

RESEARCH ARTICLE

Reconstruction of the molecular evolution of Usutu virus in Germany: Insights into virus emersion and circulation

Felicitas Bergmann¹✉, Cora M. Holicki¹✉, Friederike Michel¹✉, Sabine Bock², Nelly Scuda³, Grit Priemer⁴, Susanne Kenklies⁵, Timo Siempelkamp⁶, Jasmin Skuballa⁷, Claudia Sauerwald⁸, Louise Herms⁹, Aemero Muluneh¹⁰, Martin Peters¹¹, Andreas Hlinak², Martin H. Groschup^{1,12}, Balal Sadeghi¹*✉, Ute Ziegler^{1,12}*

1 Institute of Novel and Emerging Infectious Disease, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany, **2** Berlin-Brandenburg State Laboratory, Frankfurt (Oder), Germany, **3** Bavarian Health and Food Safety Authority, Erlangen, Germany, **4** State Office of Agriculture, Food Safety and Fisheries Mecklenburg-Vorpommern (LALLF), Rostock, Germany, **5** State Office for Consumer Protection Saxony-Anhalt (LAV), Stendal, Germany, **6** Thuringian State Office for Consumer Protection, Bad Langensalza, Germany, **7** State Institute for Chemical and Veterinary Analysis Karlsruhe, Karlsruhe, Germany, **8** Department of Veterinary Medicine, Hessian State Laboratory, Gießen, Germany, **9** Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Hannover, Germany, **10** Saxon State Laboratory of Health and Veterinary Affairs, Dresden, Germany, **11** Chemical and Veterinary Investigation Office (CVUA), Arnsberg, Germany, **12** German Center of Infection Research (DZIF), Partner site Hamburg-Lübeck-Borstel-Riems, Greifswald-Insel Riems, Germany

✉ These authors contributed equally to this work.

* Current address: Saxon State Laboratory of Health and Veterinary Affairs, Dresden, Germany

* balal.sadeghi@fli.de (B.S.); ute.ziegler@fli.de (U.Z.)



OPEN ACCESS

Citation: Bergmann F, Holicki CM, Michel F, Bock S, Scuda N, Priemer G, et al. (2023) Reconstruction of the molecular evolution of Usutu virus in Germany: Insights into virus emersion and circulation. *PLoS Negl Trop Dis* 17(10): e0011203. <https://doi.org/10.1371/journal.pntd.0011203>

Editor: Michael R. Holbrook, NIAID Integrated Research Facility, UNITED STATES

Received: March 2, 2023

Accepted: September 7, 2023

Published: October 2, 2023

Copyright: © 2023 Bergmann et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All data generated or analysed during this study are included in this published article and its [Supporting Information files](#). The consensus sequences have been submitted to the NCBI database (GenBank with the accession numbers: OR166135-OR166169 and OR141529-OR141606).

Funding: This research was funded by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2819113919 (MHG, UZ), by

Abstract

Usutu virus (USUV) is a mosquito-borne flavivirus that is widely distributed in southern and central Europe. The zoonotic virus circulates primarily between birds and mosquitoes, can, however, in rare cases infect other mammals including humans. In the past, USUV has been repeatedly associated with mass mortalities in birds, primarily blackbirds and owls. Birds commonly succumb either due to the peracute nature of the infection or due to severe encephalitis. In Germany, USUV has spread rapidly since its first detection in 2010 in mosquitoes under the presence of susceptible host and vector species. Nonetheless, there is to date limited access to whole genome sequences resulting in the absence of in-depth phylogenetic and phylodynamic analyses. In this study, 118 wild and captive birds were sequenced using a nanopore sequencing platform with prior target enrichment via amplicons. Due to the high abundance of Europe 3 and Africa 3 in Germany an ample quantity of associated whole genome sequences was generated and the most recent common ancestor could be determined for each lineage. The corresponding clock phylogeny revealed an introduction of USUV Europe 3 and Africa 3 into Germany three years prior to their first isolation in the avifauna in 2011 and 2014, respectively. Based on the clustering and temporal history of the lineages, evidence exists for the genetic evolution of USUV within Germany as well as new introductions thereof into the country.

the German Ministry of Education and Research (BMBF) grant number 01KI2026D (MHG, UZ) and by the German Center for Infection Research (DZIF); Project Number TTU 01.804 (MHG, UZ) and 01.808 (UZ). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Author summary

Usutu virus is transmitted by mosquitoes and has so far been detected in various bird species on the African and European continent. The virus was isolated for the first time near the Usutu River in Swaziland, South Africa in 1959. Around the middle of the 20th century, the virus was introduced to Europe by migratory birds. In Germany, the virus was initially detected in 2010 in mosquitoes and in 2011 in birds and was associated with numerous bird deaths in the following years. To better understand the viral genome, several platforms are available to date to generate whole genome sequences. In this manuscript, a portable real-time sequencer was used to obtain Usutu virus genomes from birds sampled from 2017 to 2021 throughout the country. The subsequent phylogenetic analysis of these sequences showed that two Usutu virus lineages are prevalent in Germany and distributed nationwide. These two lineages could in turn be divided into two and three subclusters, respectively. It could be demonstrated that the time lag between the detection of the Usutu lineages in birds and the most recent common ancestor is very short, only a few years long, indicating the success of the stringent surveillance efforts in place.

Introduction

Usutu virus (USUV) is an arbovirus which belongs to the *Flaviviridae* family, genus *Flavivirus*. Its positive sense single stranded RNA genome, of 11,064 nucleotides, encodes a single poly-protein which is cleaved by viral and host proteases into three structural (C, prM, E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [1,2]. USUV was first isolated in Swaziland, Africa in 1959 from a mosquito [3]. USUV circulates in an enzootic cycle between mosquitoes as vectors and birds as reservoir and amplifying hosts. Mosquitoes belonging to the *Culex pipiens* complex represent the main vector [4].

Extremely susceptible bird species such as passerine species including blackbirds (*Turdus merula*), or house sparrows (*Passer domesticus*), and birds of prey like great grey owls (*Strix nebulosa*) serve as natural hosts [5–8]. So far, USUV was found primarily in association with die-offs of susceptible birds, primarily blackbirds in Germany [9]. These mass mortality events have occurred in wild birds all over Central Europe (e.g., in Austria [7], Hungary [6], Switzerland [10], Italy [11], Czech Republic [12], and Germany [8]) since the first reported outbreak in Vienna, Austria in 2001 [13] (Fig 1). In retrospect, the first known occurrence in Europe dates back to 1996 in Italy, in association with a blackbird mortality event [14]. However, Engel et al. 2016 [15] assumed that the virus was probably already introduced earlier, between the 1950s and 1960s, via bird migration from Africa into Western and Central Europe, followed by a rapid geographic spread of the virus.

The virus also has a zoonotic potential and can infect humans, in rare cases causing severe neurological symptoms [16–23]. Humans as well as other susceptible mammalian species are considered dead-end hosts as they can become infected but cannot sustain the transmission cycle. USUV has in the past been found in horses [24,25], dogs [26], deer [27], wild boar [28], bats [29], squirrels [30], and rodents [31].

The genetic variability of USUV was studied by phylogenetic studies targeting partial sequences, especially of the envelope E and NS5 genes, and whole genome sequences [15,32–35,4,36]. These analyses resulted in the distinguishment of eight distinct lineages, which cluster together according to their geographic origin of detection: Africa 1–3 and Europe 1–5. Migratory birds from Africa are thought to have brought different USUV lineages to Europe at

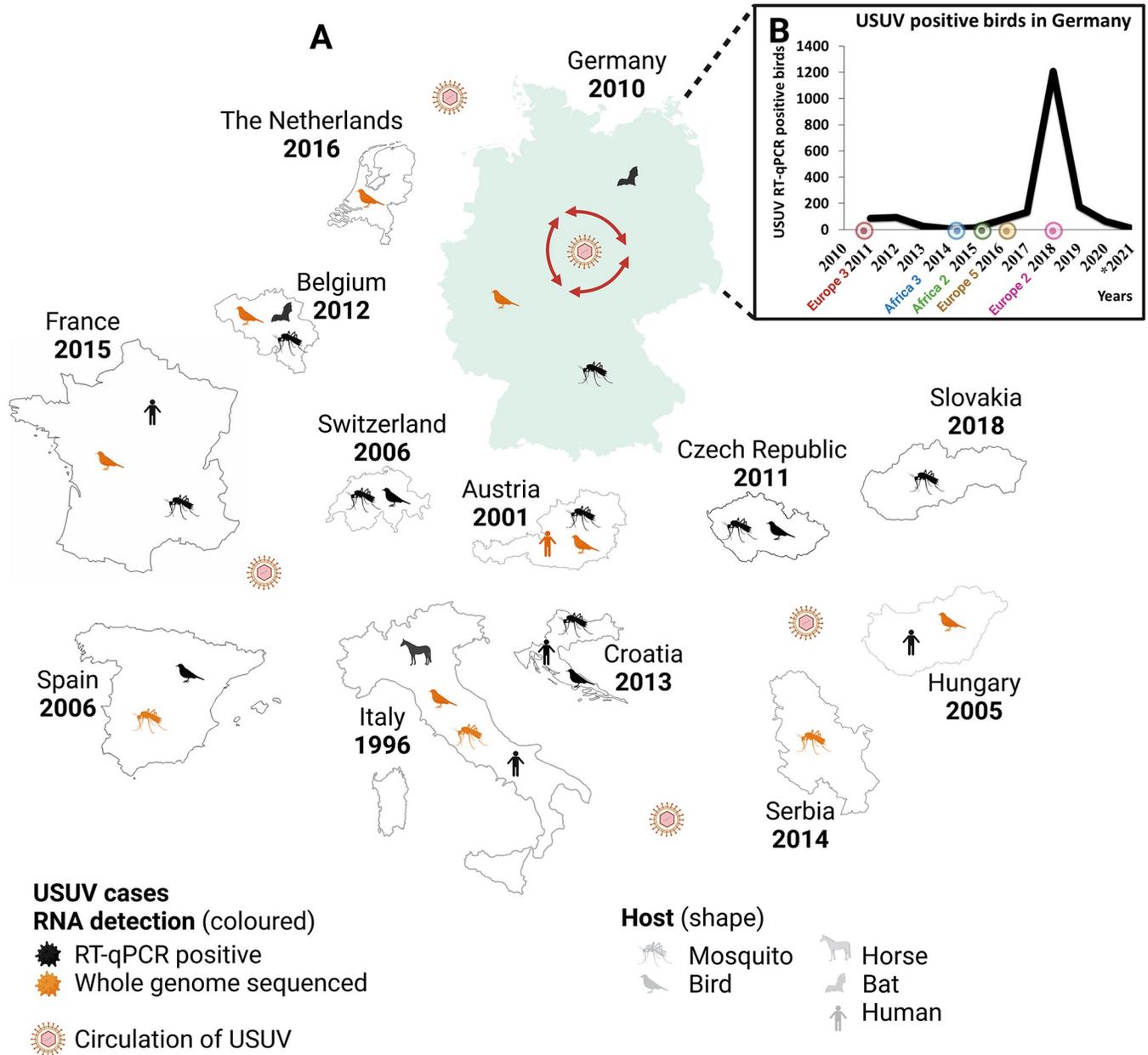


Fig 1. (A) Geographic distribution of USUV-RNA detection throughout Europe. The first detected USUV-positive case per country is depicted in the figure. In addition, the icons for mosquito, bird, horse, bat, and human indicate in which species USUV-RNA has been detected so far and whether a corresponding whole genome sequence is available (in orange). (B) The graph shows all USUV-positive birds detected in Germany since 2011, highlighting the first occurrence of each lineage in the following years, with the exception of USUV Europe 3, which was first detected in 2010 in a mosquito pool. *Results not finalized and only based on dead bird surveillance (updated on 21th February 2023). Created with [BioRender.com](https://www.biorender.com) (Agreement number: NB25GJXN1B).

<https://doi.org/10.1371/journal.pntd.0011203.g001>

independent time points, resulting in the dispersal of distinct USUV lineages. As evidenced by phylogenetic analyses these lineages amplified and evolved independently. The genetic heterogeneity of the European lineages is, therefore, most likely due to local evolution rather than new introductions by long-distance migratory birds [15]. By comparison, widespread migration patterns and multiple introductions of virus variants from different geographic areas of origin resulted in the African lineages [15]. Nonetheless, in the literature the assignment and

nomenclature of USUV lineages/strains has not been standardized. It has also not been conclusively determined whether the different lineages have an influence on host and vector affinity [15].

Circulating in Europe, USUV naturally did not stop at the boundary to Germany (Fig 1A). In Germany, initial surveillance efforts for flaviviruses focused on the serological detection of WNV antibodies [37,38]. USUV-antibodies were solely determined in the further clarification of flavivirus cross-reactivity [37]. However, since 2009 molecular surveillance programs in mosquitoes and birds were implemented, followed by several introductions into the country [39–46]. The first USUV isolate (lineage Europe 3) was documented in Germany in 2010 from a pool of *Culex pipiens* biotype *pipiens* mosquitoes in the south of Frankfurt, in Weinheim [39]. Subsequently, fatal cases in wild and captive birds, mainly Eurasian blackbirds and owls, were reported from the north of the Upper Rhine valley and adjacent areas of the Palatinate and the Neckar valley to the southwest of Germany [8,43]. Around the same time (2014), a new USUV lineage (Africa 3) was introduced into the north of Germany, in Bonn, where only one case in a blackbird was detected [47,48]. In Berlin in 2015, the lineage Africa 2 was detected for the first time in two juvenile great grey owls [47]. In 2016, USUV (lineage Europe 3, Africa 3, and Africa 2) continued to spread, with numerous cases reported in the southwest, northwest, and east of Germany concurrent with the first detection of Europe 5 in central-western North Rhine-Westphalia [44,49,33]. In addition, a further spread to neighbouring countries to the west could be confirmed [33]. In the Federal State of Saxony in 2018, USUV lineage Europe 2 was detected for the first time [45]. Until 2018, the virus had spread nationwide with five USUV lineages present (Africa 2, Africa 3, Europe 2, Europe 3, and Europe 5) (Fig 1B).

To date, the circulation of USUV has been reported (based on serological and molecular evidence) in many countries in and around Europe: Tunisia [50], Morocco [51], Israel [52], Greece [53], France [54], Spain [55], Poland [56], Hungary [6], Czech Republic [57], Serbia [35], the United Kingdom [58], Croatia [59], the Netherlands [60], Switzerland [10], Italy [11], and Germany [39]. Ongoing efforts to elucidate the USUV phylogenetic scenario in Europe, reported the co-circulation of Europe 3 and Africa 3 in the Netherlands [36], Europe 3 and Africa 2 and 3 in France [54], and Europe 1–3 and Africa 3 in the Czech Republic [61]. In Germany, there is an evident co-circulation of the USUV lineages Europe 2 and 3, and Africa 2 and 3 in 2017 and 2018 [45]. A recently published study based on partial sequences from 2019 and 2020, could confirm the ongoing circulation of USUV lineages Europe 2 and 3 as well Africa 3 in the country [46].

So far, only a small number of USUV whole genome sequences from Germany are publicly available, making it difficult to determine the precise time point of USUV introduction into the country. In addition, it is not clear whether the virus was introduced once or whether several independent introductions took place. Third-generation sequencing technologies like Nanopore MinION sequencing, a sequencing platform implemented in this study, enable new and more accessible ways of studying infectious diseases. It can be used to clarify the origin, transmission routes, and ecology of emerging viral diseases, to tackle unanswered fundamental questions [62–66]. Therefore, in this study 118 USUV genomes from wild and captive birds were sequenced using Nanopore MinION to further unravel the occurrence and spread of USUV in Germany from 2017 to 2021. Whole genome sequencing (WGS) produces a greater and more complete data set as compared to partial sequencing and is therefore a promising tool in gaining an in-depth understanding of USUV introduction events into Germany and in characterizing the evolutionary history of USUV.

Material and methods

Ethics statement

Veterinarians, wild bird rescue centres, and zoological facilities supplied bird carcasses for necropsy. As such, birds were not sacrificed for this study. Residual blood material was available from birds collected primarily for veterinary examination, diagnostic purposes, specific treatments, and for the evaluation of treatment successes. The handling of birds and the sampling of blood was performed in strict accordance with national and European legislation, namely the EU council directive 86/609/EEC for the protection of animals. It was solely done by trained veterinarians.

Samples

The blood and organ samples of 118 wild and captive birds represent a subset of the samples that were routinely collected throughout Germany in the frame of ongoing monitoring bird studies in close collaboration with local state laboratories. All collected samples are analysed for USUV using standardised molecular and serological assays. For a detailed overview of the recent USUV situation in Germany please see [44–46,49,8,33,48,47]. From 2019 to 2021, a total of 6,671 birds were tested for USUV. The samples were recorded in a database for the detection of USUV-RNA in birds, which was established in 2019 with the aim of providing the public health authorities with a nationwide overview [46]. Unfortunately, prior to 2019, there was no comparable database and the number of tested birds in addition to those that were part of the study described by Michel et al. 2019 [45] can only be estimated for 2017 and 2018 and are most likely higher in reality (Table 1).

The 118 samples included in this study were submitted to the national reference laboratory (NRL) for West Nile virus (WNV) and USUV at the Friedrich-Loeffler-Institut (FLI). For WGS, USUV RNA-positive samples were selected primarily on the basis of their geographic location in order to represent a comprehensive picture of Germany. However, many of the recorded USUV-positive samples were examined by regional veterinary laboratories of the Federal States or other institutions that did not send their samples to the FLI and these could therefore not be included in the study. From the available samples, the cycle threshold (Ct) values and to some extent also the lineages, as already determined by partial sequencing [46,45,67], were used as further decision criteria. Throughout all five years, USUV isolates from the two different bird orders *Passeriformes* and *Strigiformes* were identified and sequenced. In 2019, USUV was additionally found and sequenced in *Anseriformes* and *Columbiformes* (Table 2). Viral RNA of blood and organ samples was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Cell culture supernatant samples were extracted using the Viral RNA Mini Kit (Qiagen) following the manufacturer's instructions. Analysis of

Table 1. Number of molecularly tested birds per year, from the live and dead bird surveillance, including USUV RT-qPCR positive results and number of samples sequenced [68,69,45,70].

| Year | Total Tested Birds | USUV RT-qPCR Positive | Samples Sequenced |
|--------------|--------------------|-----------------------|-------------------|
| 2017 | NA | 132 | 6 |
| 2018 | NA | 1,208 | 45 |
| 2019 | 2,202 | 177 | 37 |
| 2020 | 3,086 | 64 | 25 |
| 2021* | 1,383 | 12 | 5 |
| Total | 6,671 | 1,593 | 118 |

NA = not available

*Results not finalized and only based on dead bird surveillance (updated on 21st February 2023).

<https://doi.org/10.1371/journal.pntd.0011203.t001>

Table 2. Overview of sample set.

| | 2017 | 2018 | 2019 | 2020 | 2021 |
|-----------------------------------|------------------------------------|--|---|--|------------------------------------|
| Total (Wild/Captive Birds) | 6 (3/3) | 45 (34/11) | 37 (29/8) | 25 (22/3) | 5 (1/4) |
| Species Order | <i>Passeriformes, Strigiformes</i> | <i>Passeriformes, Strigiformes</i> | <i>Passeriformes, Strigiformes, Columbiformes, Anseriformes</i> | <i>Passeriformes, Strigiformes</i> | <i>Passeriformes, Strigiformes</i> |
| Organs/Body Fluids | Brain, CNS, liver, spleen | Blood, brain, CNS, liver, pool of organs, spleen | Blood, brain*, CNS, kidney, liver, pool of organs, spleen | Blood, brain*, pool of organs, RNA, spleen | Blood, brain, liver |
| Federal States | NW, NI, SN | BE, BY, MV, NI, NW, SL, ST | BB, BE, BY, HE, NI, NW, SL, SN, ST, TH | BB, BE, BW, HE, NI, SN, ST | MV, NI, ST |
| Lineages | Africa 3, Europe 3 | Africa 3, Europe 3 | Africa 3, Europe 3, Europe 2 | Africa 3, Europe 3, Europe 2 | Africa 3, Europe 3 |

* Selected samples were either diluted 1:5 in phosphate buffered saline (PBS) or were passaged in cell culture prior to RNA extraction.

Abbreviations: CNS: central nervous system; BB: Berlin Brandenburg; BE: Berlin; BW: Baden-Württemberg; BY: Bavaria; HE: Hesse; NI: Lower Saxony; MV: Mecklenburg-West Pomerania; NW: North Rhine-Westphalia; SL: Saarland; SN: Saxony; ST: Saxony-Anhalt; TH: Thuringia

<https://doi.org/10.1371/journal.pntd.0011203.t002>

extracted RNA was performed using real-time reverse transcription PCR (RT-qPCR) assays specific for USUV, as described by Jöst et al. 2011 [39]. Samples with Ct values between 12.09 and 32.79, covering almost all Federal States of Germany, were included in this study. Detailed information of each sample is provided in S1 Table. Four of the sequenced birds (GenBank accession numbers: OP422562–OP422565) were already published in a next-generation sequencing (NGS) methodological publication by Holicki et al. 2022 [67].

Nanopore sequencing and data analysis

Nanopore sequencing was performed as described by Holicki et al. 2022 [67]. In short, RNA was reverse transcribed using the SuperScript IV First-Strand cDNA Synthesis Reaction Kit (Cat. no. 18091050; Invitrogen by Thermo Fisher Scientific, Darmstadt, Germany) with random primers as previously described by Quick et al. 2017 [71]. Followed by an USUV-specific multiplex PCR which was performed with two separate mixes of primer pairs using AccuPrime Taq DNA Polymerase High Fidelity (Cat. no. 12346–086; Invitrogen) [72]. MinION sequencing was carried out following the manufacturer's instructions using the 1D Native barcoding genomic DNA Kit (Nanopore, EXP-NBD104 and SQK-LSK109, Oxford Nanopore-technology (ONT)) on a Spot-ON flow cell (R9.4.1; ONT). Twelve samples were multiplexed per flow cell. Fast5 raw data reads were demultiplexed using Guppy v4.5.4 [73]. Primers were trimmed and reads were quality controlled to a minimal length of 200 base pairs (bp) and reads with a minimum quality of 7 were considered for further analysis. For consensus sequence generation, an alignment against the selected USUV reference genomes v23 [74] was performed using KMA [75] and Minimap2 [76]. Successful sequencing was defined as achieving an alignment of 80% or higher in the applied target enrichment approach, ensuring effective capture and sequencing of a significant portion of the target regions. Sequencing efforts maintained a minimum coverage of 30x, meaning that each targeted region was sequenced an average of 30 times. This robust amplicon depth ensured the generation of reliable and high-quality data for subsequent downstream analysis. Consensus sequences were visualized with Geneious Prime 2021.0.1 (Biomatters Ltd., Auckland, New Zealand).

USUV genome phylogenetic analyses

USUV sequences of the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) were screened and all available full length USUV genomes from Germany up to

August 24, 2022 were downloaded [77]. All full length USUV genomes including the newly sequenced USUV genomes were aligned using MUSCLE [78] and manually inspected. The maximum likelihood phylogenetic analysis was conducted using General Time Reversible (GTR) + gamma (G) model with 1,000 bootstraps in MEGA v11 [79] and finalized trees were reconstructed with FigTree v.1.4.3 [80]. To investigate patterns of genetic variation and sub-clustering within our sequence dataset, a Principal Component Analysis (PCA) was conducted using the FactoMineR package in R [81]. PCA was then applied to the preprocessed dataset to reduce its dimensionality and visualize the inherent structure. The resulting principal components were used as input for subsequent clustering analysis.

Estimating time to the most recent common ancestor (TMRCA)

For evolutionary dynamic analyses and to determine the age of the most recent common ancestors, the Bayesian Markov chain Monte Carlo (MCMC) method was performed using BEAST v2.6.6 package [82]. All USUV whole genome sequences generated in this study as well as German whole genome sequences from NCBI were used. In these analyses, a GTR + gamma (G) substitution model and a strict clock model were applied [83]. MCMC was set to 100,000,000 generations (sampling every 2,500 steps). Log files were analysed in Tracer v1.7.1 to check effective sampling size (ESS) values (>200 indicated sufficient sampling). The maximum clade credibility (MCC) tree was generated in the Tree Annotator v1.8.4, with a default burn-in of 10%. The MCC tree was visualized in the FigTree program v1.4.3 [80].

Geolocation of USUV strains sequenced in this study

Geographic information system (GIS) analysis of USUV-positive birds used for sequencing was performed using ArcGIS ArcMap 10.8.1, license number: ESU748788721 (ESRI, Redlands, CA, USA) and open data from GeoBasis-DE/BKG 2022 [84] using the following link: https://daten.gdz.bkg.bund.de/produkte/vg/vg250_ebenen_1231/aktuell/vg250_12-31.utm32s.shape.ebenen.zip.

Results

USUV genomic sequencing

For WGS, USUV RNA-positive samples were sequenced using ONT (Figs 2 and 3). The median Ct value of the USUV-positive birds was 19.6. S1 Table provides an overview of the total reads and some quality parameters of the sequencing results (coverage, mean read quality, and identity levels) of all samples. The average number of NGS reads obtained from amplicons was approx. 250,000 and genome assembly was performed for all samples with covering >85% of the genome. The total accuracy rates of the USUV genome sequences were 94.2–98.3% with a threshold depth of 100x.

Phylogenetic analysis of USUV in Germany

The phylogenetic analysis displays the co-circulation of USUV lineages Europe 2, Europe 3, and Africa 3 in Germany, with whole genome sequences of lineage Europe 2 only present in 2019 and 2020 (Fig 3). USUV lineage Africa 3 was detected almost as often as Europe 3, while Europe 2 was only sequenced in 9% of the cases (Fig 4). Africa 3 and Europe 3 were distributed throughout the country, whereas Europe 2 was only found in the eastern part of Germany, in the Federal States Berlin, Brandenburg, and Saxony (Fig 3). USUV lineages Europa 5 and Africa 2 were not part of this study as samples thereof were not available for sequencing.

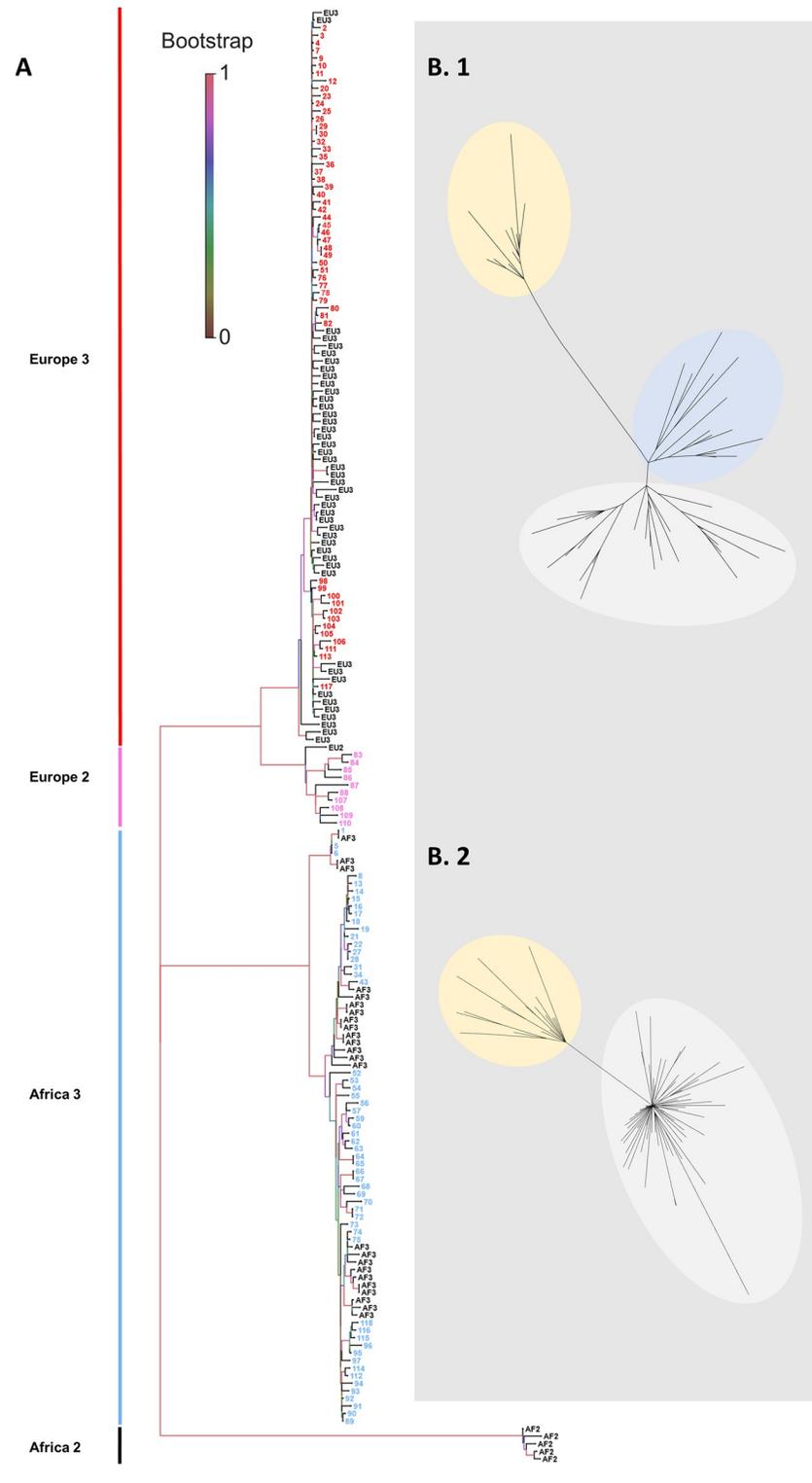


Fig 2. Phylogeny of the sequenced USUV isolates from 2017–2021. (A) Samples were numbered consecutively and coloured according to the lineages. Reference genomes are marked as EU3 (Europe 3), EU2 (Europe 2), A3 (Africa 3), and A2 (Africa 2). Detailed information to each sample number can be found in [S1 Table](#) including GenBank accession numbers and the years in which the samples were detected. Scale bars indicate the mean number of nucleotide substitutions per site. (B. 1) Cluster analyses suggesting three subclusters belonging to lineage Europe 3 and

(B. 2) two subclusters belonging to lineage Africa 3. (The supplementary file (S1 Table) provides comprehensive details, including sequence assignments, for the identified subclusters.)

<https://doi.org/10.1371/journal.pntd.0011203.g002>

Molecular clock phylogeny of USUV lineages detected in Germany

The molecular clock phylogeny of USUV lineages was performed to determine the time to the most recent common ancestors of the USUV lineages Europe 3, Africa 3, and Europe 2. The estimated time to TMRCA of lineage Europe 3 was determined to be around 2008 (between 2006–2010, 95% confidence interval), while the estimated time of lineage Africa 3 was shown

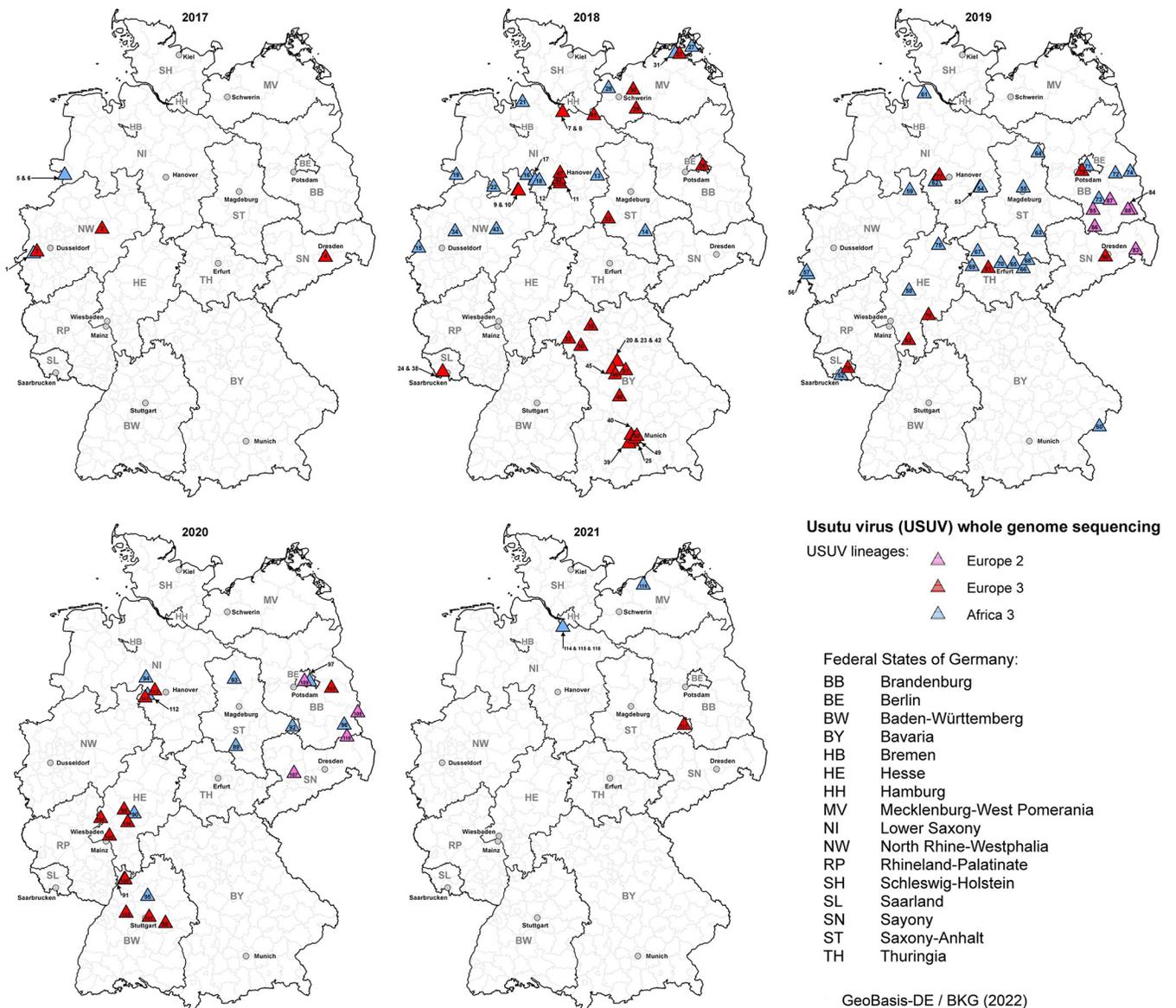


Fig 3. Geographic distribution of whole genome sequences of USUV in Germany from 2017 to 2021. The different USUV lineages are depicted as coloured triangles: pink = Europe 2, red = Europe 3, and light blue = Africa 3 with the appropriate sample number (detailed information to each sample in S1 Table). GIS analysis was performed using ArcGIS ArcMap 10.8.1, license number: ESU748788721 (ESRI, Redlands, CA, USA) and open data from GeoBasis-DE/BKG 2022 [84] using the following link: https://daten.gdz.bkg.bund.de/produkte/vg/vg250_ebenen_1231/aktuell/vg250_12-31.utm32s.shape.ebenen.zip.

<https://doi.org/10.1371/journal.pntd.0011203.g003>

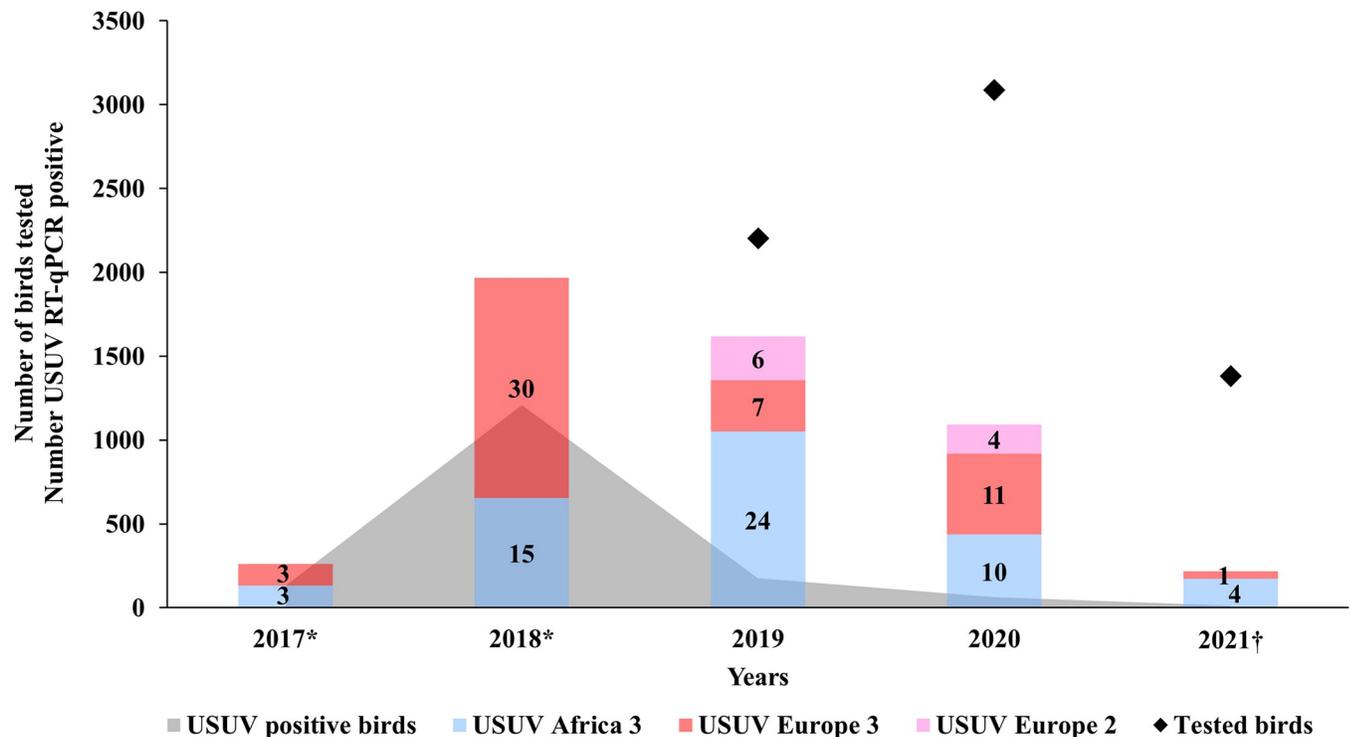


Fig 4. Overview of the distribution of the different USUV lineages in Germany (2017–2021) (depicted in blue, red, and pink with the total number per lineage) with regard to the total number of live and dead birds molecularly tested (depicted with black diamonds) and those tested RT-qPCR positive for USUV (depicted in grey). * Number of tested birds not recorded in 2017 and 2018; † Results not finalized and only based on dead bird surveillance (updated on 21st February 2023). Bird samples with USUV lineage Europe 5 or Africa 2 were not available for this study.

<https://doi.org/10.1371/journal.pntd.0011203.g004>

to be around 2011 (between 2008–2012, 95% confidence interval), as demonstrated in Fig 5. For Europe 2 TMRCA was calculated to be around 2017 (2015–2019, 95% confidence interval). However, the result for Europe 2 should be considered with caution as the number of available sequences is currently too limited to enable the construction of a fully resolved phylogeny clustering as well as to describe the timing of branching events in phylogenetic trees (S1 Fig).

Discussion

The annual reoccurrence of USUV in Germany since 2011 is the cause of ongoing disease and death among wild and captive birds. Regular and stringent genomic surveillance of viral pathogens supports outbreak investigations by providing evidence for their transmission routes and geographic spread. Since only a few whole genome sequences of USUV from Germany are available [67,47,8,85,33,15,49], a better phylogenetic analysis is the next essential step in understanding the spread of USUV in Germany as well as in Europe. A preferred method using third-generation sequencing is a nanopore sequencing approach based on target-enrichment through amplicon generation [72,67]. This recently established protocol [67] was used to gain insight into the distribution and expansion of USUV throughout five consecutive years in Germany. This sequencing technique proved to be sensitive in sequencing the majority of the USUV-positive bird samples and produced good results up to a Ct value of 32.79. The here described study confirms that WGS using the Nanopore platform is suitable in rapidly tracking and detecting ongoing USUV infections in deceased and live birds. Due to the real-time

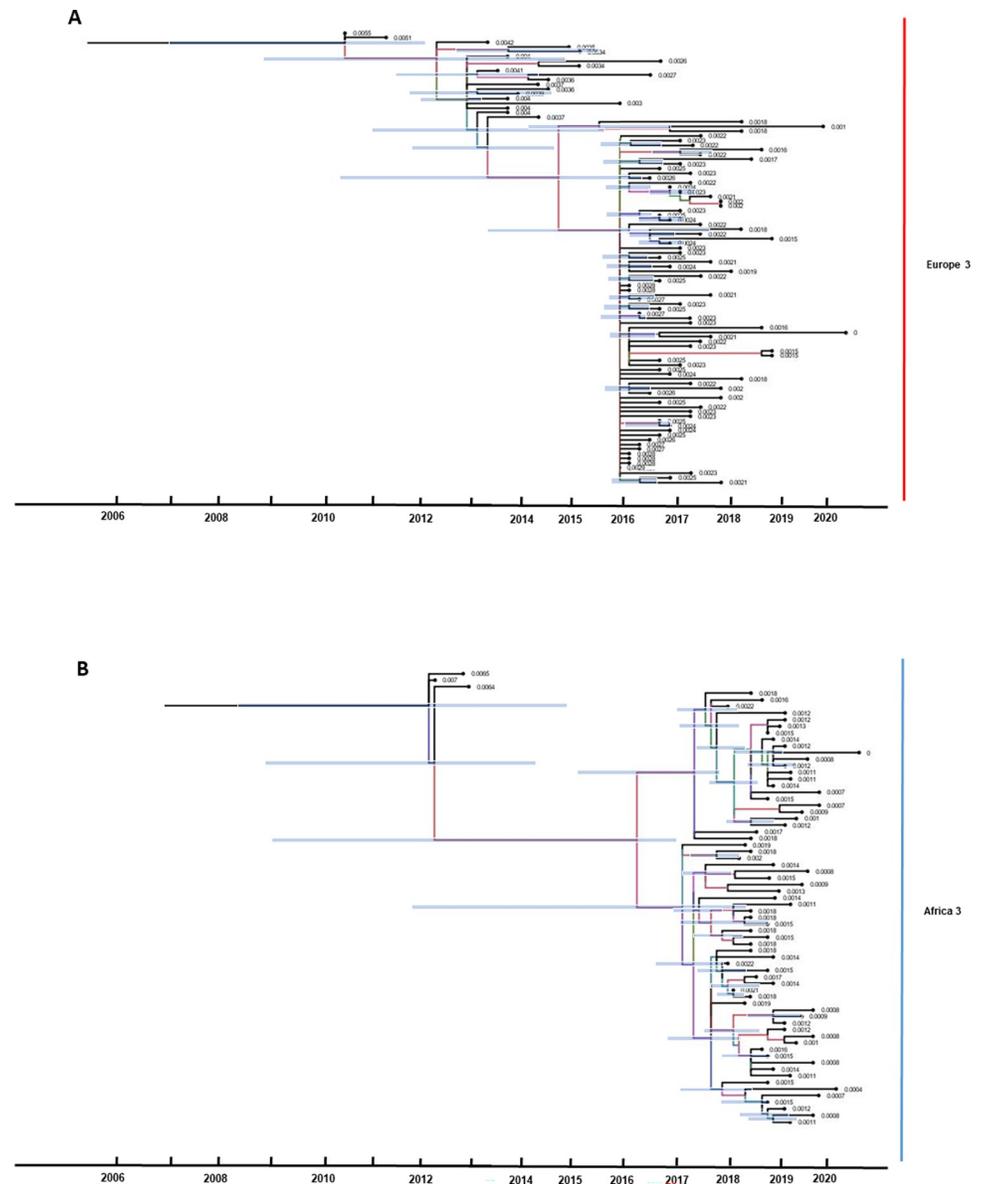


Fig 5. Molecular clock phylogeny of the complete coding sequences of USUV lineages, (A) Europe 3 and (B) Africa 3 detected in Germany. The blue bars indicate 95% confidence intervals of node dates. The branches are colored according to the sampling location of their nodes.

<https://doi.org/10.1371/journal.pntd.0011203.g005>

and user-friendly application of the Nanopore sequencing platform, it is a promising tool to supersede partial sequencing in the future. Mass parallelization of sample sets can enable fast-turnaround times without having adverse effects on the platform's sensitivity in detecting genomic variants [67].

Since the first occurrence of USUV in 2010 in a mosquito pool (one year prior to its detection in birds) [39], five USUV lineages (Europe 2, 3, 5, and Africa 2, 3) (Fig 1) have been described in Germany. Among these, the two USUV lineages Europe 3 and Africa 3 appear to have been the prevailing players in the USUV scenario in the past five years (2017–2021). Therefore, there is an imbalance in the amount of available USUV whole genome sequences for the different USUV lineages. USUV lineage Europe 3 and Africa 3 are predominant

compared to only a few whole genome sequences of Europe 2 and Africa 2 and no available whole genome sequences of Europe 5 to date (Ziegler et al. 2016, Cadar et al. 2017)(47,33). USUV lineage Europe 3, was the first USUV lineage to be detected in Germany, namely in mosquitoes in 2010 [39]. However, TMRCA of this lineage is estimated to be about two years prior to its first detection. The 95% confidence interval covers a period from 2006 to 2010 (Fig 5). Similarly, the first detection of USUV lineage Africa 3 occurred in Germany in 2014 [48,49], yet the TMRCA is estimated to have occurred prior than that, already in 2011 (Fig 5). However, to produce an even more accurate estimate, more data from other geographic areas and earlier years are needed. In contrast, Europe 2 is less frequent in Germany and it was only possible to generate whole genome sequences from 2019 and 2020 (Figs 3 and 4). It should, nonetheless, be noted that partial genome sequences were already generated from samples in 2018, when the lineage was first detected in Germany [45]. The TMRCA for Europe 2 was determined in 2017, one year prior to its actual detection. The phylodynamic analyses of TMRCA therefore provide evidence of a 1- to 3-year lag, respectively between the introduction and the first-case detection of an USUV lineage. By contrast, a large time-lag was described for USUV in the Netherlands, where 7 to 14 years were between the estimated common ancestor and the first detection of USUV lineage Africa 3 and Europe 3, respectively [36]. Likewise, the temporal windows determined for the TMRCA in that publication are broad and can vary depending on the size of the available data set and the set timeframe [36].

The phylogenetic analysis of the USUV whole genome sequences in this study reveal two subclusters for Africa 3. Furthermore, the results of the clock phylogeny (Fig 5B) suggest a long time-lag spanning several years between the first introduction of USUV Africa 3 into the country and its first large-scale occurrence in the avifauna. This could be indicative of silent evolutionary dynamics of the endemic and overwintering lineage Africa 3 (i.e., the first cluster) for several years prior to causing an outbreak with numerous detections (i.e., the second cluster). The occurrence of the outbreak can then be correlated to optimal environmental as well as host and vector conditions driving virus transmission or to the evolution of a more pathogenic strain (i.e., the second cluster). A similar phenomenon was already suggested for WNV by Zehender et al. 2017 [86] and Chaintoutis et al. 2019 [87]. They describe that quiet enzootic transmission seasons over several years often proceed virus outbreaks in animals and humans, respectively. Improving the sample matrix (vector and host species) and size as well as the temporal and geographic extent of future surveillance strategies can help on the one hand to detect quiet enzootics and on the other to not miss out on introduction events as well as epizootics. By contrast, the phylogenetic and phylodynamic (Fig 5A) analyses of USUV Europe 3 suggest three subclusters appearing within a shorter time frame. This temporal connection gives the impression of multiple introduction events of USUV Europe 3 into Germany in the past creating the three separate clusters. However, to accurately unravel this phenomenon future studies must be performed phylogenetically analysing USUV also on a European scale. Alternatively, it is also plausible that the other clusters derived from another undetected USUV Europe 3 isolate endemic in Germany.

In Germany as well as in the Netherlands it, however, remains unclear which influence the absence of USUV surveillance programs had on the first detection of USUV in the two countries. In the case of Germany, the first mosquito monitoring study took place in 2009 at the same sampling site as in 2010, when the first infected mosquito pool was found in Weinheim in the Upper Rhine Valley [39]. Prior to 2009, first surveillance efforts were limited to serological investigations, restricted locally [37,38], and often not primarily focused on flaviviruses (at most testing for WNV [88]). Therefore, it is possible that individual USUV infections occurred before but as they were not accompanied by mass mortalities of birds remained undetected. Serological studies prior to 2009 show neutralising antibodies against USUV in wild birds yet

no molecular investigations were performed [37,38]. Furthermore, when working with BEAST-analyses one must always keep in mind that a TMRCA is only an estimation of an introduction event based strongly on the quantity and quality of the available data. Caution is always needed when interpreting these results as the inclusion of further samples can reveal different results. This was for example verified by the sequencing of an ancient hepatitis B virus which yielded new data on the evolution of hepatitis B, that was not apparent when only evaluating recent sequences [89].

On a European scale, USUV was isolated for the first time in 2001 in Austria (“Strain Vienna”; USUV Europe 1) [13] and retrospectively in 1996 in Italy (also USUV Europe 1) [14]. However, molecular clock analyses revealed that the first entry of USUV (classified as Europe 1) into central Europe was estimated to have occurred already in Spain in the late 20th century, with a virus closely related to USUV from Senegal. Furthermore, the lineages Europe 2 and 3 were calculated to have their origin in Austria in 1993 and Italy in 2007, respectively [15]. Since then, USUV has spread throughout Europe with lineage Europe 4 detected in Italy from a sampling set from 2010–2014 with its estimated TMRCA dating back to 2003–2005 [34]. Europe 5 was detected for the first time in Germany in 2016 [33] and so far, no TMRCA has been calculated for this lineage. The lineages Africa 2 and 3 descended from multiple independent introductions from sub-Saharan as indicated by analyses from Spain [15].

A few publications have analysed the geographic flow of the USUV genome throughout Europe, with the majority of available whole genome sequences from Italy, the Netherlands, and Germany. Italy is considered an “USUV-donor” to neighbouring countries. Especially north-western Italy appears to have played a key role in the transfer of USUV to central Europe (from Switzerland to Germany to France and Belgium) and eastern Italy to central and eastern Europe (from Austria to Hungary to Serbia) [90]. It must however be kept in mind that other publications have described an USUV spread in the other direction, i.e., from Austria to Italy and Germany [15,34,4]. Analyses performed with USUV sequences from the Netherlands have confirmed an USUV-circulation between the Netherlands, Germany, and Belgium [36]. USUV Europe 3 is less frequent in the Netherlands and is most likely periodically re-introduced from neighboring countries. By contrast the prevailing Africa 3 lineage was probably introduced into the Netherlands from Germany in 2016, overwintered there and has since then become enzootic.

Flavivirus transmission dynamics are influenced by environmental and biological factors affecting the host as well as the vector of a virus. The population density of amplifying/reservoir species and the species immune fitness towards specific pathogens also has a significant influence on virus maintenance and spread in the environment [91,92]. Equally, population dynamics of mosquito vectors can play a role in virus transmission and are among others affected by population density, urbanisation, humidity, and temperature [93,94]. The spread of USUV may, therefore, have been favoured by the presence of beneficial environmental conditions for mosquitoes in recent years [33,95,96,8]. In 2016, for instance, an exceptionally high activity of USUV infections was observed in birds, correlating with temperature anomalies in September in Western Europe [33]. Higher temperatures shorten the extrinsic incubation period (time required for virus replication in the mosquito) which in turn influences the population dynamics of mosquitoes and as a result the vector-host contact rate [93,97,91]. A similar scenario of optimal weather conditions was observed again in Germany in 2018 with an early humid spring combined with a warm and dry summer (S2 Fig) [45,96]. This might have also paved the way for the introduction of WNV into Germany in the same year [98]. The extensive USUV outbreaks for example in 2016 and 2018 are in the literature often associated with optimal weather conditions for mosquito-borne virus transmission [99,91]. Especially the fulminant outbreak in 2018 led to a massive die-off of blackbirds, a species highly susceptible to

USUV, and consequently a decline in the species population throughout Germany [45]. The observed high seroprevalence of USUV antibodies in 2018 compared to the prior years [45] lets one hypothesize that USUV thereafter faced a higher proportion of non-naïve hosts for its replication cycle. This could help explain the absence of USUV outbreaks of a comparable magnitude in the consecutive years even though the weather conditions continued to encourage arbovirus transmission. This phenomenon was also observed after the USUV outbreak in Austria in 2001, where herd immunity of the wild bird populations protected susceptible species from a severe USUV disease in subsequent years [100].

Arboviruses such as USUV or the closely-related flavivirus WNV can overwinter in a susceptible host and/or vector species. In the past decade, the nationwide German wild bird surveillance network for zoonotic arthropod-borne viruses has consistently provided evidence for the persistence of these arboviruses in the German avifauna [38,40,43–45]. Similarly, the endemicity of both arboviruses was confirmed in findings from indigenous mosquito species, i.e., *Culex pipiens* [42,41], that are known to be vector competent for both viruses [93,101]. Increased mosquito breeding, facilitated by high summer temperatures as well as a sufficiently high water balance (S2 Fig), can lead to a longer mosquito and virus-transmission season. Taken together the overwintering of mosquitoes infected with flaviviruses [102] and the here discussed evolution of different USUV lineages within the country verify the persistence of the virus in Germany. In addition to optimal weather conditions favouring the spread of endemic USUV lineages in Germany, new introductions from neighbouring countries may have taken place, such as from the Netherlands, Czech Republic or Italy, as well as from long-distance migrants. Newly introduced strains in turn spread with ease under the favourable environmental conditions (Fig 1).

Conclusion

The study helps to understand the evolution and spread of the major USUV lineages in Germany since their first occurrence in 2010 and to classify the most recent common ancestor for the two most important lineages Europe 3 and Africa 3. For this purpose, a validated protocol was used to efficiently generate whole genome sequences using the Nanopore platform. There was a correlation between the weather conditions and the number of USUV infections detected, with 2018 displaying an exceptionally large USUV epizootic. Using clock phylogenies, the most recent common ancestors were determined for the ubiquitous USUV lineages Europe 3 and Africa 3 in Germany. These results once more emphasize the importance of a stringent surveillance strategy for USUV as well as other flaviviruses as the viruses are to date often first detected two to three years after their calculated introduction.

Supporting information

S1 Table. Detailed information on the origin of phylogenetically analysed USUV from wild and captive birds in 2017 and 2021. Sample numbers are used in Fig 1. (XLSX)

S1 Fig. Molecular clock phylogeny of the complete coding sequences of USUV lineage Europe 2 detected in Germany. Node bars indicate 95% confidence intervals of the time of TMRCA. The branches are colored according to the sampling location of their nodes. (TIF)

S2 Fig. Climatological maps of Germany displaying (A) temperature (in degrees Celsius) and (B) water balance (in millimetre) based on data collected in the summers 2017–2021 [103].

Climatological maps were downloaded from the German Weather Service [104].
(TIF)

S3 Fig. Principal Component Analysis (PCA) plot showing distinct clustering patterns of subclusters in Africa 3 lineage of USUV. Each data point represents a viral sample, and the plot provides insights into the genetic diversity and distribution of the subclusters within the lineage.

(TIF)

S4 Fig. Principal Component Analysis (PCA) plot showing distinct clustering patterns of subclusters in Europe 3 lineage of USUV. Each data point represents a viral sample, and the plot provides insights into the genetic diversity and distribution of the subclusters within the lineage.

(TIF)

Acknowledgments

The authors are very grateful to Cornelia Steffen and Katja Wittig (both from FLI) for excellent technical assistance. We are also grateful to Patrick Wysocki (FLI) for producing the maps in Fig 3. Furthermore, we thank the staff of the bird clinics/practices, rehabilitation and falconry centres, and ornithologists in the nationwide wild bird surveillance network for zoonotic arthropod-borne viruses who took and sent samples for the annual WNV and USUV monitoring project. Selected samples from the monitoring project were used additionally for whole genome sequencing in this manuscript. Therefore, we thank the following contributors, in particular: Dominik Fischer (Clinic for Birds, Reptiles, Amphibians and Fish, Justus Liebig University Giessen), Sylvia Urbaniak (Raptor Rehabilitation Centre Rhineland), Brigitte Böhm (Tiergesundheitsdienst Bayern e.V.), Mathias Sterneberg (Veterinary practice for raptor medicine, Schüttorf), Karolin Schütte (Wildlife Rescue and Conservation Center, Sachsenhagen), Pierre Grothmann (Wildlife Veterinarian, Geestland), Andreas Kirchoff (Clinic of Pathology, Gelsenkirchen), Anke Scherer (Nature and Biodiversity Conservation Union (NABU)), Claudia Szentiks (Leibniz Institute for Zoo and Wildlife Research), Niels Mensing (Veterinary practice, Magdeburg), Monika Rinder (Clinic for Birds, Small Mammals, Reptiles and Fish, Centre for Clinical Veterinary Medicine, Ludwig Maximilians University Munich), Kerstin Müller (Department of Veterinary Medicine, Small Animal Clinic, Freie Universität Berlin), Martina Schmoock Wellhausen (Veterinary medicine am Rothenbaum, Hamburg), Heike Weber (Zoological Garden Nordhorn), Florian Hansmann and Anna Schwarz (both from University of Veterinary Medicine Hannover, Department of Pathology), Arne Jung (University of Veterinary Medicine Hannover, Clinic for Birds), Bernhard Böer (Veterinary practice for raptor medicine, Salzgitter), and Heinz-Eike Lange (Veterinary practice, Würselen). We also thank the staff of the wildlife park in Cottbus and Saarbrücken.

Author Contributions

Conceptualization: Felicitas Bergmann, Cora M. Holicki, Balal Sadeghi, Ute Ziegler.

Data curation: Felicitas Bergmann, Cora M. Holicki, Balal Sadeghi, Ute Ziegler.

Formal analysis: Felicitas Bergmann, Cora M. Holicki, Balal Sadeghi, Ute Ziegler.

Funding acquisition: Martin H. Groschup, Ute Ziegler.

Investigation: Felicitas Bergmann, Cora M. Holicki, Balal Sadeghi, Ute Ziegler.

Methodology: Felicitas Bergmann, Cora M. Holicki, Balal Sadeghi, Ute Ziegler.

Project administration: Balal Sadeghi, Ute Ziegler.

Resources: Felicitas Bergmann, Cora M. Holicki, Friederike Michel, Sabine Bock, Nelly Scuda, Grit Priemer, Susanne Kenklies, Timo Siempelkamp, Jasmin Skuballa, Claudia Sauerwald, Louise Herms, Aemero Muluneh, Martin Peters, Andreas Hlinak, Balal Sadeghi, Ute Ziegler.

Software: Felicitas Bergmann, Cora M. Holicki, Balal Sadeghi, Ute Ziegler.

Supervision: Martin H. Groschup, Balal Sadeghi, Ute Ziegler.

Validation: Felicitas Bergmann, Cora M. Holicki, Balal Sadeghi, Ute Ziegler.

Visualization: Felicitas Bergmann, Cora M. Holicki, Balal Sadeghi, Ute Ziegler.

Writing – original draft: Felicitas Bergmann, Cora M. Holicki, Martin H. Groschup, Balal Sadeghi, Ute Ziegler.

Writing – review & editing: Felicitas Bergmann, Cora M. Holicki, Friederike Michel, Sabine Bock, Nelly Scuda, Grit Priemer, Susanne Kenklies, Timo Siempelkamp, Jasmin Skuballa, Claudia Sauerwald, Louise Herms, Aemero Muluneh, Martin Peters, Andreas Hlinak, Martin H. Groschup, Balal Sadeghi, Ute Ziegler.

References

1. Calisher CH, Gould EA. Taxonomy of the virus family Flaviviridae. In: Elsevier; 2003. p. 1–19 (Advances in Virus Research).
2. Bakonyi T, Gould EA, Kolodziejek J, Weissenböck H, Nowotny N. Complete genome analysis and molecular characterization of Usutu virus that emerged in Austria in 2001: comparison with the South African strain SAAR-1776 and other flaviviruses. *Virology* 2004; 328(2):301–10. <https://doi.org/10.1016/j.virol.2004.08.005> PMID: 15464850
3. Williams MC, Simpson DI, Haddow AJ, Knight EM. The Isolation of West Nile Virus From Man And of Usutu Virus From The Bird-Biting Mosquito *Mansonia Aurites* (Theobald) in The Entebbe Area of Uganda. *Ann Trop Med Parasitol* 1964; 58:367–74. <https://doi.org/10.1080/00034983.1964.11686258> PMID: 14212897
4. Roesch F, Fajardo A, Moratorio G, Vignuzzi M. Usutu Virus: An Arbovirus on the Rise. *Viruses* 2019; 11(7). <https://doi.org/10.3390/v11070640> PMID: 31336826
5. Saiz J-C, Blazquez A-B. Usutu virus: current knowledge and future perspectives. *VAAT* 2017; Volume 9:27–40.
6. Bakonyi T, Erdélyi K, Ursu K, Ferenczi E, Csörgo T, Lussy H et al. Emergence of Usutu virus in Hungary. *J Clin Microbiol* 2007; 45(12):3870–4. <https://doi.org/10.1128/JCM.01390-07> PMID: 17913929
7. Weissenböck H, Kolodziejek J, Fagner K, Kuhn R, Pfeffer M, Nowotny N. Usutu virus activity in Austria, 2001–2002. *Microbes and Infection* 2003; 5(12):1132–6. [https://doi.org/10.1016/s1286-4579\(03\)00204-1](https://doi.org/10.1016/s1286-4579(03)00204-1) PMID: 14554255
8. Becker N, Jöst H, Ziegler U, Eiden M, Höper D, Emmerich P et al. Epizootic emergence of Usutu virus in wild and captive birds in Germany. *PLoS One* 2012; 7(2):e32604. <https://doi.org/10.1371/journal.pone.0032604> PMID: 22389712
9. Lühken R, Jöst H, Cadar D, Thomas SM, Bosch S, Tannich E et al. Distribution of Usutu Virus in Germany and Its Effect on Breeding Bird Populations. *Emerg Infect Dis* 2017; 23(12):1994–2001. <https://doi.org/10.3201/eid2312.171257> PMID: 29148399
10. Steinmetz HW, Bakonyi T, Weissenböck H, Hatt J-M, Eulenberger U, Robert N et al. Emergence and establishment of Usutu virus infection in wild and captive avian species in and around Zurich, Switzerland—genomic and pathologic comparison to other central European outbreaks. *Vet Microbiol* 2011; 148(2–4):207–12. <https://doi.org/10.1016/j.vetmic.2010.09.018> PMID: 20980109
11. Manarolla G, Bakonyi T, Gallazzi D, Crosta L, Weissenböck H, Dorrestein GM et al. Usutu virus in wild birds in northern Italy. *Vet Microbiol* 2010; 141(1–2):159–63. <https://doi.org/10.1016/j.vetmic.2009.07.036> PMID: 19720475

12. Hubálek Z, Rudolf I, Čapek M, Bakonyi T, Betášová L, Nowotny N. Usutu virus in blackbirds (*Turdus merula*), Czech Republic, 2011–2012. *Transbound Emerg Dis* 2014; 61(3):273–6. <https://doi.org/10.1111/tbed.12025> PMID: 23095331
13. Weissenböck H, Kolodziejek J, Url A, Lussy H, Rebel-Bauder B, Nowotny N. Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. *Emerg Infect Dis* 2002; 8(7):652–6. <https://doi.org/10.3201/eid0807.020094> PMID: 12095429
14. Weissenböck H, Bakonyi T, Rossi G, Mani P, Nowotny N. Usutu virus, Italy, 1996. *Emerg Infect Dis* 2013; 19(2):274–7. <https://doi.org/10.3201/eid1902.121191> PMID: 23347844
15. Engel D, Jöst H, Wink M, Börstler J, Bosch S, Garigliani M-M et al. Reconstruction of the Evolutionary History and Dispersal of Usutu Virus, a Neglected Emerging Arbovirus in Europe and Africa. *mBio* 2016; 7(1):e01938–15. <https://doi.org/10.1128/mBio.01938-15> PMID: 26838717
16. Pecorari M, Longo G, Gennari W, Grottola A, Sabbatini am, Tagliazucchi S et al. First human case of Usutu virus neuroinvasive infection, Italy, August–September 2009. *Euro Surveill* 2009; 14(50). PMID: 20070936
17. Santini M, Vilbic-Cavlek T, Barsic B, Barbic L, Savic V, Stevanovic V et al. First cases of human Usutu virus neuroinvasive infection in Croatia, August–September 2013: clinical and laboratory features. *J Neurovirol* 2015; 21(1):92–7. <https://doi.org/10.1007/s13365-014-0300-4> PMID: 25361698
18. Grottola A, Marcacci M, Tagliazucchi S, Gennari W, Di Gennaro A, Orsini M et al. Usutu virus infections in humans: a retrospective analysis in the municipality of Modena, Italy. *Clin Microbiol Infect* 2017; 23(1):33–7. <https://doi.org/10.1016/j.cmi.2016.09.019> PMID: 27677699
19. Nagy A, Mezei E, Nagy O, Bakonyi T, Csonka N, Kaposi M et al. Extraordinary increase in West Nile virus cases and first confirmed human Usutu virus infection in Hungary, 2018. *Euro Surveill* 2019; 24(28). <https://doi.org/10.2807/1560-7917.ES.2019.24.28.1900038> PMID: 31311619
20. Pacenti M, Sinigaglia A, Martello T, Rui ME de, Franchin E, Pagni S et al. Clinical and virological findings in patients with Usutu virus infection, northern Italy, 2018. *Euro Surveill* 2019; 24(47). <https://doi.org/10.2807/1560-7917.ES.2019.24.47.1900180> PMID: 31771697
21. Simonin Y, Sillam O, Carles MJ, Gutierrez S, Gil P, Constant O et al. Human Usutu Virus Infection with Atypical Neurologic Presentation, Montpellier, France, 2016. *Emerg Infect Dis* 2018; 24(5):875–8. <https://doi.org/10.3201/eid2405.171122> PMID: 29664365
22. Vilbic-Cavlek T, Kaic B, Barbic L, Pem-Novosel I, Slavic-Vrzic V, Lesnikar V et al. First evidence of simultaneous occurrence of West Nile virus and Usutu virus neuroinvasive disease in humans in Croatia during the 2013 outbreak. *Infection* 2014; 42(4):689–95. <https://doi.org/10.1007/s15010-014-0625-1> PMID: 24793998
23. Cadar D, Simonin Y. Human Usutu Virus Infections in Europe: A New Risk on Horizon? *Viruses* 2023; 15(1):77.
24. Bergmann F, Trachsel DS, Stoeckle SD, Bernis Sierra J, Lübke S, Groschup MH et al. Seroepidemiological Survey of West Nile Virus Infections in Horses from Berlin/Brandenburg and North Rhine-Westphalia, Germany. *Viruses* 2022; 14(2). <https://doi.org/10.3390/v14020243> PMID: 35215837
25. Ganzenberg S, Sieg M, Ziegler U, Pfeffer M, Vahlenkamp TW, Hörügel U et al. Seroprevalence and Risk Factors for Equine West Nile Virus Infections in Eastern Germany, 2020. *Viruses* 2022; 14(6). <https://doi.org/10.3390/v14061191> PMID: 35746662
26. Montagnaro S, Piantadosi D, Ciarcia R, Loponte R, Veneziano V, Fusco G et al. Serological Evidence of Mosquito-Borne Flaviviruses Circulation in Hunting Dogs in Campania Region, Italy. *Vector Borne Zoonotic Dis* 2019; 19(2):142–7. <https://doi.org/10.1089/vbz.2018.2337> PMID: 30160624
27. García-Bocanegra I, Paniagua J, Gutiérrez-Guzmán AV, Lecollinet S, Boadella M, Arenas-Montes A et al. Spatio-temporal trends and risk factors affecting West Nile virus and related flavivirus exposure in Spanish wild ruminants. *BMC Vet Res* 2016; 12(1):249. <https://doi.org/10.1186/s12917-016-0876-4> PMID: 27829427
28. Escribano-Romero E, Lupulović D, Merino-Ramos T, Blázquez A-B, Lazić G, Lazić S et al. West Nile virus serosurveillance in pigs, wild boars, and roe deer in Serbia. *Vet Microbiol* 2015; 176(3–4):365–9. <https://doi.org/10.1016/j.vetmic.2015.02.005> PMID: 25724332
29. Cadar D, Becker N, Campos RdM, Börstler J, Jöst H, Schmidt-Chanasit J. Usutu virus in bats, Germany, 2013. *Emerg Infect Dis* 2014; 20(10):1771–3. <https://doi.org/10.3201/eid2010.140909> PMID: 25271769
30. Romeo C, Lecollinet S, Caballero J, Isla J, Luzzago C, Ferrari N et al. Are tree squirrels involved in the circulation of flaviviruses in Italy? *Transbound Emerg Dis* 2018; 65(5):1372–6. <https://doi.org/10.1111/tbed.12874> PMID: 29635877

31. Diagne MM, Ndione MHD, Di Paola N, Fall G, Bedekelabou AP, Sembène PM et al. Usutu Virus Isolated from Rodents in Senegal. *Viruses* 2019; 11(2). <https://doi.org/10.3390/v11020181> PMID: 30795524
32. Bakonyi T, Jungbauer C, Aberle SW, Kolodziejek J, Dimmel K, Stiasny K et al. Usutu virus infections among blood donors, Austria, July and August 2017—Raising awareness for diagnostic challenges. *Euro Surveill* 2017; 22(41). <https://doi.org/10.2807/1560-7917.ES.2017.22.41.17-00644> PMID: 29043962
33. Cadar D, Lühken R, van der Jeugd H, Garigliany M, Ziegler U, Keller M et al. Widespread activity of multiple lineages of Usutu virus, western Europe, 2016. *Euro Surveill* 2017; 22(4). <https://doi.org/10.2807/1560-7917.ES.2017.22.4.30452> PMID: 28181903
34. Calzolari M, Chiapponi C, Bonilauri P, Lelli D, Baioni L, Barbieri I et al. Co-circulation of two Usutu virus strains in Northern Italy between 2009 and 2014. *Infect Genet Evol* 2017; 51:255–62. <https://doi.org/10.1016/j.meegid.2017.03.022> PMID: 28341546
35. Kemenesi G, Buzás D, Zana B, Kurucz K, Krtinic B, Kepner A et al. First genetic characterization of Usutu virus from *Culex pipiens* mosquitoes Serbia, 2014. *Infect Genet Evol* 2018; 63:58–61. <https://doi.org/10.1016/j.meegid.2018.05.012> PMID: 29778766
36. Oude Munnink BB, Mürger E, Nieuwenhuijse DF, Kohl R, van der Linden A, Schapendonk CME et al. Genomic monitoring to understand the emergence and spread of Usutu virus in the Netherlands, 2016–2018. *Sci Rep* 2020; 10(1):2798. <https://doi.org/10.1038/s41598-020-59692-y> PMID: 32071379
37. Linke S, Niedrig M, Kaiser A, Ellerbrok H, Müller K, Müller T et al. Serologic evidence of West Nile virus infections in wild birds captured in Germany. *Am J Trop Med Hyg* 2007; 77(2):358–64. PMID: 17690413
38. Seidowski D, Ziegler U, Rönn JAC von, Müller K, Hüppop K, Müller T et al. West Nile virus monitoring of migratory and resident birds in Germany. *Vector Borne Zoonotic Dis* 2010; 10(7):639–47. <https://doi.org/10.1089/vbz.2009.0236> PMID: 20854016
39. Jöst H, Bialonski A, Maus D, Sambri V, Eiden M, Groschup MH et al. Isolation of usutu virus in Germany. *Am J Trop Med Hyg* 2011; 85(3):551–3. <https://doi.org/10.4269/ajtmh.2011.11-0248> PMID: 21896821
40. Ziegler U, Seidowski D, Globig A, Fereidouni SR, Ulrich RG, Groschup MH. Sentinel birds in wild-bird resting sites as potential indicators for West Nile virus infections in Germany. *Arch Virol* 2010; 155(6):965–9. <https://doi.org/10.1007/s00705-010-0618-z> PMID: 20437062
41. Kampen H, Holicki CM, Ziegler U, Groschup MH, Tews BA, Werner D. West Nile Virus Mosquito Vectors (Diptera: Culicidae) in Germany. *Viruses* 2020; 12(5). <https://doi.org/10.3390/v12050493> PMID: 32354202
42. Scheuch DE, Schäfer M, Eiden M, Heym EC, Ziegler U, Walther D et al. Detection of Usutu, Sindbis, and Batai Viruses in Mosquitoes (Diptera: Culicidae) Collected in Germany, 2011–2016. *Viruses* 2018; 10(7).
43. Ziegler U, Jöst H, Müller K, Fischer D, Rinder M, Tietze DT et al. Epidemic Spread of Usutu Virus in Southwest Germany in 2011 to 2013 and Monitoring of Wild Birds for Usutu and West Nile Viruses. *Vector Borne Zoonotic Dis* 2015; 15(8):481–8. <https://doi.org/10.1089/vbz.2014.1746> PMID: 26273809
44. Michel F, Fischer D, Eiden M, Fast C, Reuschel M, Müller K et al. West Nile Virus and Usutu Virus Monitoring of Wild Birds in Germany. *Int J Environ Res Public Health* 2018; 15(1). <https://doi.org/10.3390/ijerph15010171> PMID: 29361762
45. Michel F, Sieg M, Fischer D, Keller M, Eiden M, Reuschel M et al. Evidence for West Nile Virus and Usutu Virus Infections in Wild and Resident Birds in Germany, 2017 and 2018. *Viruses* 2019; 11(7). <https://doi.org/10.3390/v11070674> PMID: 31340516
46. Ziegler U, Bergmann F, Fischer D, Müller K, Holicki CM, Sadeghi B et al. Spread of West Nile Virus and Usutu Virus in the German Bird Population, 2019–2020. *Microorganisms* 2022; 10(4). <https://doi.org/10.3390/microorganisms10040807> PMID: 35456857
47. Ziegler U, Fast C, Eiden M, Bock S, Schulze C, Hoepfer D et al. Evidence for an independent third Usutu virus introduction into Germany. *Vet Microbiol* 2016; 192:60–6. <https://doi.org/10.1016/j.vetmic.2016.06.007> PMID: 27527765
48. Cadar D, Bosch S, Jöst H, Börstler J, Garigliany M-M, Becker N et al. Putative Lineage of Novel African Usutu Virus, Central Europe. *Emerg Infect Dis* 2015; 21(9):1647–50. <https://doi.org/10.3201/eid2109.142026> PMID: 26291923
49. Sieg M, Schmidt V, Ziegler U, Keller M, Höper D, Heenemann K et al. Outbreak and Cocirculation of Three Different Usutu Virus Strains in Eastern Germany. *Vector Borne Zoonotic Dis* 2017; 17(9):662–4. <https://doi.org/10.1089/vbz.2016.2096> PMID: 28816628

50. Ben Hassine T, Massis F de, Calistri P, Savini G, BelHaj Mohamed B, Ranen A et al. First detection of co-circulation of West Nile and Usutu viruses in equids in the south-west of Tunisia. *Transbound Emerg Dis* 2014; 61(5):385–9. <https://doi.org/10.1111/tbed.12259> PMID: 25065813
51. Durand B, Haskouri H, Lowenski S, Vachiere N, Beck C, Lecollinet S. Seroprevalence of West Nile and Usutu viruses in military working horses and dogs, Morocco, 2012: dog as an alternative WNV sentinel species? *Epidemiol Infect* 2016; 144(9):1857–64. <https://doi.org/10.1017/S095026881600011X> PMID: 26838515
52. Mannasse B, Mendelson E, Orshan L, Mor O, Shalom U, Yeger T et al. Usutu Virus RNA in Mosquitoes, Israel, 2014–2015. *Emerg Infect Dis* 2017; 23(10):1699–702. <https://doi.org/10.3201/eid2310.171017> PMID: 28930008
53. Chaintoutis SC, Dovas CI, Papanastassopoulou M, Gewehr S, Danis K, Beck C et al. Evaluation of a West Nile virus surveillance and early warning system in Greece, based on domestic pigeons. *Comp Immunol Microbiol Infect Dis* 2014; 37(2):131–41. <https://doi.org/10.1016/j.cimid.2014.01.004> PMID: 24503179
54. Eiden M, Gil P, Ziegler U, Rakotoarivony I, Marie A, Frances B et al. Emergence of two Usutu virus lineages in *Culex pipiens* mosquitoes in the Camargue, France, 2015. *Infect Genet Evol* 2018; 61:151–4. <https://doi.org/10.1016/j.meegid.2018.03.020> PMID: 29592838
55. Bakonyi T, Busquets N, Nowotny N. Comparison of complete genome sequences of Usutu virus strains detected in Spain, Central Europe, and Africa. *Vector Borne Zoonotic Dis* 2014; 14(5):324–9. <https://doi.org/10.1089/vbz.2013.1510> PMID: 24746182
56. Bażanów B, van Jansen Vuren P, Szymański P, Stygar D, Fracka A, Twardoń J et al. A Survey on West Nile and Usutu Viruses in Horses and Birds in Poland. *Viruses* 2018; 10(2). <https://doi.org/10.3390/v10020087> PMID: 29462983
57. Rudolf I, Bakonyi T, Šebesta O, Mendel J, Peško J, Betášová L et al. Co-circulation of Usutu virus and West Nile virus in a reed bed ecosystem. *Parasit Vectors* 2015; 8:520. <https://doi.org/10.1186/s13071-015-1139-0> PMID: 26459018
58. Buckley A, Dawson A, Moss SR, Hinsley SA, Bellamy PE, Gould EA. Serological evidence of West Nile virus, Usutu virus and Sindbis virus infection of birds in the UK. *J Gen Virol* 2003; 84(Pt 10):2807–17. Available from: URL: <https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.19341-0>. <https://doi.org/10.1099/vir.0.19341-0> PMID: 13679615
59. Barbic L, Vilibic-Cavlek T, Listes E, Stevanovic V, Gjenero-Margan I, Ljubin-Sternak S et al. Demonstration of Usutu virus antibodies in horses, Croatia. *Vector Borne Zoonotic Dis* 2013; 13(10):772–4. <https://doi.org/10.1089/vbz.2012.1236> PMID: 23808977
60. Rijks JM, Kik ML, Slaterus R, Foppen R, Stroo A, IJzer J et al. Widespread Usutu virus outbreak in birds in the Netherlands, 2016. *Euro Surveill* 2016; 21(45). <https://doi.org/10.2807/1560-7917.ES.2016.21.45.30391> PMID: 27918257
61. Höning V, Palus M, Kaspar T, Zemanova M, Majerova K, Hofmannova L et al. Multiple Lineages of Usutu Virus (Flaviviridae, Flavivirus) in Blackbirds (*Turdus merula*) and Mosquitoes (*Culex pipiens*, *Cx. modestus*) in the Czech Republic (2016–2019). *Microorganisms* 2019; 7(11). <https://doi.org/10.3390/microorganisms7110568> PMID: 31744087
62. Faria NR, Kraemer MUG, Hill SC, Goes de Jesus J, Aguiar RS, Iani FCM et al. Genomic and epidemiological monitoring of yellow fever virus transmission potential. *Science* 2018; 361(6405):894–9. <https://doi.org/10.1126/science.aat7115> PMID: 30139911
63. Faria NR, Quick J, Claro IM, Thézé J, Jesus JG de, Giovanetti M et al. Establishment and cryptic transmission of Zika virus in Brazil and the Americas. *Nature* 2017; 546(7658):406–10. <https://doi.org/10.1038/nature22401> PMID: 28538727
64. Faria NR, Da Azevedo RdSS, Kraemer MUG, Souza R, Cunha MS, Hill SC et al. Zika virus in the Americas: Early epidemiological and genetic findings. *Science* 2016; 352(6283):345–9. <https://doi.org/10.1126/science.aaf5036> PMID: 27013429
65. Hill SC, Neto de Vasconcelos J, Granja BG, Thézé J, Jandondo D, Neto Z et al. Early Genomic Detection of Cosmopolitan Genotype of Dengue Virus Serotype 2, Angola, 2018. *Emerg Infect Dis* 2019; 25(4):784–7. <https://doi.org/10.3201/eid2504.180958> PMID: 30882320
66. Naveca FG, Claro I, Giovanetti M, Jesus JG de, Xavier J, Iani FCdM et al. Genomic, epidemiological and digital surveillance of Chikungunya virus in the Brazilian Amazon. *PLoS Negl Trop Dis* 2019; 13(3):e0007065. <https://doi.org/10.1371/journal.pntd.0007065> PMID: 30845267
67. Holicki CM, Bergmann F, Stoek F, Schulz A, Groschup MH, Ziegler U et al. Expedited retrieval of high-quality Usutu virus genomes via Nanopore sequencing with and without target enrichment. *Front. Microbiol.* 2022; 13. Available from: URL: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.1044316>.

68. Ziegler U, Eiden M, Fast C, Keller M, Groschup MH. Tiergesundheitsjahresbericht 2017: Usutu-Virus-Infektion (USUV)—Usutu virus infection: Friedrich-Loeffler-Institut; 2017 [cited 2022 Nov 10]. Available from: URL: https://www.openagrar.de/rsc/viewer/openagrar_derivate_00019140/SD201845870.pdf?page=1.
69. Ziegler U, Eiden M, Fast C, Keller M, Groschup MH. Tiergesundheitsjahresbericht 2018: Usutu-Virus-Infektion (USUV)—Usutu virus infection: Friedrich-Loeffler-Institut; 2018. Available from: URL: https://www.openagrar.de/rsc/viewer/openagrar_derivate_00025589/SD201954064.pdf?page=1.
70. Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit. Datensammlung West-Nil-Fieber zur Virusinfektion bei Vögeln [cited 2022 Dec 14]. Available from: URL: <https://westnil-fieber.fli.de/anmeldung/?returnurl=%2fgib-daten-ein%2f>.
71. Quick J, Grubaugh ND, Pullan ST, Claro IM, Smith AD, Gangavarapu K et al. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nat Protoc* 2017; 12(6):1261–76. <https://doi.org/10.1038/nprot.2017.066> PMID: 28538739
72. Oude Munnink BB, Kik M, Bruijn ND de, Kohl R, van der Linden A, Reusken CBEM et al. Towards high quality real-time whole genome sequencing during outbreaks using Usutu virus as example. *Infect Genet Evol* 2019; 73:49–54. <https://doi.org/10.1016/j.meegid.2019.04.015> PMID: 31014969
73. Wick RR, Judd LM, Holt KE. Performance of neural network basecalling tools for Oxford Nanopore sequencing. *Genome Biol* 2019; 20(1):129. <https://doi.org/10.1186/s13059-019-1727-y> PMID: 31234903
74. Goodacre N, Aljanahi A, Nandakumar S, Mikailov M, Khan AS. A Reference Viral Database (RVDB) To Enhance Bioinformatics Analysis of High-Throughput Sequencing for Novel Virus Detection. *mSphere* 2018; 3(2). <https://doi.org/10.1128/mSphereDirect.00069-18> PMID: 29564396
75. Clausen PTLC Aarestrup FM, Lund O. Rapid and precise alignment of raw reads against redundant databases with KMA. *BMC Bioinformatics* 2018; 19(1):307. <https://doi.org/10.1186/s12859-018-2336-6> PMID: 30157759
76. Li H. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* 2018; 34(18):3094–100. <https://doi.org/10.1093/bioinformatics/bty191> PMID: 29750242
77. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. *Nucleic Acids Res* 2010; 38(Database issue):D46–51. <https://doi.org/10.1093/nar/gkp1024> PMID: 19910366
78. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004; 32(5):1792–7. <https://doi.org/10.1093/nar/gkh340> PMID: 15034147
79. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol Biol Evol* 2021; 38(7):3022–7. <https://doi.org/10.1093/molbev/msab120> PMID: 33892491
80. FigTree version 1.4.3.; 2012. Available from: URL: <http://tree.bio.ed.ac.uk/software/figtree/>.
81. Lê S, Josse J, Husson F. FactoMineR: An R Package for Multivariate Analysis. *J. Stat. Soft.* 2008; 25(1).
82. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007; 7:214. <https://doi.org/10.1186/1471-2148-7-214> PMID: 17996036
83. Drummond AJ, Rambaut A, Shapiro B, Pybus OG. Bayesian coalescent inference of past population dynamics from molecular sequences. *Mol Biol Evol* 2005; 22(5):1185–92. <https://doi.org/10.1093/molbev/msi103> PMID: 15703244
84. GeoBasis-DE. Available from: URL: <https://gdz.bkg.bund.de/index.php/default/open-data.html>.
85. Störk T, Le Roi M de, Haverkamp A-K, Jesse ST, Peters M, Fast C et al. Analysis of avian Usutu virus infections in Germany from 2011 to 2018 with focus on dsRNA detection to demonstrate viral infections. *Sci Rep* 2021; 11(1):24191. <https://doi.org/10.1038/s41598-021-03638-5> PMID: 34921222
86. Zehender G, Veo C, Ebranati E, Carta V, Rovida F, Percivalle E et al. Reconstructing the recent West Nile virus lineage 2 epidemic in Europe and Italy using discrete and continuous phylogeography. *PLoS One* 2017; 12(7):e0179679. <https://doi.org/10.1371/journal.pone.0179679> PMID: 28678837
87. Chaintoutis SC, Papa A, Pervanidou D, Dovas CI. Evolutionary dynamics of lineage 2 West Nile virus in Europe, 2004–2018: Phylogeny, selection pressure and phylogeography. *Molecular Phylogenetics and Evolution* 2019; 141:106617. Available from: URL: <https://www.sciencedirect.com/science/article/pii/S1055790319303288>. <https://doi.org/10.1016/j.ympev.2019.106617> PMID: 31521822
88. Hlinak A, Mühle RU, Werner O, Globig A, Starick E, Schirrmeyer H et al. A virological survey in migrating waders and other waterfowl in one of the most important resting sites of Germany. *J Vet Med B Infect Dis Vet Public Health* 2006; 53(3):105–10. <https://doi.org/10.1111/j.1439-0450.2006.00935.x> PMID: 16629720
89. Mühlemann B, Jones TC, Damgaard PdB, Allentoft ME, Shevnina I, Logvin A et al. Ancient hepatitis B viruses from the Bronze Age to the Medieval period. *Nature* 2018; 557(7705):418–23. <https://doi.org/10.1038/s41586-018-0097-z> PMID: 29743673

90. Zecchin B, Fusaro A, Milani A, Schivo A, Ravagnan S, Ormelli S et al. The central role of Italy in the spatial spread of USUTU virus in Europe. *Virus Evol* 2021; 7(1):veab048. <https://doi.org/10.1093/ve/veab048> PMID: 34513027
91. Rubel F, Brugger K, Hantel M, Chvala-Mannsberger S, Bakonyi T, Weissenböck H et al. Explaining Usutu virus dynamics in Austria: model development and calibration. *Prev Vet Med* 2008; 85(3–4):166–86. <https://doi.org/10.1016/j.prevetmed.2008.01.006> PMID: 18314208
92. Durand B, Balança G, Baldet T, Chevalier V. A metapopulation model to simulate West Nile virus circulation in Western Africa, Southern Europe and the Mediterranean basin. *Vet Res* 2010; 41(3):32. <https://doi.org/10.1051/vetres/2010004> PMID: 20167194
93. Holicki CM, Scheuch DE, Ziegler U, Lettow J, Kampen H, Werner D et al. German *Culex pipiens* biotype *molestus* and *Culex torrentium* are vector-competent for Usutu virus. *Parasit Vectors* 2020; 13(1):625. <https://doi.org/10.1186/s13071-020-04532-1> PMID: 33380339
94. Holicki CM. Mosquito-borne Flaviviruses: Vector and Avian Host Susceptibility for West Nile Virus and Usutu Virus in Germany. Hannover: Tierärztliche Hochschule Hannover; 2020.
95. Brugger K, Rubel F. Simulation of climate-change scenarios to explain Usutu-virus dynamics in Austria. *Prev Vet Med* 2009; 88(1):24–31. <https://doi.org/10.1016/j.prevetmed.2008.06.023> PMID: 18715663
96. Semenza JC, Suk JE. Vector-borne diseases and climate change: a European perspective. *FEMS Microbiol Lett* 2018; 365(2). <https://doi.org/10.1093/femsle/fnx244> PMID: 29149298
97. Chvala S, Bakonyi T, Bukovsky C, Meister T, Brugger K, Rubel F et al. Monitoring of Usutu virus activity and spread by using dead bird surveillance in Austria, 2003–2005. *Vet Microbiol* 2007; 122(3–4):237–45. <https://doi.org/10.1016/j.vetmic.2007.01.029> PMID: 17346908
98. Ziegler U, Lühken R, Keller M, Cadar D, van der Grinten E, Michel F et al. West Nile virus epizootic in Germany, 2018. *Antiviral Res* 2019; 162:39–43. <https://doi.org/10.1016/j.antiviral.2018.12.005> PMID: 30550796
99. Weidinger P, Kolodziejek J, Bakonyi T, Brunthaler R, Erdélyi K, Weissenböck H et al. Different dynamics of Usutu virus infections in Austria and Hungary, 2017–2018. *Transbound Emerg Dis* 2020; 67(1):298–307. <https://doi.org/10.1111/tbed.13351> PMID: 31505099
100. Meister T, Lussy H, Bakonyi T, Sikutová S, Rudolf I, Vogl W et al. Serological evidence of continuing high Usutu virus (Flaviviridae) activity and establishment of herd immunity in wild birds in Austria. *Vet Microbiol* 2008; 127(3–4):237–48. <https://doi.org/10.1016/j.vetmic.2007.08.023> PMID: 17869454
101. Holicki CM, Ziegler U, Răileanu C, Kampen H, Werner D, Schulz J et al. West Nile Virus Lineage 2 Vector Competence of Indigenous *Culex* and *Aedes* Mosquitoes from Germany at Temperate Climate Conditions. *Viruses* 2020; 12(5). <https://doi.org/10.3390/v12050561> PMID: 32438619
102. Kampen H, Tews BA, Werner D. First Evidence of West Nile Virus Overwintering in Mosquitoes in Germany. *Viruses* 2021; 13(12). <https://doi.org/10.3390/v13122463> PMID: 34960732
103. Constant O, Bollere K, Clé M, Barthelemy J, Foulongne V, Chenet B et al. Evidence of Exposure to USUV and WNV in Zoo Animals in France. *Pathogens* 2020; 9(12). <https://doi.org/10.3390/pathogens9121005> PMID: 33266071
104. Deutscher Wetterdienst. Climatological maps of Germany: Federal Ministry for Digital and Transport; 2022 [cited 2022 Nov 8]. Available from: URL: <https://www.dwd.de/EN/ourservices/klimakartendeutschland/klimakartendeutschland.html?nn=495490>.