences (n = 2478) that passed our quality control,

with the command “FastTreeDbl -nt -gtr -gamma -log logfile -pseudo”. A maximum likelihood phylogeny of the subset dataset (n = 368) was reconstructed by using RAxMLv8.2.12 [44], with commands “-p 1989 -m GTRGAMMAI -x 2483 -# 100 -f a -T 20”. Clades were annotated using the resulting topology when having bootstrap supports ≥90%.

8.4.5.5 Molecular Clock

BEASTv2.7 [45] was used to obtain phylogenies of the sequences using different sets of priors and models (Table S8.3) to explore their effect and influence on our phylogenetic reconstruction. Our subsampled dataset of 368 genomes was employed to reconstruct the overall genome evolution of WNV. The topologies of all trees obtained were compared together and with the maximum-likelihood tree to test the robustness of the reconstruction.

8.4.5.6 Phylogeographic and Phylodynamic Analysis of the Italian Clade of WNV L2

A clade comprising almost all Italian sequences of WNV was found in each tree, with bootstrap = 100 and posterior probability = 1. The sequences belonging to this Italian clade (n = 74) were re-annotated, including the name of the specific region of origin and, for those lacking an exact date, a refined estimation of the collection date based on the median of the collection dates of the sequences with complete metadata. The previous steps (alignment, trimming, model selection and tree building with RAxML and BEAST2) were therefore repeated. Phylogeography was reconstructed by using continuous traits (latitudinal and longitudinal coordinates for each sequence) in BEASTv1.10.4 [46]. When the exact position was not available, we approximated the location using the coordinates of the municipality from which the sample was collected (for detailed information about coordinates please contact the authors). We divided the analysis into two different partitions (one for sequence data and the other for continuous coordinates) and used the Cauchy RRW substitution model for our location

partition, with bivariate traits representing latitude and longitude, adding random jitter to the tips (jitter window size: 0.01). For the location partition, we selected the option to reconstruct states for all ancestors. We employed an uncorrelated relaxed clock with a log-normal distribution and a coalescent Bayesian skyline tree prior. We set a MCMC length of 500 × 106 generations, sampling every 50,000 steps. Convergence was assessed using Tracerv1.7.1 [47], ensuring that all parameters were above a significant threshold of ESS (>200). In parallel, a phylogeographic reconstruction using discrete characters was carried out using BEAST2 for the same dataset and under the same set of parameters (excluding the modelling of the character state), obtaining posterior probability estimates for each location at each node. To characterise the dynamic of the viral population in Italy, coalescent Bayesian skyline and birth–death skyline serial analyses were performed using different sets of models and parameters implemented in BEAST2 (see Table S8.3). The birth–death skyline serial model made it possible to estimate the effective reproductive number (Re), which is the average number of secondary infections caused by an infected individual at a given time during the epidemic.

8.4.6 Ecological and Epidemiological Modelling

In [28], the authors presented a model based on climatic and environmental factors (daytime and nighttime land surface temperature, normalised difference vegetation index, and surface soil moisture) that produces, two weeks in advance, risk maps for WNV circulation throughout Italy using a decision-tree-based ensemble machine learning algorithm (XGBoost, https://xgboost.readthedocs.io/en/latest/build.html, accessed on 20 October 2022).

This model, initially based on 2017–2019 data, was later updated and calibrated using the additional 2020 epidemic data and here applied for a comparison between risk maps in 2021 and 2022.

8.5 Results

8.5.1 Epidemiological Scenario

Based on all the data collected by the national surveillance plan between 2011 and 2021 in Italy (see Table S8.1), WNV cases recorded in mosquito pools, birds, horses, and humans were mapped as shown in Figure 8.2.

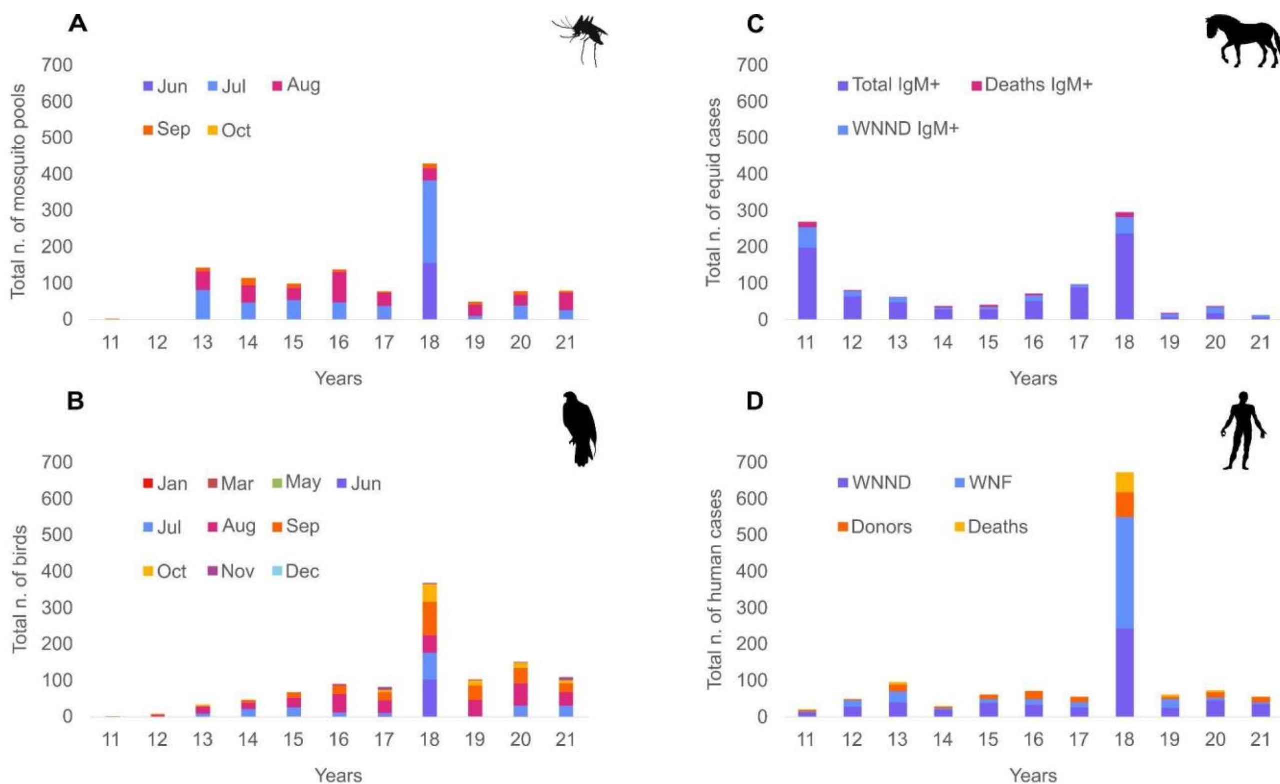

Figure 8.2 Mapped WNV cases recorded in mosquito pools, birds, horses, and humans

Total yearly number (from 2011 to 2021) of: (A) WNV L2 rt-PCR positive mosquito pools; (B) WNV L2 rt-PCR positive wild and target birds; (C) Total WNV IgM+ cases, IgM+ West Nile neuroinvasive disease (WNND), and IgM+ deaths among equids, not lineage specific; (D) Human WNND, West Nile fever (WNF), donors, deaths, and molecular and/or serological positive tests. For (A,B), the temporal distribution is shown per month and year, with cases reported in the months of: January (Jan); March (Mar); May; June (Jun); July (Jul); August (Aug); September (Sep); October (Oct); November (Nov); and December (Dec). Different

colours representing each month are displayed in the legend. In (C,D), the temporal distribution is shown per year.

WNV L2 typically circulates in the environment among birds and mosquitoes between July and October, with peak activities shown in August (mosquitoes, birds) and September (birds). In mosquito vectors, an increased viral transmission was observed in 2013, 2016, and 2018, a tendency also confirmed by human data (not lineage-specific). The 2018 epidemic season was characterised by a strong recrudescence in mosquitoes, birds, horses, and human cases, with earlier and higher incidence of infections already registered in the month of June. In recent years (2016–2021), an increased viral transmission in birds was observed in November. Serological studies (not lineage-specific) conducted among equids highlighted an intense viral circulation in these animals in 2011.

8.5.2 Genetic Scenario

8.5.2.1 Genome Sequence Analysis

Illumina sequencing produced an average total number of raw reads per sample of about 1,647,235, and the average total numbers of trimmed reads is 1,618,180. The numbers of mapped reads (151 nucleotides [nt] in length) ranged from 252,431 to 289,804, with coverage depth ranging from 1267× to 6858×. Overall, consensus sequences were characterised by complete WNV L2 whole genomes. The 45 WNV L2 whole genome sequences obtained at the CESME were uploaded to BankIT NCBI (https://submit.ncbi.nlm.nih.gov/about/bankit/, accessed on 20 October 2022) in May 2021 and March 2022.

8.5.2.2 World Scale Phylogenomics of WNV

Phylogenetic reconstructions for the worldwide dataset comprising 368 sequences were similar across maximum-likelihood and Bayesian methods. For Bayesian trees, evolutionary rates and divergence

times were different according to the specific combination of models used (see Table S8.3 and Figure S8.1). The majority of WNV L2 Italian genomes (74 out of a total of 95 included in the analysis) clustered together in a large clade (Figure 8.3, highlighted in red), which was retrieved with high support in each tree that was reconstructed, independently of the framework and of the models employed (see Table S8.3).

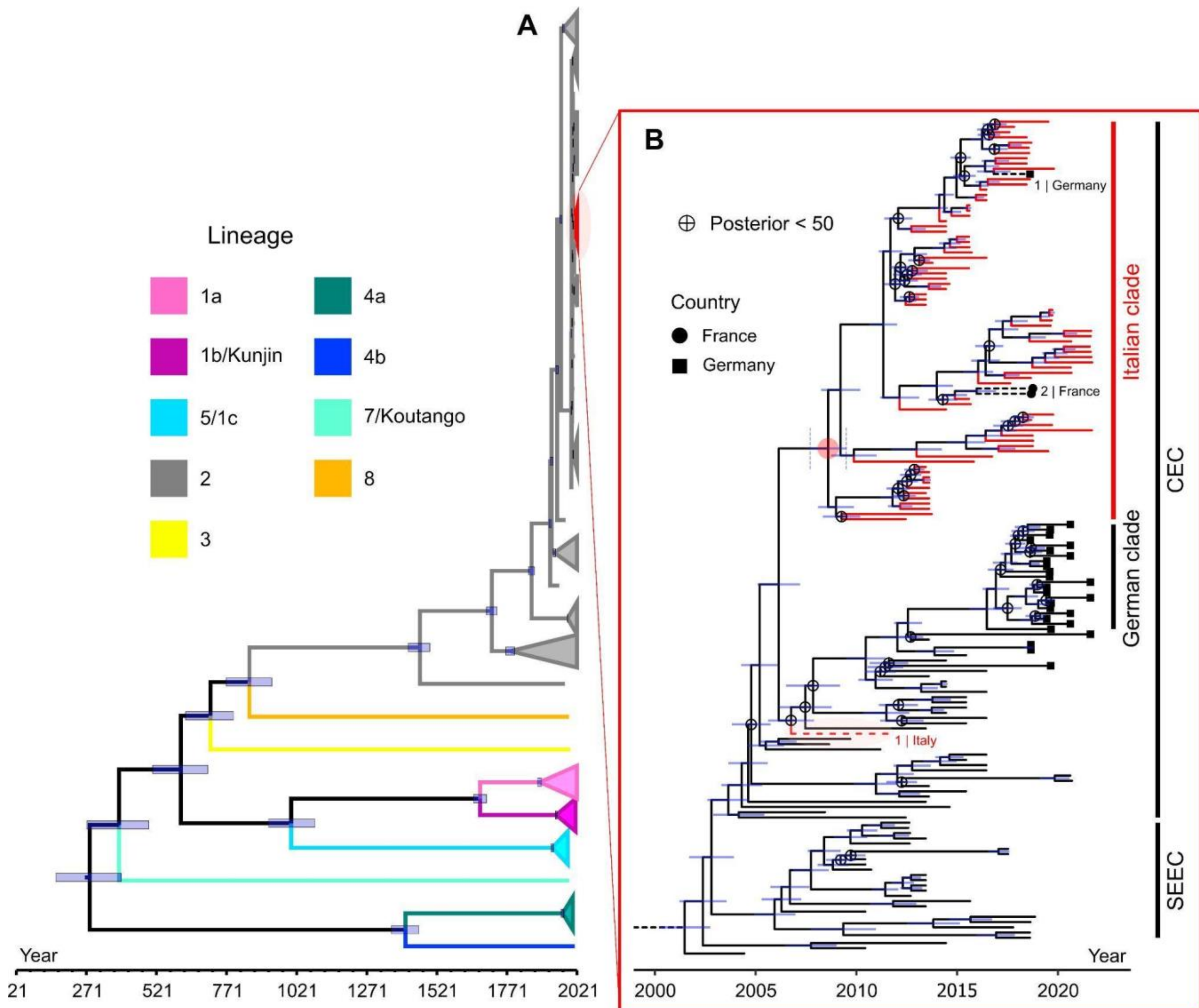

Figure 8.3 West Nile virus worldwide time tree

(A) Worldwide time tree of 368 WNV genome sequences from 44 different countries. WNV lineages are indicated with different colours. Uncertain dates are represented with blue bars showing the 95% HPDs. Nodes

defining the major lineages have all posteriors > 70 (in most cases > 90). A clade which includes the majority of the WNV L2 sequences from Italy is highlighted in red, inside the general WNV L2 subtree (in grey). (B) A magnification of the tree, showing the position of the Italian clade of WNV L2 among the central European clade (CEC). A WNV L2 genome sampled in Italy (JN858070.1, sampled in 2011 in the city of Ancona) and not belonging to the identified clade is shown with a red dotted line. The south-eastern European clade (SEEC), sister of the CEC, is also shown in the tree. Nodes with posterior probability < 50 are indicated with a circled cross.

This group also included two sequences from France (MT863560.1, MT863561.1) and one from Germany (MH910045.1), both from the year 2018. The genomes from France clustered with sequences mostly from Piedmont, while the German sequence was part of a group of sequences from Veneto and Sardinia. Only one Italian sequence (JN858070.1, sampled in 2011 in the city of Ancona) of WNV L2 was not included in the clade, forming a poorly supported group with the German clade of the virus and other sequences from Austria and Slovakia (Figure 8.3B).

8.5.2.3 Phylodynamic and Phylogeographic Analysis of the WNV L2 Italian Clade

Phylogenies built for the identified monophyletic Italian clade were consistent among methods and models, always showing the presence of the same four main well supported clades (Figure 8.4).

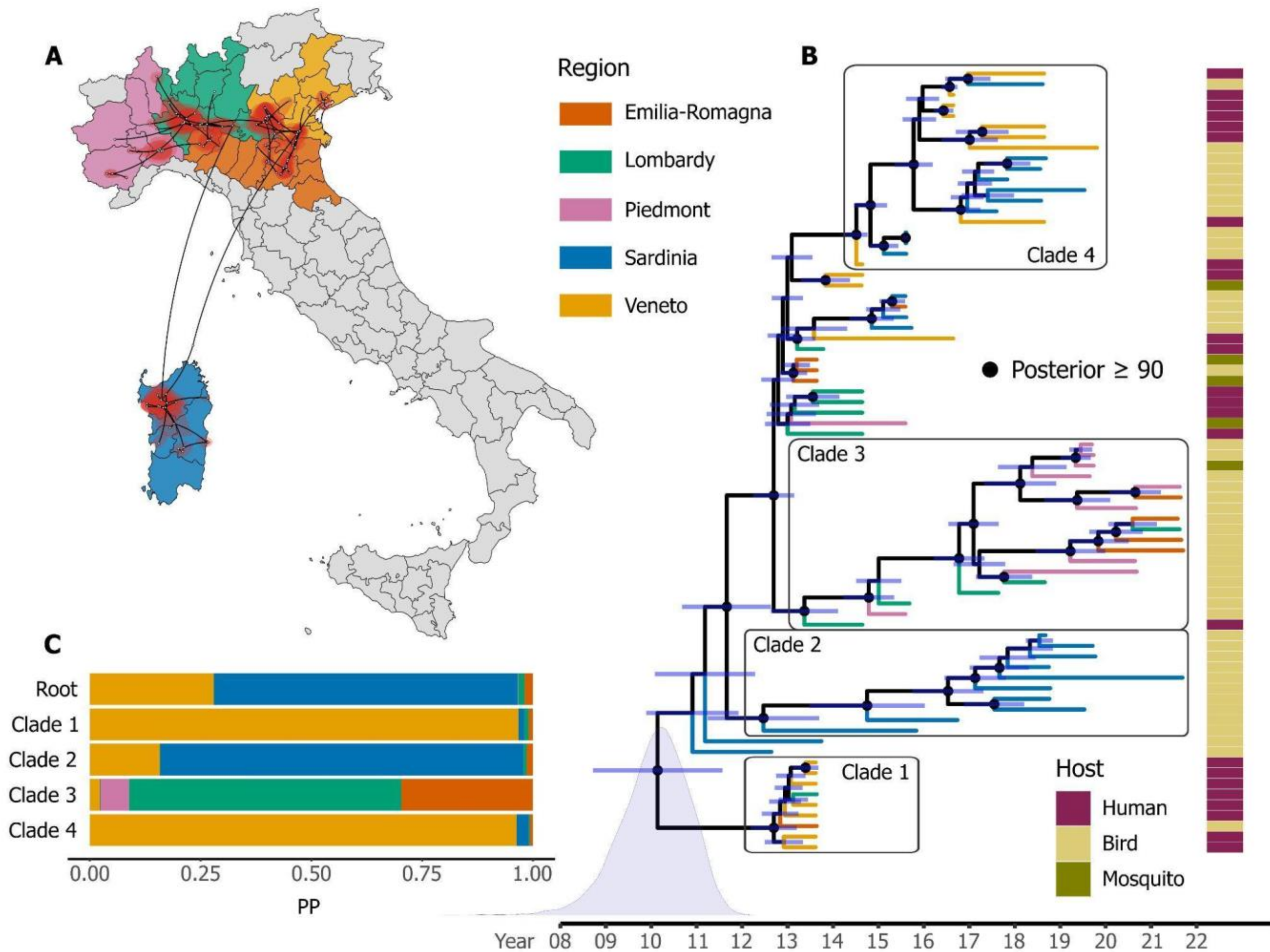

Figure 8.4 Phylogeographic analysis of WNV L2 sequences, part of the identified Italian clade

Colours indicate samples originating from five different Italian regions where the virus was detected. (A) The geographic position and the connection between all genomes analysed are shown. (B) Uncertain dates are represented with light blue bars showing the 95% HPDs. Nodes with high posterior probabilities (≥90) are indicated with black dots. The host from which the sample was reconstructed is indicated in a column on the right with bars of different colours. The blue curve at the root represents the posterior density. (C) A table showing posterior probabilities for the location state of the root and the four highly supported clades.

Clade 1, at the base of the tree, contained variants that were not sampled anymore in the following years. A group of sequences only from Sardinia defines clade 2. Clade 3 included sequences from northern Italian regions (Piedmont, Emilia-Romagna, and Lombardy), while clade 4 had genomes

from Veneto and Sardinia. Some sequences cannot be placed steadily in a group (their position changes among different trees) and come from different regions. The diffusion of the virus in the early years seemed to have started from two main areas (Sardinia and Veneto; Figures S8.2 and S8.3, Videos S8.1 and S8.2), moving further towards nearby regions in the following seasons and bursting in 2018 with an unusually large epidemic. The state of the character at the root suggests that the last common ancestor of these sequences was in Sardinia (posterior probability = 68.59), even though the signal is not highly supported and competes with the one from the Veneto region (posterior probability = 27.9). The ancestors of the other clades have a clearer geographic location (Veneto for clade 1 and 4, Sardinia for clade 2, Emilia-Romagna for clade 3).

Bayesian skyline plot analysis was used to investigate population dynamics of WNV L2 circulating in Italy. Plots reconstructed under both coalescent (Figure S8.4) and birth–death models (Figure 8.5) gave similar results, indicating an increase in the circulation of the virus for the 2013 and 2018 epidemic seasons.

Figure 8.5 Birth–death skyline serial analysis of WNV L2 sequences of the identified Italian clade

Reproductive number (Re) is plotted for a timespan that goes from 2012 to 2021. (Re) > 1 indicates a growth of the epidemic.

A more modest growth is observed in 2016 and 2021, whilst a decline is shown for all the other years. The estimates of the Re were in line with this scenario (Figure 8.5). The peaks of 2013 and 2018 are preceded by a phase of some months in which population growth seems to increase constantly.

8.5.3 Epidemiological and Ecological Modelling

The eco-climatic model developed by Candeloro et al. [28] confirms the presence of suitable conditions for WNV L2 2022 early spread. Compared to 2021, the 2022 epidemic season is characterised by a higher probability of WNV circulation and by an earlier start of the vector season (1–1.5 months). The most endangered regions are considered Emilia-Romagna, Lombardy, and Veneto, where the median values of WNV circulation suitability range from 0.56 to 0.84 already at the end of June. Results are shown in Figure 8.6.

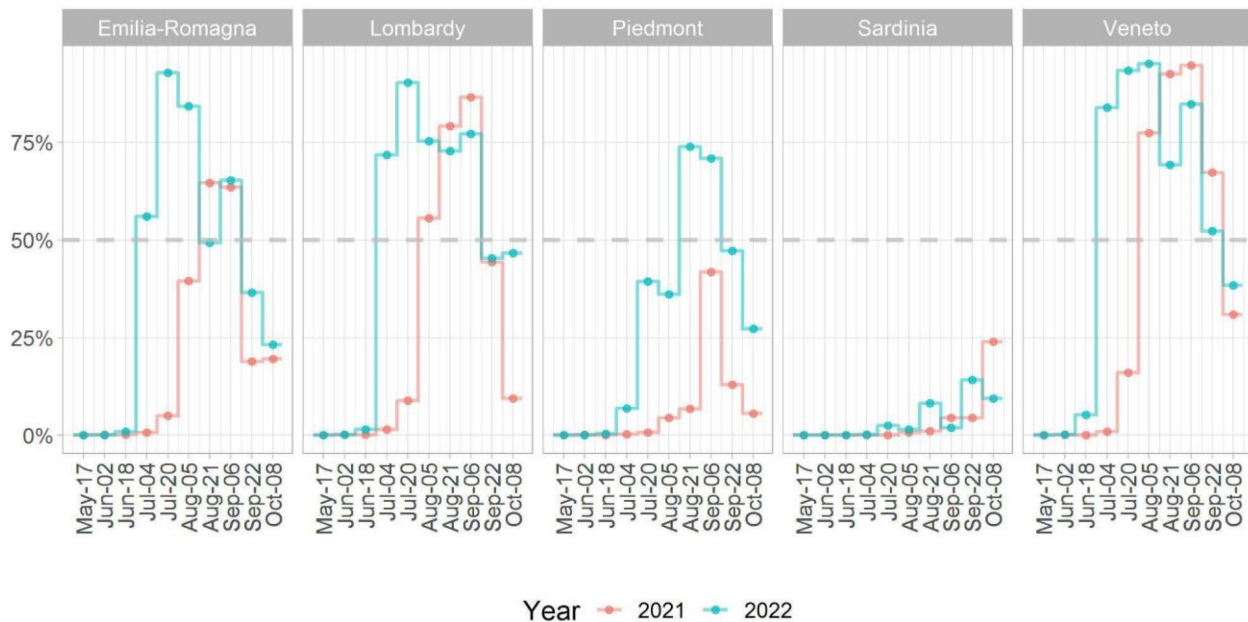

Figure 8.6 WNV circulation suitability

Median values of WNV circulation suitability (probability ranging from 0 to 100%) in five Italian regions during the epidemic season in 2021 (orange line) and 2022 (light blue line). The spatial distribution of the probability across Italy is accessible through the web application https://mapserver.izs.it/gis_wn_predictions/, accessed on 20 October 2022, in which it is possible to view the risk map for WNV throughout the Italian territory with forecast periods of 16 days.

According to these predictions, preliminary data analysis performed on notified outbreaks in SIMAN occurred in the 2022 epidemic season, which confirmed an earlier and wider WNV circulation in Italy compared to 2021.

8.6 Discussion

Italy is one of the European countries reporting the highest numbers of human and animal WND cases. WNV L1 constantly circulated in the area from 2008 until 2011. Since 2012, the epidemiological

scenario has been primarily dominated by WNV L2—at least until 2022, when L1 and L2 WNV strains surprisingly started co-circulating again, with numerous infections and co-infections reported in humans, horses, birds, and mosquitoes [27], (https://westnile.izs.it/j6_wnd/periodicalItalyDocs?docYear=2022, accessed on 20 October 2022).

Since its first appearance, WNV L2 has been reported every year in Italy. Monitoring activities detected WNV L2 infections in 10 out of 20 Italian regions (Emilia-Romagna, Lombardy, Piedmont, Liguria, Veneto, Friuli-Venezia Giulia, Umbria, Tuscany, Lazio, and Sardinia), as well as IgM positive horses in 12 regions (Emilia-Romagna, Friuli-Venezia Giulia, Veneto, Piedmont, Sardinia, Lombardy, Tuscany, Lazio, Basilicata, Puglia, Calabria, and Sicily), evidencing a wide viral diffusion all over the country. Northern Italy and Sardinia stand out as the most affected areas and are nowadays considered endemic (Figure 8.1), (IZS-Teramo Annual Epidemiological Bulletins, https://westnile.izs.it/j6_wnd/wndItalia, https://westnile.izs.it/j6_wnd/wndItaliaPeriodici, accessed on 20 October 2022). According to the data, WNV L2 typically circulates between July and October in Italy, with peaks observed in the months of August and September. However, variations in WNV transmission patterns are not unusual in the country: (i) the 2018 and 2022 epidemic seasons were characterised by an earlier and increased incidence of transmission, with infections detected as early as June [27], (IZS-Teramo Annual Epidemiological Bulletins, Figure 8.2); (ii) in the last years (2016–2021), an increasing number of cases have been reported in November, showing a right shift in WND epidemiological curve's tail-end (Results, Figure 8.2); (iii) WNV-L2 strains have been detected among birds in December and January (2017, 2019, 2022), even in low-risk areas (i.e., Umbria region—2019, 2022) (IZS-Teramo Annual Epidemiological Bulletins) [9,35]; and (iv) an elevated number of WND cases (serological findings, not lineage specific) were observed in horses in 2011, likely due to WNV L1 infection, the most prevalent lineage circulating in Italy in that year [29], or vaccine seroconversion.

Differences in the WNV transmission patterns in the WND epidemics also emerged clearly from the investigation on the population dynamics of the WNV L2 clade inferred using Bayesian skyline plots, under both a coalescent (Figure S8.4) and a birth–death model (Figure 8.5). The results derived from this investigation in fact highlighted, in consistency with the observed surveillance activity data, the increased viral transmission which occurred during the 2013, 2016, and 2018 epidemic seasons (IZS-Teramo Annual Epidemiological Bulletins). According to the Bayesian skyline plots, in case of favourable events (e.g., temperature and humidity), the vector and viral population abnormal growth might be evident before summer, well ahead of the epidemic season.

All reconstructed trees gave comparable results despite the method used. All confirmed the overall scenario described in previous studies, in which different lineages of WNV were defined [17]. As revealed by the molecular clocks and phylogeographic analysis, the first introduction of L2 strains in the Italian territory likely occurred between 2008 and 2011 in Sardinia or Veneto (Figure 8.4; Figures S8.2 and S8.3; Supplementary Videos S8.1 and S8.2). In line with other studies [48], in all our phylogenetic reconstructions WNV L2 Italian genomes tended to cluster together within the Central and Eastern European clades (Figure 8.3), excluding any recent external introduction of the virus, but at the same time confirming the existence of an endemicity status of WNV L2 in Italy. Viral endemicity is most probably assured by the establishment of an endemic cycle through resident birds and vector competent mosquitoes and by overwintering strategies put in place by the virus to survive the winter season [35,49,50].

Phylogenetic analysis indicated the presence of a main WNV L2 Italian group, which was highly supported across all our different phylogenies. Our phylogeographic analysis and ancestral character state reconstruction suggest that the origin of the sequences included in this group was most likely in Sardinia, with a posterior probability of 68.59 for this region as ancestral location. This is an

interesting possible scenario that had not been suggested by previous studies, but which could be the result of a bias due to our sequence dataset, which only includes data for complete genomes and excludes the partial genome sequences obtained in northeast Italy in 2011 [26]. As described by previous studies, the most probable scenario is a southward expansion of the WNV L2 from Central European countries [19,51] to north-eastern regions of Italy—possibly to the Veneto region (for which the support in our ancestral character state reconstruction is 27.9), later expanding to other regions [26,51], with a genetic flow probably sustained by birds migrating either along the south-eastern migration route from Europe and western Asia to Africa, or along short migration routes from Central to Southern Europe [26]. Our molecular clock displayed in Figure 8.2 also suggests this to be the most likely scenario, as the sister group and other closely related genomes of the Italian L2 clade are from central European countries (Austria, Germany, Slovakia, Czech Republic, Serbia, and Hungary), as can be checked by looking at the full tree used to generate Figure 8.2 (see the file "wnv_world368_clock_trimmed_tipd_gtr_gamma4_strict_cc.tre" in Data Availability Statement section, https://figshare.com/articles/dataset/West_Nile_virus_lineage_2_tree_files/21518541/1, accessed on 20 October 2022). Further studies are required to clarify the first introductory events of this lineage in Italy.

Our phylogeographic analysis shows four main Italian groups that were highly supported, regardless of the framework (maximum-likelihood or Bayesian) and on the models used: (i) clade 1, characterised by sequences mostly from Veneto that became extinct in 2013–2014, as previously described in the literature [20]; (ii) clade 2, including only Sardinian strains, which probably arrived in Italy via infected birds from neighbouring countries [52] and then possibly spread to north-western Italy, indicating a possible local variant from Sardinia; (iii) clade 3, with genomes circulating in Emilia-Romagna and Piedmont, with a few sequences from Lombardy; and iv) clade 4, including mostly genomes from Veneto and Sardinia. The group division is mostly related to the diverse regions

of Italy, as also presented in the results (Figure 8.4), suggesting initial viral interregional circulation followed by a local establishment and adaptation to diverse eco-climatic conditions and maintenance hosts, which lead to strain genetic diversity and constant intra-regional WNV L2 circulation.

These findings underline the importance of the national surveillance plan and the urgent need for the development of mathematical models able to predict, early on, the WNV behaviour in the following vector season [28,53]. The eco-climatic model developed by Candeloro et al. [28], which is based on environmental covariates such as daytime and nighttime land surface temperature, normalised difference vegetation index, and surface soil moisture, is capable of generating risk maps for WNV spatial distribution probability throughout the Italian territory with 16 day-forecast periods (https://mapserver.izs.it/gis_wn_predictions/, accessed on 20 October 2022). In the 2022 WNV epidemic, it was able to indicate the presence of suitable conditions for an earlier (1–1.5 months) and wider spread of WNV in Italy (in particular, in Emilia-Romagna, Lombardy and Veneto regions (Results, Figure 8.6).

Despite the progress achieved in understanding the ecology and dynamics of West Nile virus in Italy, there are still significant knowledge gaps, especially on the role of different bird hosts as reservoirs and amplifiers of the infection and on the immune response of different species, which could affect the viral circulation among hosts and vectors. Therefore, new investigations to clarify the transmission and dynamics of viral spread are called for, as is the presence of overwintering phenomena that might allow a constant circulation of the virus from one season to the next.

8.7 Conclusions

Our findings show that WNV L2 can persist in Italy, indicating an endemic circulation, which is probably sustained or amplified by several different reservoir species. Contrary to WNV L1, which

mostly circulated in Italy between 2008 and 2011, and re-appeared on the Italian territory in the 2020–2022 epidemic seasons, WNV L2 has been constantly circulating in many regions of Italy. The substantial amount of WNV L2 data collected between 2011 and 2021 underline the fundamental importance of the Italian surveillance system in understanding the epidemiological scenario and in early detection of viral circulation (also in WNV-low risk areas and especially in regions presenting wild populations of birds subject to WNV infection). Additional epidemiological plans for active surveillance of animals received at rescue centres in Italy might also be important to promptly identify diseased birds, as suggested by Giglia et al. [9]. Deepening the knowledge on WNV ecology and transmission in wildlife would help the set-up of predictive epidemiological models for a better understanding of the viral dynamics in order to detect local spreads early on and to support the prompt implementation of response measures.

8.8 Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15010035/s1, Figure S8.1: Molecular clocks of WNV genomes belonging to all known lineages; Figure S8.2: Phylogeography of WNV L2 in Italy for the seasons from 2010 to 2021; Figure S8.3: Phylogeography of WNV L2 in Italy for the seasons from 2010 to 2021; Figure S8.4: Coalescent Bayesian Skyline analysis of WNV L2 sequences; Table S8.1: Molecular and serological tests among bird, mosquitoes, horses, and humans; Table S8.2: Metadata of WNV sequences included in the study; Table S8.3: Dataset of models used; Video S8.1: Phylogeographic analysis of WNV L2 sequences_1; Video S8.2: Phylogeographic analysis of WNV L2 sequences_2; File S8.1: Group authorship list.

8.9 Author Contributions

Conceptualization, G.M. (Giulia Mencattelli), F.M. and G.S.; methodology, G.M. (Giulia Mencattelli), A.S. and G.S.; validation, F.M., G.S., A.S., G.M. (Giulia Mencattelli) and G.S.; formal analysis, A.S., L.C., C.I., A.C. and G.M. (Giulia Mencattelli); investigation, L.T., A.D.G., A.P., V.C. and G.M. (Giulia Mencattelli); resources, G.S.; data curation, F.I., A.S., L.C., C.I., A.C., V.C., A.P. and G.M. (Giulia Mencattelli); writing G.S., A.S. and G.M. (Giulia Mencattelli); original draft preparation, G.M. (Giulia Mencattelli), A.S. and G.S.; writing—review and editing, F.M., F.I., C.I., A.C., O.R.-S., A.R., R.R., N.S., A.S., G.M. (Giovanni Marini), C.I. and G.S.; visualization, A.S., G.M. (Giulia Mencattelli), F.I., L.T., A.D.G., A.P., V.C., L.C., C.I., A.C., R.R., G.M. (Giovanni Marini), F.M., N.S., D.M., M.G.P., A.R., O.R.-S., West Nile working group and G.S.; supervision, O.R.-S., F.M., N.S., R.R., A.R. and G.S.; project administration, G.S.; funding acquisition, G.S. All authors have read and agreed to the published version of the manuscript.

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8.11 Data Availability Statement

The data presented in this study, including sequence alignments and tree files, are openly available in FigShare at https://figshare.com/articles/dataset/WNV_L2_sequence_alignments/21518529, and https://figshare.com/articles/dataset/West_Nile_virus_lineage_2_tree_files/21518541/1, respectively, both accessed on 20 October 2022. All scripts used to perform this analysis are available

at the https://github.com/andrea-silverj/WNV-L2_IT GitHub repository, accessed on 20 October 2022.

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8.13 Conflicts of Interest

All the authors declare no conflict of interest.

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CHAPTER 9. West Nile Virus and Usutu Virus: A Post-Mortem Monitoring Study in Wild Birds from Rescue Centers, Central Italy

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by

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9.1 Abstract

West Nile virus (WNV) and Usutu virus (USUV) are mosquito-borne flaviviruses that have been associated with neurological diseases in humans and wild birds. Wild bird rescue centers are potential significant hotspots for avian infection surveillance, as recognized in the Italian Integrate National Surveillance Plan for Arboviruses. Here we report the results of a post-mortem active monitoring study conducted from November 2017 to October 2020 on animals hosted in five wild bird rescue centers of Central Italy. Five hundred seventy-six (n = 576) wild birds were tested by real-time polymerase chain reaction (RT-PCR) for the presence of WNV or USUV RNA fragments. No birds tested positive for USUV RNA (n = 0; 0.00%). Evidence of WNV RNA (Ct value = 34.36) was found in one bird (n = 1; 0.17%), an adult little grebe (*Tachybaptus ruficollis* subsp. ruficollis), that tested WNV positive in December 2019. This study highlights the strategic role of wildlife rescue centers in monitoring both the introduction and circulation of avian emerging zoonotic diseases. In addition, the presence of WNV during the cold season evidences the possible role of birds in overwintering mechanisms in the Italian territory and requires further investigations.

9.2 Keywords

Arboviruses; West Nile; Usutu; monitoring; wild birds; Central Italy

9.3 Introduction

The family Flaviviridae includes several viral species [1]. Most recent outbreaks have been caused by West Nile and Usutu viruses, two species belonging to the Flavivirus genus. Included in the Japanese Encephalitis group, they are genetically closely related and share many traits of their transmission cycle which involves birds (amplifying host) and mosquitoes (vector). Mammals, including humans, might be infected through mosquito bites and represent dead-end hosts of these infections. When

infected, humans usually develop flu-like syndrome, although sometimes cases of neuroinvasive disease can occur [2,3,4]. In Italy, WNV and USUV infections, sometimes associated with clinical neuroinvasive disease, have been reported in wild birds, horses and humans since the late 1990s [4,5,6,7,8,9,10,11,12,13]. Since 2020, an integrated National Plan for Prevention, Surveillance and Response to Arbovirus (PNA) has been in place as a five-year plan [14] and includes, among other things, the activity of WNV and USUV. PNA aims to early detect viral circulation in Italy to minimize the risk of human infections. In the plan, the Italian territory has been classified in three types of areas (high, low and minimum risk) according to the epidemiological–environmental conditions and, consequently, the transmission risk: (1) High risk areas are those where WNV is circulating or has circulated in at least one of the 5 previous years. (2) Low risk areas are those where WNV has never been or has been rarely reported and where eco-climatic conditions are favorable to viral circulation. (3) Minimum risk areas are those where WNV has never been reported and where eco-climatic conditions are not suitable for WNV circulation [14]. In all three types of areas, the surveillance includes monitoring of wild bird mortality. Wild bird rescue centers (WRCs) are scattered in the territory. They are the main providers of medical support in emergency and wild diseased animal care and may represent an important source of information on WNV and USUV circulation and their virulence characteristics. This study aims to examine carcasses from WRCs of Central Italy to monitor the circulation and introduction of WNV and USUV. Additionally, to obtain possible new insights on the role of wild birds in facilitating the overwintering of USUV and WNV in the study area, bird samples were collected and examined throughout the year.

9.4 Materials and Methods

9.4.1 Animals

Carcasses of wild birds (n = 576) collected by 5 rescue centers located in Umbria, Latium, and Tuscany (Central Italy) from November 2017 to October 2020 were used in this study. All birds died spontaneously or were humanely euthanized for clinical conditions compromising animal welfare (e.g., gunshot lesions, head trauma and fractures). The animals were submitted for necropsy at the Department of Veterinary Medicine, University of Perugia, Umbria, Italy. During necropsy, the cadaver condition was scored as follows: Code 1 (absent autolysis), Code 2 (mild autolysis), Code 3 (moderate autolysis), Code 4 to Code 5 (marked autolysis or corruption, respectively) [15]. For animals with Codes 1 to 3, tissue samples (1 cm3) collected during the necropsy were submitted to RT-PCR, histopathology, and immunohistochemistry analyses to investigate either the presence of WNV or USUV or eventual related lesions; for birds with cadaver condition Code 4 or 5, the collected tissues were submitted only for RT-PCR analyses [16]. Ethical approval was not required for this study.

9.4.2 WNV and USUV Molecular Detection

Samples of heart, liver, spleen, kidney, and brain were collected, stored at −80 °C and sent to the WOAH (formerly OIE) Reference Laboratory for West Nile Fever at the Istituto Zooprofilattico Sperimentale “G. Caporale” (Teramo, Italy) for molecular detection of WNV and USUV RNA by RT-PCR analyses. Tissues (brain, heart, liver, spleen, and kidney) were homogenized in phosphate-buffered saline solution. Briefly, after tissue homogenization, RNA extraction was performed by using the MagMAX CORE Nucleic Acid Purification KIT (Applied Biosystem, Termofisher Scientific, Life technologies corporation, Austin, TX, USA), following the manufacturer’s instructions.

The extracted RNA was amplified as described in the literature, using a double RT-PCR approach, one aiming for simultaneous detection of WNV lineages 1 and 2 [16] and the other aiming for detection of all WNV lineages [17]. For USUV, the RT-PCR was performed as previously described by Cavrini et al., 2011 [18].

9.4.3 Histopathology and Immunohistochemistry (IHC)

For animals with Code 1 to 3, routine histological examination was performed on formaline-fixed paraffin-embedded (FFPE) tissues collected during necropsy. For the immunohistochemistry, 3 µm sections were obtained from FFPE tissues; the sections were deparaffinized and rehydrated in alcohols. Endogenous peroxidases were blocked by 3% H2O2 in methanol incubation for 10 min and antigen was retrieved by proteinase K digestion for 10 min at 37 °C. A goat serum block was applied before incubation with the primary antibody. For primary incubation, anti-WNV serum (FLI, Jena, Germany) was used with 1:1700 dilution. As secondary antibody, Bright vision 1 step detection system anti-rabbit HRP was used, with the aminoethyl carbazole (AEC) as substrate. For USUV, IHC was performed as previously described [19]. Primary polyclonal Rabbit Anti-CD3 antibody was used at a dilution of 1:200 (A0452, Dako, Italy) to mark T-cells as a common player of adaptive immunity to intracellular agents in inflammatory infiltrates [20]. As secondary antibody, Mouse Envision HRP kit was used (Ab93697; Abcam, Italy) with the AEC as substrate.

9.4.4 Geographical Distribution Analysis

To map geographical distribution of the rescued animals, collection sites were registered, and coordinates recorded. If the exact collection site was not available, data of the rescue center were used. To visualize the geographical distribution of the collected animals and the location of identified cases,

the open-source Quantum Geographic Information System (QGIS®) (v. 3.16.10) was used. The geographical distribution of sampling sites and positive case location is shown in Figure 9.1.

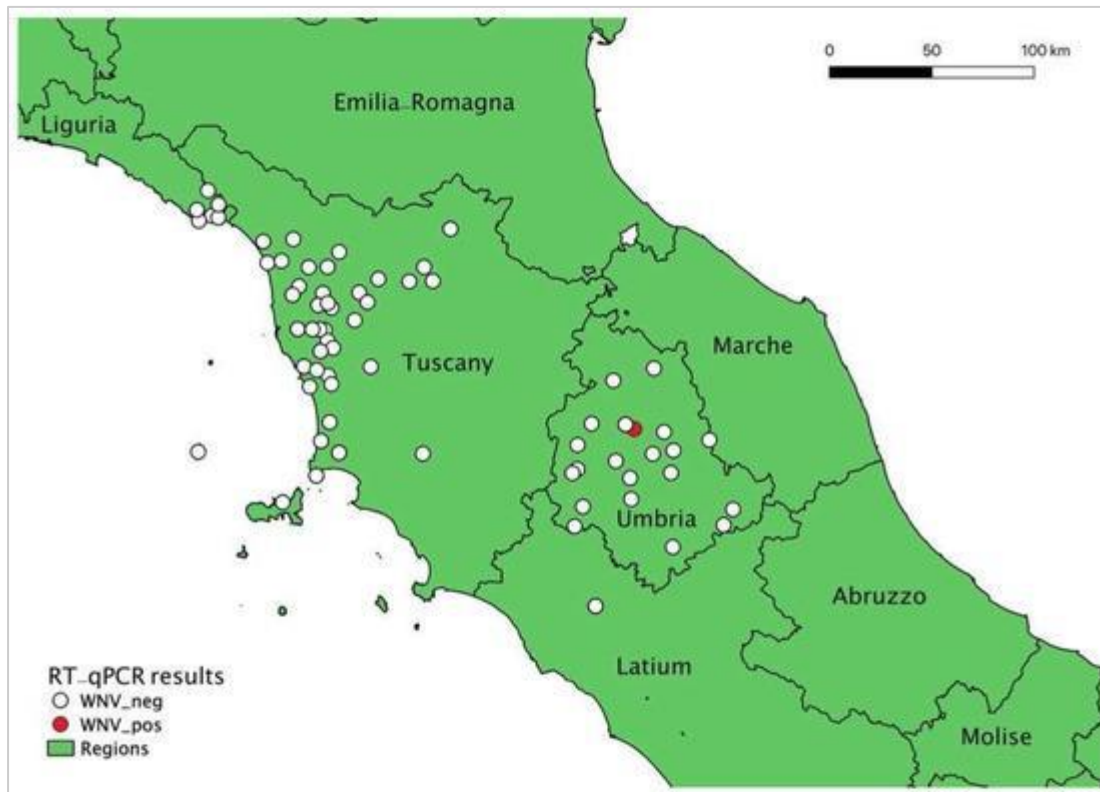

Figure 9.1 Geographical distribution of sampling sites and positive case location (QGIS map).

The map shows the sites of wild birds sampling (white dots) distributed in Umbria, Lazio, Tuscany, and the location of the WNV-positive little grebe (Tachybaptus ruficollis subsp. ruficollis) (red dot).

9.5 Results

One out of 576 (0.17%) wild birds tested for the presence of WNV and USUV RNA was found positive for WNV (Ct value: 34.36), while none of the wild birds tested were positive for USUV (Table 9.1). The only WNV-positive bird was a little grebe (*Tachybaptus ruficollis* subsp. ruficollis) belonging to the Order Podicipediformes, Family Podicipedidae. The bird was found on the street side

in the Umbria region at the end of December 2019 and admitted to the Veterinary Teaching Hospital of the Department of Veterinary Medicine of the University of Perugia (Italy). It showed depression and difficulties in its movements, partially justified by the presence of a complete luxation of the 3rd phalanx of the 3rd digit, confirmed by radiography. The bird died spontaneously during the day after admission. At necropsy, a mild hepatomegaly and multiorgan congestion were observed. At microscopy, the heart showed mild multifocal loss of cardiomyocytes and interstitial oedema. In the kidneys, tubulo-interstitial nephritis and tubular necrosis were observed, while in the brain, scattered lymphocytes were seen in the meninges and perivascular neuroparenchyma. At immunohistochemistry, leukocyte infiltration consisted of CD3-positive lymphocytes. WNV and USUV antigens were not detected. Additional data regarding the number and percentages of birds collected for the three regions, and for the four seasons of each year of monitoring, are reported in the Supplementary Materials (Figures S9.1, S9.2 and Table S9.1).

Table 9.1 Summary of examined avian species, migratory behavior and WNV RT-PCR results. Data on USUV RT-PCR are not included as results were negative for all the birds

Order	Family	Species	Migratory/Resident	WNV RT-PCR
Accipitriformes	Accipitridae	Booted eagle (*Hieraaetus pennatus*)	L	0/1
		Golden eagle (*Aquila chrysaetos*)	R/S	0/2
		Northern goshawk (*Accipiter gentilis*)	R/S	0/3
		Indian vulture (*Gyps indicus*)	R	0/1
		Short-toed snake eagle (*Circaetus gallicus*)	L	0/2
		Western marsh harrier (*Circus aeruginosus*)	P	0/1

		European honey buzzard (*Pernis apivorus*)	L	0/6
		Rough-legged buzzard (*Buteo lagopus*)	S	0/1
		Common buzzard (*Buteo buteo*)	S	0/37
		Harris's hawk (*Parabuteo unicinctus*)	R	0/1
		Eurasian sparrowhawk (*Accipiter nisus*)	P	0/21
Anseriformes	Anatidae	Mute swan (*Cygnus olor*)	R	0/1
		Mallard (*Anas platyrhynchos*)	R/S	0/1
Apodiformes	Apodidae	Common swift (*Apus apus*)	L	0/16

Bucerotiformes	Upupidae	Eurasian hoopoe (*Upupa epops*)	R	0/1
Caprimulgiformes	Caprimulgidae	European nightjar (*Caprimulgus europaeus*)	L	0/2
Charadriiformes	Scolopacidae	Woodcocks (*Scolopax rusticola*)	L	0/2
	Laridae	Common gull (*Larus canus*)	R/S	0/28
		Yellow-legged gull (*Larus michahellis*)	R	0/9
Columbiformes	Columbidae	Common wood pigeon (*Columba palumbus*)	P	0/4
		Rock dove (*Columba livia*)	R	0/10

		Eurasian collared dove (*Streptopelia decaocto*)	R	0/7
Coraciiformes	Meropidae	European bee-eater (*Merops apiaster*)	L	0/3
	Alcedinidae	Kingfisher (*Alcedo atthis*)	S	0/1
Falconiformes	Falconidae	Lanner falcon (*Falco biarmicus*)	R	0/1
		Peregrine falcon (*Falco peregrinus*)	R/L	0/8
		Kestrel (*Falco tinnunculus*)	R/L	0/43
		Eurasian hobby (*Falco subbuteo*)	L	0/4
		Merlin (*Falco columbarius*)	R/L	0/1

Gruiformes	Rallidae	Eurasian coot (*Fulica atra*)	R/P	0/1
Passeriformes	Corvidae	Carrion crow (*Corvus corone*)	R	0/25
		Eurasian magpie (*Pica pica*)	R	0/93
		Eurasian jay (*Pica pica*)	R	0/4
		Western jackdaw (*Coloeus monedula*)	R/P	0/2
	Fringillidae	Canary (*Serinus canaria*)	R	0/12
		European goldfinch (*Carduelis carduelis*)	R/P	0/1
		Hawfinch (*Coccothraustes coccothraustes*)	R/S	0/1

		European greenfinch (*Chloris chloris*)	R	0/1
	Hirundinidae	Common house martin (*Delichon urbicum*)	L	0/5
		Barn swallow (*Hirundo rustica*)	L	0/6
	Muscicapidae	European robin (*Erithacus rubecula*)	R/P	0/8
	Passeridae	House sparrow (*Passer domesticus*)	R	0/17
	Phylloscopidae	Common chiffchaff (*Phylloscopus collybita*)	L	0/1
	Sturnidae	Common starling (*Sturnus vulgaris*)	R/S	0/6

	Sylviidae	Eurasian blackcap (*Sylvia atricapilla*)	P	0/4
	Turdidae	Eurasian blackbird (*Turdus merula*)	R/P	0/16
		Song thrush (*Turdus philomelos*)	P	0/7
Pelecaniformes	Ardeidae	Grey heron (*Ardea cinerea*)	P	0/7
		Cattle egret (*Bubulcus ibis*)	S/L	0/5
		Heron NI	-	0/3
		Eurasian bittern (*Botaurus stellaris*)	P	0/1
Piciformes	Picidae	European green woodpecker (*Picus viridis*)	R	0/7

Podicipediformes	Podicipedidae	Little grebe (*Tachybaptus ruficollis*)	R/S	0/1
Strigiformes	Strigidae	Tawny owl (*Stirx aluco*)	R	0/22
		Eurasian scops owl (*Otus scops*)	L	0/3
		Little owl (*Athene noctua*)	R	0/72
		Long-eared owl (*Asio otus*)	P	0/15
		Eurasian eagle-owl (*Bubo bubo*)	R	0/1
		NI owl	-	0/2
	Tytonidae	Barn owl (*Tyto alba*)	R	0/6
Suliformes	Phalacrocoraci dae	Great cormorant (*Phalacrocorax carbo*)	P/L	0/2

NI = Not further identifiable; L = long-distance migrant, S = short-distance migrant, P = partial migrant, Irr = irregular migrant, R = resident.

9.6 Discussion

Epornitic mosquito-borne zoonotic flaviviruses associated with cases in animals and humans have significantly increased their impact on public health in the last decades [21,22]. Their continuous circulation throughout Europe have raised the attention and supported the development of National Surveillance Systems to early detect virus circulation and limit its spread [6]. For mosquito-borne zoonotic flavivirus circulation, birds, being the amplifying hosts, are the major target of surveillance strategies [21,23]. This study reports a post-mortem monitoring on wild birds admitted in WRCs of Central Italy between November 2017 and October 2020. According to the WNV risk classification areas as established in the PNA [14], the WRCs involved in this study were located in the low (Umbria) and in the high-risk areas (Latium and Tuscany). WNV lineage 2 was detected in a little grebe. The little grebe is in the IUCN Red List of Threatened Species (2019) reported as a least-concern species for the risk of extinction. It inhabits small and shallow wetlands, and it is considered resident in Italy. Being a resident species year-round implies that it has probably been infected within the rescue area. In other words, it implies that the infection likely occurred in Umbria. As far as we know, this was the first evidence of WNV circulation in Umbria, an area classified as low risk for WNV circulation. However, looking further, finding a WNV-positive bird in Umbria was somewhat expected, as USUV, which shares the same ecological niche of WNV, has been repeatedly detected by the PNA in this region [24]. Conversely, finding a WNV-positive case at the end of December, when vectors are not flying, was rather surprising. In this case, the histological lesions were mild, antigen could not be detected, and the Ct values were quite high (34.36 cycles). These results, on the one hand, exclude the hypothesis of a recent infection, but on the other hand, they might instead suggest the presence of a persistent infection [25]. Persistent infection has been defined as the detection of a virus in host tissues after viremia has receded [26]. The persistent viral load in organs of birds, and in particular in those belonging to prey species like the little grebe, might sustain the

WNV transmission to predators (e.g., birds of prey) months after the mosquito season [27,28]. The little grebe was the only bird out of 576 (0.17%) found positive for WNV RT-qPCR. None of the birds tested positive for USUV RT-qPCR. The lack of WNV and USUV infection cases in high-risk areas (Latium and Tuscany) also during low vector activity periods, additionally support the idea previously suggested in the literature that, contrarily to vectors, birds seem have a minor role in the overwintering of these viruses [29]. Additionally, the high number of negative results and of those found in the high-risk areas might be indicative of the low virulence of most of the WNV and USUV strains circulating in these regions. In the same year of WNV detection in the little grebe, no WNV was detected in mosquitoes as reported from the reports of the OIE national reference lab in Italy (https://www.epicentro.iss.it/westnile/bollettino (accessed on 19 August 2022)). For this reason, our results demonstrate that, even if not ideal to be used alone in a surveillance program because of rescuing only diseased or injured animals, WRCs can be very useful to the National Surveillance Plan in the early detection of the introduction and circulation of emerging zoonotic diseases, improving its sensitivity, expanding the period of surveillance by including the winter months, and providing important information on the virulence of the circulating strains. As a final point, considering that the little grebe is a threatened species, we cannot underestimate that WNV might play a role in this species' loss.

9.7 Conclusions

For the first time, a case of WNV infection in Umbria (Central Italy), a region currently classified as low-risk area for WNV, was detected during a low-vector activity season (winter). This result suggests that the active monitoring performed through the National Plan of Surveillance on wild birds from wild bird rescue centers can help to better detect the introduction and circulation of WNV. The lack of detection of USUV circulation among mosquitoes and birds needs to be monitored to keep assuring

early detection in humans and animals. To ensure WNV circulation and infections of animals and humans are kept under control, additional systematic epidemiological plans of active surveillance on animals received at rescue centers in Italy are highly recommended.

9.8 Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14091994/s1, Table S9.1. Summary of the birds examined for each season in each year of monitoring, Figure S9.1. Clustered bar chart on the percentages of birds examined in each season per year. For the year 2017 and 2021 the distribution of the birds examined for each season was affected from the starting and ending period of the study. For the year 2019, year of detection of WNV in the little grebe, the monitoring was more uniformly distributed throughout the four seasons. In general, for 2018, 2019, and 2020, most birds were examined in autumn and summer, Figure S9.2. Pie chart on the number and percentages of birds examined in each region. The majority of birds examined were collected in Umbria, site of the were the post-mortem investigation and sampling was performed.

9.9 Author Contributions

Conceptualization and design of the work, data collection and analysis, manuscript draft-preparation, G.G.; Data collection and analysis, manuscript draft-preparation and revision, G.M.; Data collection and analysis, manuscript-revision, E.L., G.A. and M.G.; supervision, manuscript draft-revision, A.G. and J.M.A.v.d.B.; Conceptualization and design of the work, supervision, manuscript draft-revision, G.S. and M.T.M. All authors have read and agreed to the published version of the manuscript.

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9.11 Institutional Review Board Statement

The study did not require ethical approval.

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9.13 Conflicts of Interest

The authors declare no conflict of interest.

9.14 References

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CHAPTER 10. GENERAL DISCUSSION

The following general discussion summarizes the content and principal findings of the thesis, discusses some relevant issues, and draws overall conclusions.

10.1 Recapitulation

Chapter 1 introduces Arthropod-Borne Diseases with a special focus on West Nile virus and elucidates the three main objectives of the thesis.

Chapter 2 provides the state of art on WNV investigation carried out in Africa, characterizing the geographical distribution within specific host and vector populations in the Continent (objective 1).

Chapter 3 describes the development and evaluation of a whole genome amplicon-based sequencing approach for WNV L1 and L2 strains (objectives 2 & 3).

Chapter 4 describes WNV phylogenetic and phylogeographical patterns between Africa and Europe, uncovering the viral dynamics of WNV L1 and L2 strains between the two Continents (objective 2).

Chapters 5 –9 define the genetic structure and epidemiology of Italian WNV L1 and L2 strains, giving an insight into the viral circulation dynamics in the Italian territory (objective 3).

Chapter 10 discusses the major finding of the thesis and identifies future perspectives.

10.2 Main findings

In this Section, I discuss the main findings of the thesis in relation to the aim set out at the beginning of the thesis, detailed for each chapter.

Chapter 2. The aim was to characterize the geographical distribution within specific host and vector populations in Africa. Since its discovery in 1937 in Uganda [3], little is known about WNV distribution in Africa, especially due to the difficulty of its diagnosis with clinical signs usually similar to those of many *Flaviviruses* and other pathogens co-circulating in the Continent [4], and to the lack of appropriate fundings, resources and surveillance systems of many African countries [5]. Despite this, Africa is still the Continent where the number of tourists is expected to double from 2010 to 2030 [6], accelerating the contact between travelers and endemic areas for WNV disease [7]. Moreover, it is the Continent hosting millions of migratory birds [8], which could act as amplifiers and spread the virus from native areas to new ones. For all these reasons I considered very important to fill current knowledge gaps on WNV circulation in the African Continent.

Results provide new insights on: (i) the presence of WNV in many mosquito species in Africa, with viral isolation reported in 46 species naturally infected; the low number of vector-competent studies in the Continent, with only eight experimentations conducted in Madagascar, Algeria, Morocco, Tunisia, Senegal, Kenya, and South Africa so far [2,9–14]; (ii) the vectorial capacity of *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. vansomereni*, *Cx. univittatus*, *Cx. theileri*, and *Cx. neavei* mosquitoes in the African Continent [2,9,12,14–16]; (iii) the WNV parenteral infection in *O. savignyi* and vertical and horizontal transmission in *A. arboreus* ticks in Egypt [17,18]. In addition, chapter 2 points out the existence of WNV vectorial competency in diverse arthropods, and the need for more vector-competent studies to be conducted in these animals. It also stresses the presence of numerous seroprevalence studies in humans and other animals, providing evidence of WNV circulation in at least 28 African countries, and the lack of knowledge for 19 countries. Moreover, it shows the existence of possible bias in many of these studies due to the non-specificity of the methods used, leading to possible cross-reactivity with other *Flaviviruses* widely diffused in the Continent. Finally, it highlights the co-circulation of the following four WNV lineages in Africa:

L1, most diffused in Western-Northern African countries, with evidence reported in Senegal, Ivory Coast, Morocco, Algeria, Tunisia, Egypt, Central African Republic, Kenya, and South Africa [1,2,19–23];

L2, most diffused in Southern African countries, with reports in Congo, Uganda, Tanzania, Mozambique, Namibia, Botswana, Madagascar, South Africa, Senegal, Djibouti, CAR, and Kenya [1,2,19,23–29];

L7, nowadays known as a different virus called KOUTV, diffused in Senegal, Gabon, Niger, and Somalia [30];

putative L8, detected only in Senegal so far [2] and considered a good candidate for a future WNV vaccine due to its low viraemia [1].

Chapter 3. Of the four lineages present in Africa, only two - L1 and L2 - spread to Europe [21]. Many studies suggested a possible introduction of WNV L1 in Italy and France in 1998 and 2000 [31,32], respectively, from Morocco [33,34], while other studies stressed the unknown origin of WNV L2 [35,34], which appeared for the first time in Hungary in 2004 [21]. These areas are along the migratory routes of birds migrating along the African-Eurasian flyways [8], stressing their importance in viral introduction and spread in Europe. To describe WNV phylogenetic and phylogeographical patterns between Africa and Europe, we designed two novel WNV L1 and L2 set of primers for an amplicon-based sequencing approach.

Chapter 3 describes the development and evaluation of the two set of primers which made possible: (i) the direct, highly specific, and low-cost sequencing of clinical samples, under resource-limited conditions too; (ii) the production of a consistent dataset of WNV L1 and WNV L2 genome sequences obtained from samples collected in Italy and Senegal between 2006 and 2022.

By integrating our dataset into a wider dataset of WNV L1 and WNV L2 worldwide genomes with detailed geographical observations, we performed novel phylogenetic and phylogeographic inference reconstructions.

Chapter 4. This work aimed at better understanding of the migratory patterns of WNV L1 and L2 between Africa and Europe. It stresses the existence of WNV L1 and L2 transmission events among diverse African countries. Furthermore, it proposes WNV L1 strain spread through an ideal line that connects Senegal, Morocco, Spain, Southern France, and Northern Italy between the 1980s and 1990s, and WNV L2 spread from South Africa to Hungary through DRC around the 2000s. Also, it confirms the existence of sporadic WNV L1 back introductions between Western-Mediterranean European countries (Italy, France, and Spain) and Northern-Western Africa (Morocco and Senegal), as recently suggested by Ndione et al. [37]. Finally, it points out the overlapping of bird migratory patterns between breeding and nonbreeding countries in Europe and Africa [6] with WNV L1 and L2 viral dispersal dynamics.

While in Africa no clinical signs are usually detected [2], the efficiency of the surveillance systems of many European countries uncovers the WNV serious impact on public and animal health [34,35]. In Europe, Italy is one of the most seriously affected countries [36–45], with nearly 300 WNND cases and 37 deaths in humans and WNV L1-L2 positivities reported in 45 horses and 349 wild and target birds, in the 2022 epidemic season only (https://westnile.izs.it/j6_wnd/docBolletItaPeriodico?annoDocumento=2022).

Chapters 5 – 9. This part of the work aimed at investigating WNV L1 and L2 viral dynamics in Italy, giving deep insights into the epidemiology and genetic features of the two viral strains. It stresses a different behavior of the two strains, showing: (i) WNV L1 first introduction in Italy from Northern-Western African countries around 1985; (ii) possible sporadic back and-forth re-introductions

between these areas; and (iii) silent periods with unnoticed circulation lasting sometimes for more than 10 years in Italy. The contents of these chapters also evidence: (iv) WNV L2 first introduction in Italy between 2008 and 2010 from Central-Eastern Europe, where the strain was possibly first introduced in Hungary from South Africa through DRC around the 2000s; (v) initial viral interregional circulation and local establishment and adaptation to diverse eco-climatic conditions in ten out of 20 Italian regions; (vi) constant intra-regional circulation throughout the time, with no significant novel introductory events from other countries in recent years.

In addition, chapters 5 – 9 show: (vii) the existence of WNV L1 preferred corridors connecting Italy and Western-Mediterranean countries, such as France, Spain, and Portugal; (viii) the current presence of two diverse WNV L1 strains in Italy, one in the North-East, and one intra-regionally in the Campania region; (ix) an increasing incidence of neurological disease cases in humans in 2022, possibly due to new genetic mutations, previously highlighted by Barzon et al. [38]; (x) the existence of co-infections with WNV L2 in birds and mosquitoes.

These chapters also point out (xi) the occurrence of WNV overwintering mechanisms in Italy, probably supported by bird-to-bird, rodent-to-bird, or mosquito-to-bird transmission routes, which contributes to WNV local endemization and viral transmission from one epidemic season to the next.

Furthermore, chapters 5 – 9 highlight for both strains: (xii) an introduction from Africa to Europe through long-distance migratory birds; (xiii) a continuous transmission between Western Mediterranean countries, probably sustained by short-distance migratory birds; (xiv) the presence of both strain interregional circulation and local endemization in some Italian regions, supported by resident wild birds and vector competent mosquitoes mainly belonging to the Culex genus; (xv) the possibility of new re-introduction or unnoticed silent circulation events. Finally, they show the crucial importance of the surveillance system and the strategic role of wildlife rescue centers in monitoring

both the introduction and circulation of avian emerging zoonotic diseases in Italy. Specifically, chapter 8 validates a predictive model identifying the presence of suitable conditions for 2022 earlier and wider spread in Italy, stressing the importance of this type of model for the early warning detection of WNV outbreaks.

10.3 Conclusion and future perspectives

We built a new consistent dataset of complete L1 and L2 genome sequences available that might be a reference for future investigations and would help in shaping public health novel strategies to reduce WNV impact. We pointed out the existence of knowledge gaps in many African countries, stressing the strong need for coordinated surveillance activities and investments in research within Africa, and for further phylogenetics, ecological and epidemiological studies to be conducted. Moreover, we highlighted the spread of the virus between Africa and Europe and the circulation between diverse European countries through long and short-distance bird migration, respectively. We evidenced the presence of a strong transmission cycle and overwintering mechanisms mostly involving resident birds and vector-competent mosquitoes in some Italian regions.

Despite the progress achieved in understanding the ecology and dynamics of WNV, there are still significant knowledge gaps, especially on: (i) the role of different bird hosts as reservoirs and amplifiers of the infection and on the immune response of different species, which could affect the viral circulation among hosts and vectors either in Europe and in Africa; (ii) the relation between new conservative amino acid point mutation and increased virulence in humans and other animals. Further research is needed in this direction, both in Italy and Africa.

Finally, we emphasized the importance of including wildlife rescue centers in the National surveillance plan, to better monitor both the introduction and circulation of avian emerging zoonotic

diseases also during wintertime, and the importance of coordinated surveillance activities implementation between Africa and Europe.

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