

## **PLOS Neglected Tropical Diseases**

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

Short title: Molecular evolution of Usutu virus in Germany

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## **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 associated with mass mortalities in birds, formerly 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.

52 Nonetheless, there is to date limited access to whole genome sequences resulting in the absence of in-  
53 depth phylogenetic and phylodynamic analyses. In this study, 118 wild and captive birds were screened  
54 using a nanopore sequencing platform with prior target enrichment via amplicons. Due to the high  
55 abundancy of Europe 3 and Africa 3 in Germany an ample quantity of associated whole genome  
56 sequences was generated and the most recent common ancestor could be determined for each lineage.  
57 The corresponding clock phylogeny revealed an introduction of USUV Europe 3 and Africa 3 into  
58 Germany three years prior to their first isolation in the avifauna in 2011 and 2014, respectively. Based  
59 on the clustering and temporal history of the lineages, evidence exists for the genetic evolution of USUV  
60 within Germany as well as new introductions thereof into the country.  
61

## 62 Introduction

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

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

80 The virus also has a zoonotic potential and can infect humans, in rare cases causing severe neurological  
81 symptoms (16–23). Humans as well as other susceptible mammalian species are considered dead-end  
82 hosts as they can become infected but cannot sustain the transmission cycle. USUV has in the past been  
83 found in horses (24, 25), dogs (26), deer (27), wild boar (28), bats (29), squirrels (30), and rodents (31).  
84 The genetic variability of USUV was studied by phylogenetic studies targeting partial sequences,  
85 especially of the envelope E and NS5 genes, and whole genome sequences (15, 32–35, 4, 36). These  
86 analyses resulted in the declaration of eight distinct lineages, which cluster together according to their  
87 geographic origin of detection: Africa 1-3 and Europe 1-5. Migratory birds from Africa are thought to  
88 have brought different USUV lineages to Europe at independent time points, resulting in the dispersal  
89 of distinct USUV lineages. As evidenced by phylogenetic analyses these lineages amplified and evolved  
90 independently. The genetic heterogeneity of the European lineages is, therefore, most likely due to in  
91 situ evolution rather than new introductions by long-distance migratory birds (15). By comparison,  
92 widespread migration patterns and multiple introductions of virus variants from different geographic  
93 areas of origin resulted in the African lineages (15). Nonetheless, in the literature the assignment and  
94 nomenclature of USUV lineages/strains has not been standardized. It has also not been conclusively  
95 determined whether the different lineages have an influence on host and vector affinity (15).

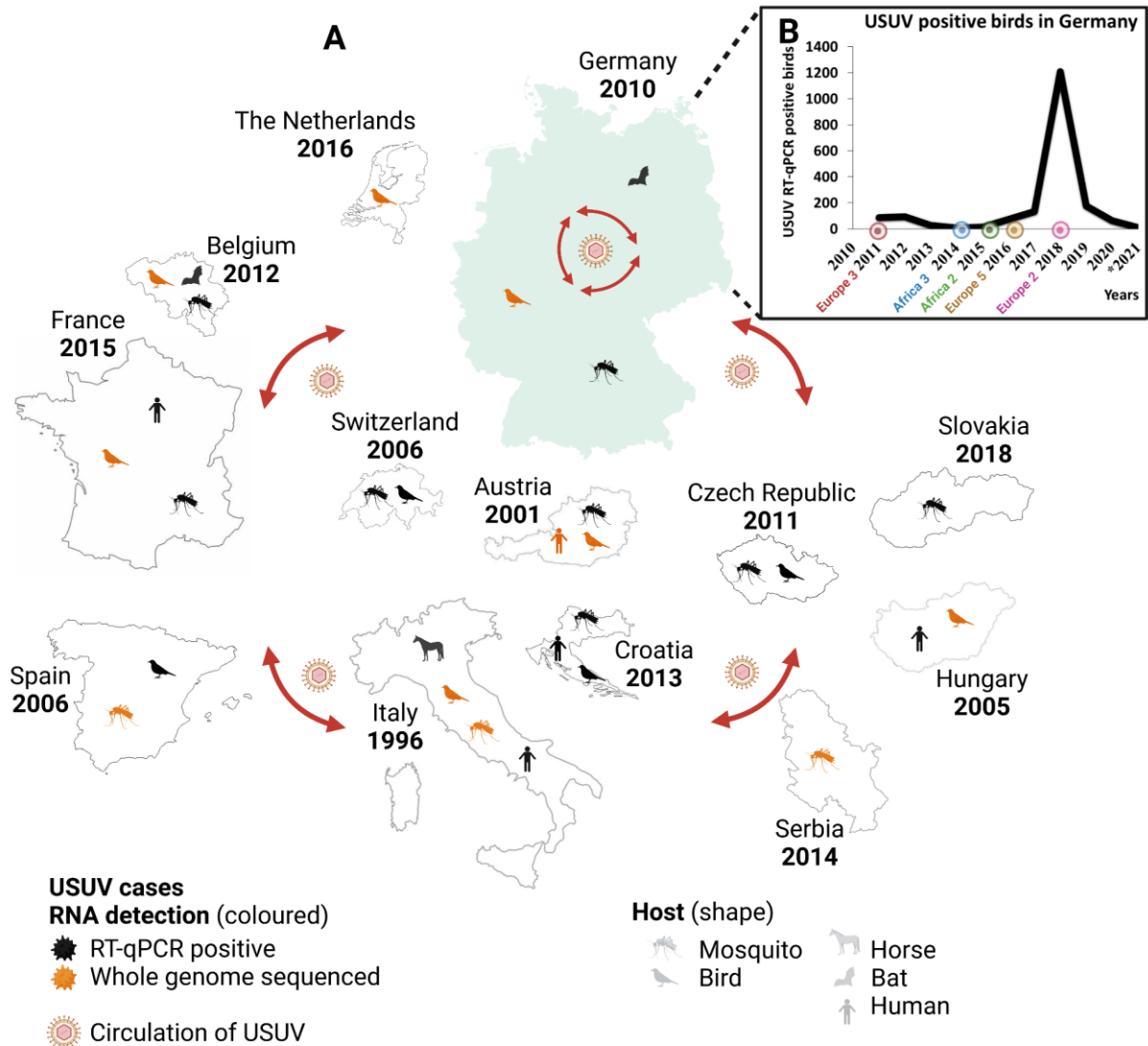
96 Circulating in Europe, USUV did not stop at the boundary to Germany (Fig 1 (A)). In Germany, initial  
97 surveillance efforts for flaviviruses focused on the serological detection of WNV antibodies (37, 38).  
98 USUV-antibodies were solely determined in the further clarification of flavivirus cross-reactivity (37).  
99 However, since 2009 molecular surveillance programs in mosquitoes and birds were implemented,  
100 followed by several introductions into the country (39–46). The first USUV isolate (lineage Europe 3)  
101 was documented in Germany in 2010 from a pool of *Culex pipiens* biotype *pipiens* mosquitoes in the  
102 south of Frankfurt, in Weinheim (39). Subsequently, fatal cases in wild and captive birds, mainly  
103 Eurasian blackbirds and owls, were reported from the north of the Upper Rhine valley and adjacent areas  
104 of the Palatinate and the Neckar valley to the Southwest of Germany (8, 43). Around the same time

105 (2014), a new USUV lineage (Africa 3) was introduced into the north of Germany, in Bonn, where only  
106 one case in a blackbird was detected (47, 48). In Berlin in 2015, the lineage Africa 2 occurred for the  
107 first time in two juvenile great grey owls (47). In 2016, USUV (lineage Europe 3, Africa 3, and Africa  
108 2) continued to spread, with numerous cases reported in the southwest, northwest, and east of Germany  
109 concurrent with the first detection of Europe 5 in central-western North Rhine-Westphalia (44, 49, 33).  
110 In addition, a further spread to neighbouring countries to the west could be confirmed (33). In the Federal  
111 State of Saxony in 2018, USUV lineage Europe 2 was detected for the first time (45). Until 2018, the  
112 virus had spread nationwide with five USUV lineages present (Africa 2, Africa 3, Europe 2, Europe 3,  
113 and Europe 5) (Fig 1 (B)).

114 To date, the circulation of USUV has been reported (based on serological and molecular evidence) in  
115 many countries in and around Europe: Tunisia (50), Morocco (51), Israel (52), Greece (53), France (54),  
116 Spain (55), Poland (56), Hungary (6), Czech Republic (57), Serbia (35), the United Kingdom (58),  
117 Croatia (59), the Netherlands (60), Switzerland (10), Italy (11), and Germany (39). Ongoing efforts to  
118 elucidate the USUV phylogenetic scenario in Europe, reported the co-circulation of Europe 3 and Africa  
119 3 in the Netherlands (36), Europe 3 and Africa 2 and 3 in France (54), and Europe 1-3 and Africa 3 in  
120 the Czech Republic (61). In Germany, there is an evident co-circulation of the USUV lineages Europe  
121 2 and 3, and Africa 2 and 3 in 2017 and 2018 (45). A recently published study based on partial sequences  
122 from 2019 and 2020, could confirm the ongoing circulation of USUV lineages Europe 2 and 3 as well  
123 Africa 3 in the country (46).

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

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 139 **Fig 1 (A) Geographic distribution of USUV-RNA detection throughout Europe. The first detected**  
 140 **USUV-positive case per country is depicted in the figure. In addition, the icons for mosquito, bird,**  
 141 **horse, bat, and human indicate in which species USUV-RNA has been detected so far and whether**  
 142 **a corresponding whole genome sequence is available (in orange). (B) The graph shows all USUV-**  
 143 **positive birds detected in Germany since 2011, highlighting the first occurrence of each lineage in**  
 144 **the following years, with the exception of USUV Europe 3, which was first detected in 2010 in a**  
 145 **mosquito pool. \*Results not finalized and only based on dead bird surveillance (updated on 21<sup>th</sup>**  
 146 **February 2023). Created with BioRender.com.**  
 147

## 148 Material and Methods

### 149 Samples

150 Blood and organ samples of 118 wild and captive birds were collected in the frame of ongoing  
 151 monitoring bird studies and in close collaboration with the local state laboratories in Germany and  
 152 submitted to the national reference laboratory (NRL) for West Nile virus (WNV) and USUV at the  
 153 Friedrich-Loeffler-Institut (FLI). From 2019 to 2021, a total of 3,762 birds were tested for USUV. The  
 154 samples were recorded in a database for the detection of USUV-RNA in birds, which was established  
 155 in 2019 with the aim of providing the public health authorities with a nationwide overview (46).  
 156 Unfortunately, prior to 2019, there was no comparable database and the number of tested birds in  
 157 addition to those that were part of the study described by Michel et al. 2019 (45) can only be estimated  
 158 for 2017 and 2018 and are most likely higher in reality (Table 1).

159 For WGS, USUV RNA-positive samples were selected primarily on the basis of their geographic  
 160 location in order to represent a comprehensive picture of Germany. The cycle threshold (Ct) values and  
 161 to some extent also the lineages, as already determined by partial sequencing (46, 45, 67), were used as  
 162 further decision criteria. Throughout all five years, USUV isolates from the two different bird orders  
 163 *Passeriformes* and *Strigiformes* were identified and sequenced. In 2019, USUV was additionally found  
 164 and sequenced in *Anseriformes* and *Columbiformes* (Table 2). Viral RNA of blood and organ samples  
 165 was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's  
 166 protocol. Cell culture supernatant samples were extracted using the Viral RNA Mini Kit (Qiagen)  
 167 following the manufacturer's instructions. Analysis of extracted RNA was performed using real-time  
 168 reverse transcription PCR (RT-qPCR) assays specific for USUV, as described by Jöst et al. 2011 (39).  
 169 Samples with Ct values between 12.09 and 32.79, covering almost all Federal States of Germany, were  
 170 included in this study. Detailed information of each sample is provided in S1 Table. Four of the  
 171 sequenced birds (GenBank accession numbers: OP422562-OP422565) were already published in a next-  
 172 generation sequencing (NGS) methodological publication by Holicki et al. 2022 (67).  
 173

174 **Table 1. Number of molecularly tested birds per year, from the live and dead bird surveillance,**  
 175 **including USUV RT-qPCR positive results and number of samples sequenced (68, 69, 45, 70).**  
 176

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
<b>Total</b>	<b>6,671</b>	<b>1,593</b>	<b>118</b>

177 NA= not available

178 \*Results not finalized and only based on dead bird surveillance (updated on 21th February 2023).  
 179

180 **Table 2. Overview of sample set.**

	2017	2018	2019	2020	2021
<b>Total (Wild/Captive Birds)</b>	6 (3/3)	45 (34/11)	37 (29/8)	25 (22/3)	5 (1/4)
<b>Species Order</b>	<i>Passeriformes</i> , <i>Strigiformes</i>	<i>Passeriformes</i> , <i>Strigiformes</i>	<i>Passeriformes</i> , <i>Strigiformes</i> , <i>Columbiformes</i> , <i>Anseriformes</i>	<i>Passeriformes</i> , <i>Strigiformes</i>	<i>Passeriformes</i> , <i>Strigiformes</i>
<b>Organs</b>	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
<b>Federal States</b>	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
<b>Lineages</b>	Africa 3, Europe 3	Africa 3, Europe 3	Africa 3, Europe 3, Europe 2	Africa 3, Europe 3, Europe 2	Africa 3, Europe 3

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

182 Abbreviation: CNS: central nervous system; BB: Berlin Brandenburg; BE: Berlin; BW: Baden-Württemberg; BY: Bavaria; HE: Hesse; NI:

183 Lower Saxony; MV: Mecklenburg-West Pomerania; NW: North Rhine-Westphalia; SL: Saarland; SN: Saxony; ST: Saxony-Anhalt; TH:

184 Thuringia  
 185  
 186

## 187 Nanopore sequencing and data analysis

188 Nanopore sequencing was performed as described by Holicki et al. 2022 (67). In short, RNA was reverse  
 189 transcribed using the SuperScript IV First-Strand cDNA Synthesis Reaction Kit (Cat. no. 18091050;  
 190 Invitrogen by Thermo Fisher Scientific, Darmstadt, Germany) with random primers as previously  
 191 described by Quick et al. 2017 (71). Followed by an USUV-specific multiplex PCR which was  
 192 performed with two separate mixes of primer pairs using AccuPrime Taq DNA Polymerase High  
 193 Fidelity (Cat. no. 12346-086; Invitrogen). MinION sequencing was carried out following the  
 194 manufacturer's instructions using the 1D Native barcoding genomic DNA Kit (Nanopore, EXP-NBD104

195 and SQK-LSK109, Oxford Nanopore-technology (ONT)) on a Spot-ON flow cell (R9.4.1; ONT).  
196 Twelve samples were multiplexed per flow cell. Fast5 raw data reads were demultiplexed using Guppy  
197 v4.5.4 (72). Primers were trimmed and reads were quality controlled to a minimal length of 200 base  
198 pairs (bp) and reads with a minimum quality of 7 were considered for further analysis. For consensus  
199 sequence generation, an alignment against the selected USUV reference genomes v23 (73) was  
200 performed using KMA (74) and Minimap2 (75). Consensus sequences were visualized with Geneious  
201 Prime 2021.0.1 (Biomatters Ltd., Auckland, New Zealand).

202

## 203 **USUV genome phylogenetic analyses**

204 USUV sequences of the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA)  
205 were screened and all available full length USUV genomes from Germany up to August 24, 2022 were  
206 downloaded (76). All full length USUV genomes including the newly sequenced USUV genomes were  
207 aligned using MUSCLE (77) and manually inspected. The maximum likelihood phylogenetic analysis  
208 was conducted using General Time Reversible (GTR) model with 1,000 bootstraps in MEGA v11 (78)  
209 and finalized trees were reconstructed with FigTree v.1.4.3 (79).

210

## 211 **Estimating time to the most recent common ancestor (TMRCA)**

212 For evolutionary dynamic analyses and to determine the age of the most recent common ancestors, the  
213 Bayesian Markov chain Monte Carlo (MCMC) method was performed using BEAST v2.6.6 package  
214 (80). In these analyses, a GTR + gamma (G) substitution model and a strict clock model were applied  
215 (81). MCMC was set to 100,000,000 generations (sampling every 2,500 steps). Log files were analysed  
216 in Tracer v1.7.1 to check effective sampling size (ESS) values (>200 indicated sufficient sampling). The  
217 maximum clade credibility (MCC) tree was generated in the Tree Annotator v1.8.4, with a default burn-  
218 in of 10%. The MCC tree was visualized in the FigTree program v1.4.3 (79).

219

## 220 **Geolocation of USUV strains sequenced in this study**

221 GIS analysis of USUV-positive birds used for sequencing, was performed using ArcGIS ArcMap 10.8.1  
222 (ESRI, Redlands, CA, USA) and open data from GeoBasis-DE/BKG 2022 (82).

223

## 224 **Ethical statement**

225 Veterinarians, wild bird rescue centres, and zoological facilities supplied bird carcasses for necropsy.  
226 Residual blood material was available from birds collected primarily for veterinary examination,  
227 diagnostic purposes, specific treatments, and the effectiveness of a treatment.

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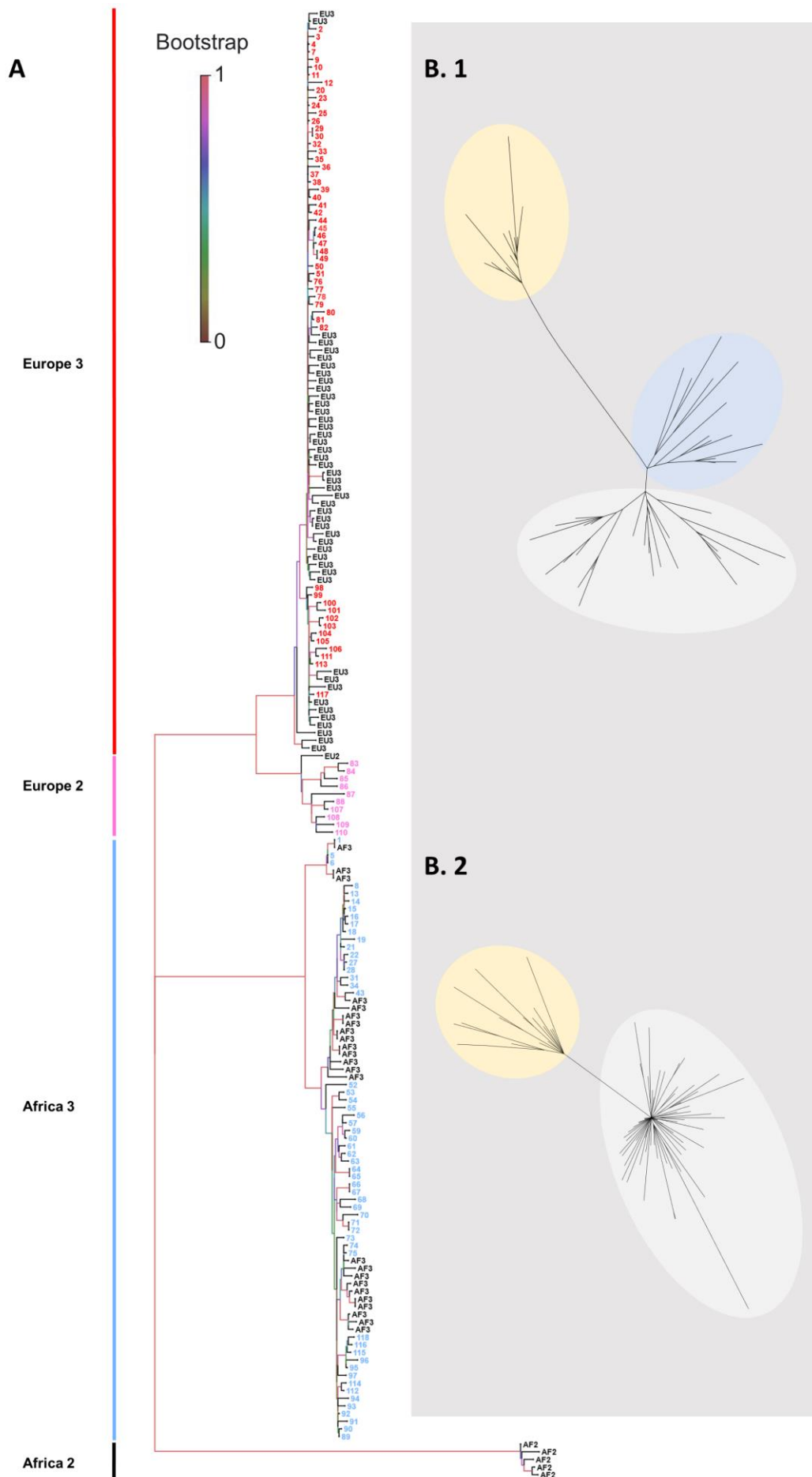
## 230 **Results**

### 231 **USUV genomic sequencing**

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

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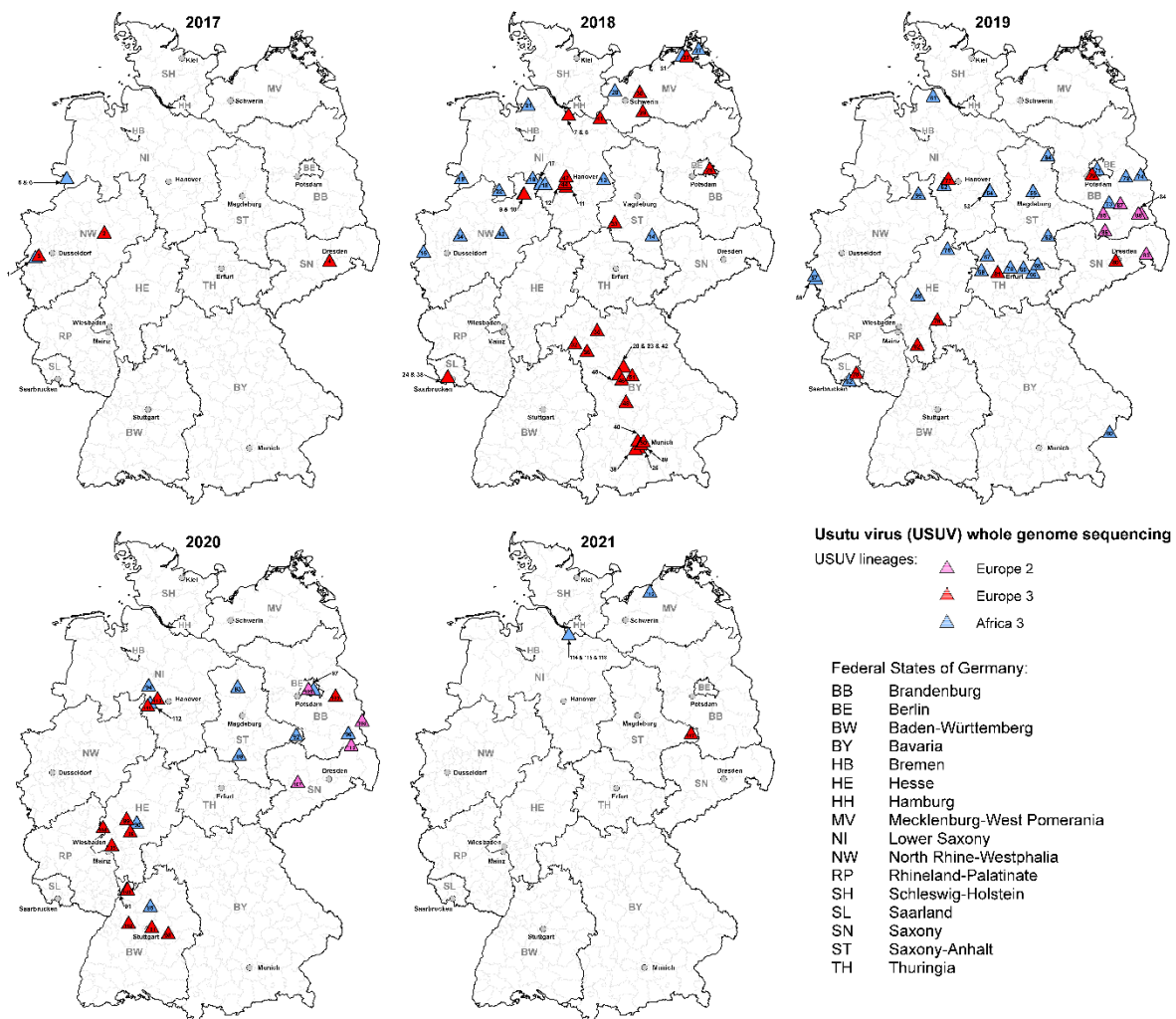
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241 **Fig 2. Phylogeny of the sequenced USUV isolates from 2017–2021**  
 242 (A) Samples were numbered consecutively and coloured according to the lineages. Reference  
 243 genomes are marked as EU3 (Europe 3), EU2 (Europe 2), A3 (Africa 3), and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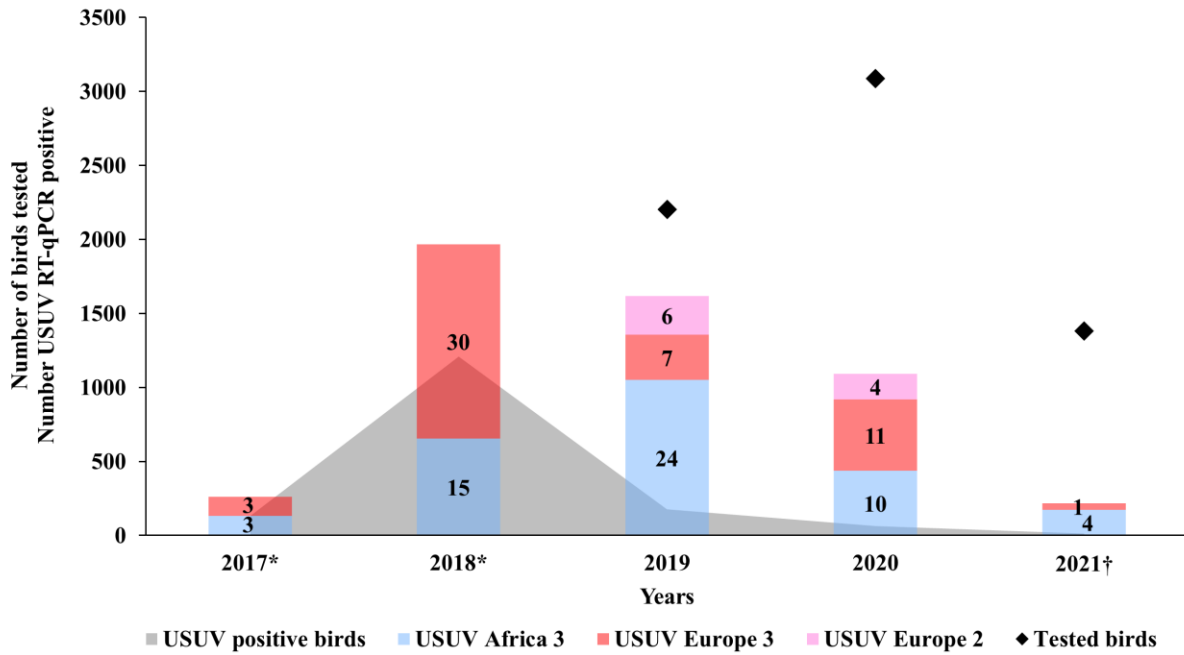
## 250 Phylogenetic analysis of USUV in Germany

251 The phylogenetic analysis displays the co-circulation of USUV lineages Europe 2, Europe 3, and Africa  
 252 3 in Germany, with whole genome sequences of lineage Europe 2 only present in 2019 and 2020 (Fig  
 253 3). USUV lineage Africa 3 was detected almost as often as Europe 3, while Europe 2 was only sequenced  
 254 in 9% of the cases (Fig 4). Africa 3 and Europe 3 were distributed throughout the country, whereas  
 255 Europe 2 was only found in the eastern part of Germany, in the Federal States Berlin, Brandenburg, and  
 256 Saxony (Fig 3). USUV lineages Europa 5 and Africa 2 were not part of this study.



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 259 **Fig 3. Geographic distribution of whole genome sequences of USUV in Germany from 2017 to**  
 260 **2021. The different USUV lineages are depicted as coloured triangles: pink = Europe 2, red =**  
 261 **Europe 3, and light blue = Africa 3 with the appropriate sample number (detailed information to**  
 262 **each sample in S1 Table).**  
 263

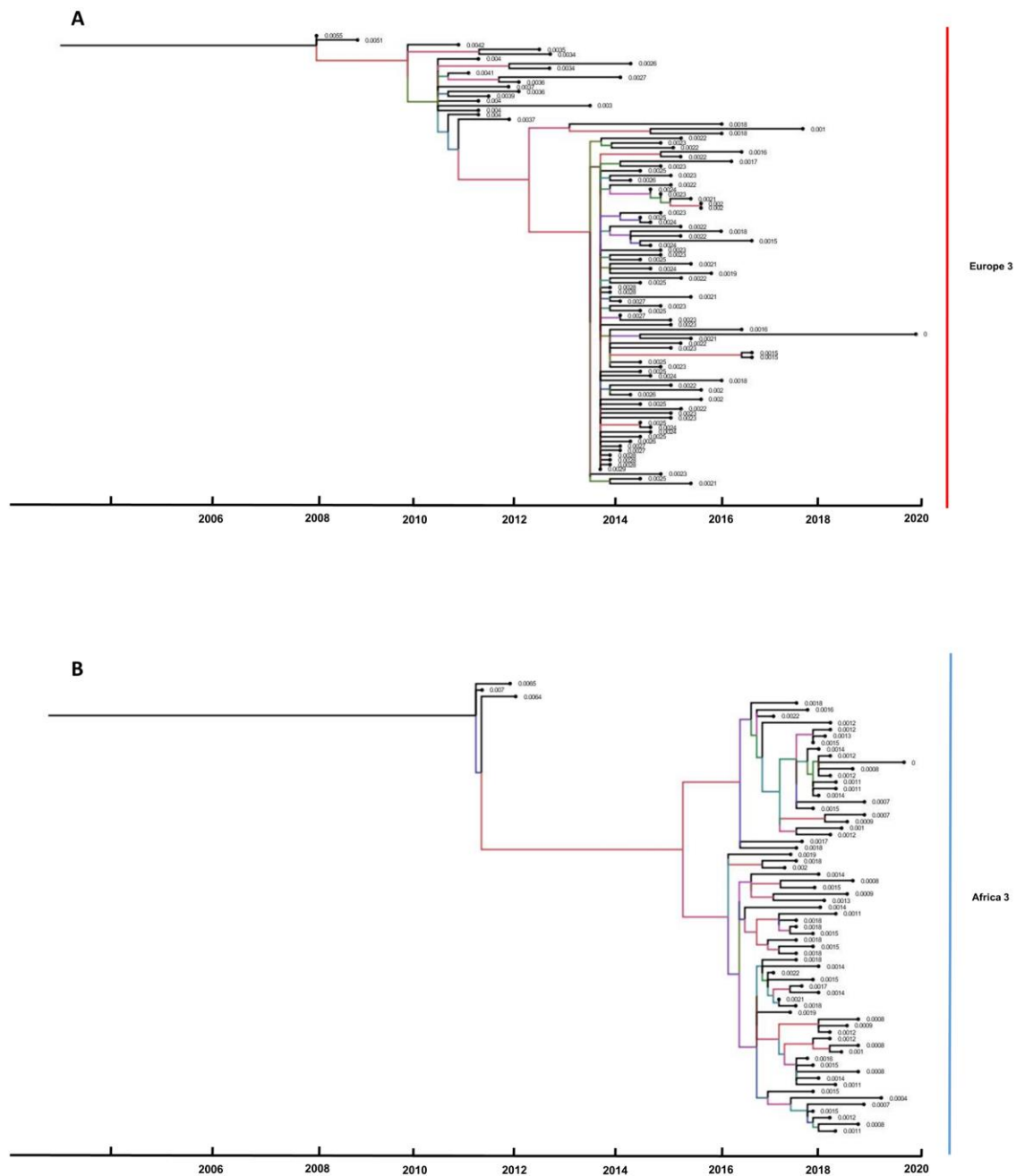




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265 **Fig 4. Overview of the distribution of the different USUV lineages in Germany (2017–2021)**  
266 (depicted in blue, red, and pink with the total number per lineage) with regard to the total number  
267 of live and dead birds molecularly tested (depicted with black diamonds) and those tested RT-  
268 qPCR positive for USUV (depicted in grey). \* Number of tested birds not recorded in 2017 and  
269 2018; † Results not finalized and only based on dead bird surveillance (updated on 21th February  
270 2023). Bird samples with USUV lineage Europa 5 or Africa 2 were not available for this study.  
271

## 272 **Molecular clock phylogeny of USUV lineages detected in Germany**

273 The molecular clock phylogeny of USUV lineages was performed to determine the time to the most  
274 recent common ancestors of the USUV lineages Europe 3, Africa 3, and Europe 2. The estimated time  
275 to TMRCA of lineage Europe 3 was determined to be around 2008 (between 2006–2010, 95%  
276 confidence interval), while the estimated time of lineage Africa 3 was shown to be around 2011 (between  
277 2008–2012, 95% confidence interval), as demonstrated in Fig 5. For Europe 2 TMRCA was calculated  
278 to be around 2017 (2015–2019, 95% confidence interval). However, the result for Europe 2 should be  
279 considered with caution as the number of available sequences is currently too limited to enable the  
280 construction of a fully resolved phylogeny clustering as well as to describe the timing of branching  
281 events in phylogenetic trees (S1 Fig).  
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**Fig 5. Molecular clock phylogeny of the complete coding sequences of USUV lineages, (A) Europe 3 and (B) Africa 3 detected in Germany. Node bars indicate 95% confidence intervals of the time of TMRCA. The branches are coloured according to the sampling location of their nodes.**

## 290 Discussion

291 The annual reoccurrence of USUV in Germany since 2011 is the cause of ongoing disease and death  
292 among wild and captive birds. Regular and stringent genomic surveillance of viral pathogens supports  
293 outbreak investigations by providing evidence for their transmission routes and geographic spread. Since  
294 only a few whole genome sequences of USUV from Germany are available (67, 47, 8, 83, 33, 15, 49),  
295 a better phylogenetic analysis is the next essential step in understanding the spread of USUV in Germany

296 as well as in Europe. A preferred method using third-generation sequencing is a nanopore sequencing  
297 approach based on target-enrichment through amplicon generation (84, 67). This recently established  
298 protocol (67) was used to gain insight into the distribution and expansion of USUV throughout five  
299 consecutive years in Germany. This sequencing technique proved to be sensitive in sequencing the  
300 majority of the USUV-positive bird samples and produced good results up to a Ct value of 32.79. The  
301 here described study confirms that WGS using the Nanopore platform is suitable in rapidly tracking and  
302 detecting ongoing USUV infections in deceased and live birds. Due to the real-time and user-friendly  
303 application of the Nanopore sequencing platform, it is a promising tool to supersede partial sequencing  
304 in the future. Mass parallelization of sample sets can enable fast-turnaround times without having  
305 adverse effects on the platform's sensitivity in detecting genomic variants (67).

306 Since the first occurrence of USUV in 2010 in a mosquito pool (one year prior to its detection in birds)  
307 (39), five USUV lineages (Europe 2, 3, 5, and Africa 2, 3) (Fig 1) have been described in Germany.  
308 Among these, the two USUV lineages Europe 3 and Africa 3 appear to have been the prevailing players  
309 in the USUV scenario in the past five years (2017–2021). Therefore, there is an imbalance in the amount  
310 of available USUV whole genome sequences for the different USUV lineages. USUV lineage Europe 3  
311 and Africa 3 are predominant compared to only a few whole genome sequences of Europe 2 and Africa  
312 2 and no available whole genome sequences of Europe 5 to date (Ziegler et al. 2016, Cadar et al. 2017).  
313 USUV lineage Europe 3, was the first USUV lineage to be detected in Germany, namely in mosquitoes  
314 in 2010 (39). However, TMRCA of this lineage is estimated to be about two years prior to its first  
315 detection. The 95% confidence interval covers a period from 2006 to 2010 (Fig 5). Similarly, the first  
316 detection of USUV lineage Africa 3 occurred in Germany in 2014 (48, 49), yet the TMRCA is estimated  
317 to have occurred prior than that, already in 2011 (Fig 5). However, to produce an even more accurate  
318 estimate, more data from other geographic areas and earlier years are needed. In contrast, Europe 2 is  
319 less frequent in Germany and it was only possible to generate whole genome sequences from 2019 and  
320 2020 (Figs 3 and 4). It should, nonetheless, be noted that partial genome sequences were already  
321 generated from samples in 2018, when the lineage was first detected in Germany (45). The TMRCA for  
322 Europe 2 was determined in 2017, one year prior to its actual detection. The phylodynamic analyses of  
323 TMRCA therefore provide evidence of a 1- to 3-year lag, respectively between the introduction and  
324 the first-case detection of an USUV lineage. By contrast, a large time-lag was described for USUV in  
325 the Netherlands, where 7 to 14 years were between the estimated common ancestor and the first  
326 detection of USUV lineage Africa 3 and Europe 3, respectively (36). Likewise, the temporal windows  
327 determined for the TMRCA in that publication are broad and can vary depending on the size of the  
328 available data set and the set timeframe (36).

329 The phylogenetic analysis of the USUV whole genome sequences in this study reveal two subclusters  
330 for Africa 3. Furthermore, the results of the clock phylogeny (Fig 5 (B)) suggest a long time-lag spanning  
331 several years between the first introduction of USUV Africa 3 into the country and its first large-scale  
332 occurrence in the avifauna. This could be indicative of silent evolutionary dynamics of the endemic and  
333 overwintering lineage Africa 3 (i.e., the first cluster) for several years prior to causing an outbreak with  
334 numerous detections (i.e., the second cluster). The occurrence of the outbreak can then be correlated to  
335 optimal environmental as well as host and vector conditions driving virus transmission or to the  
336 evolution of a more pathogenic strain (i.e., the second cluster). A similar phenomenon was already  
337 suggested for WNV by Zehender et al. 2017 (85) and Chaintoutis et al. 2019 (86). They describe that  
338 quiet enzootic transmission seasons over several years often precede virus outbreaks in animals and  
339 humans, respectively. Improving the sample matrix (vector and host species) and size as well as the  
340 temporal and geographic extent of future surveillance strategies can help on the one hand to detect quiet  
341 enzootics and on the other to not miss out on introduction events as well as epizootics. By contrast, the  
342 phylogenetic and phylodynamic (Fig 5 (A)) analyses of USUV Europe 3 suggest three subclusters  
343 appearing within a shorter time frame. This temporal connection gives the impression of multiple  
344 introduction events of USUV Europe 3 into Germany in the past creating the three separate clusters.  
345 Alternatively, it is also plausible that the other clusters derived from another undetected USUV Europe  
346 3 isolate endemic in Germany.

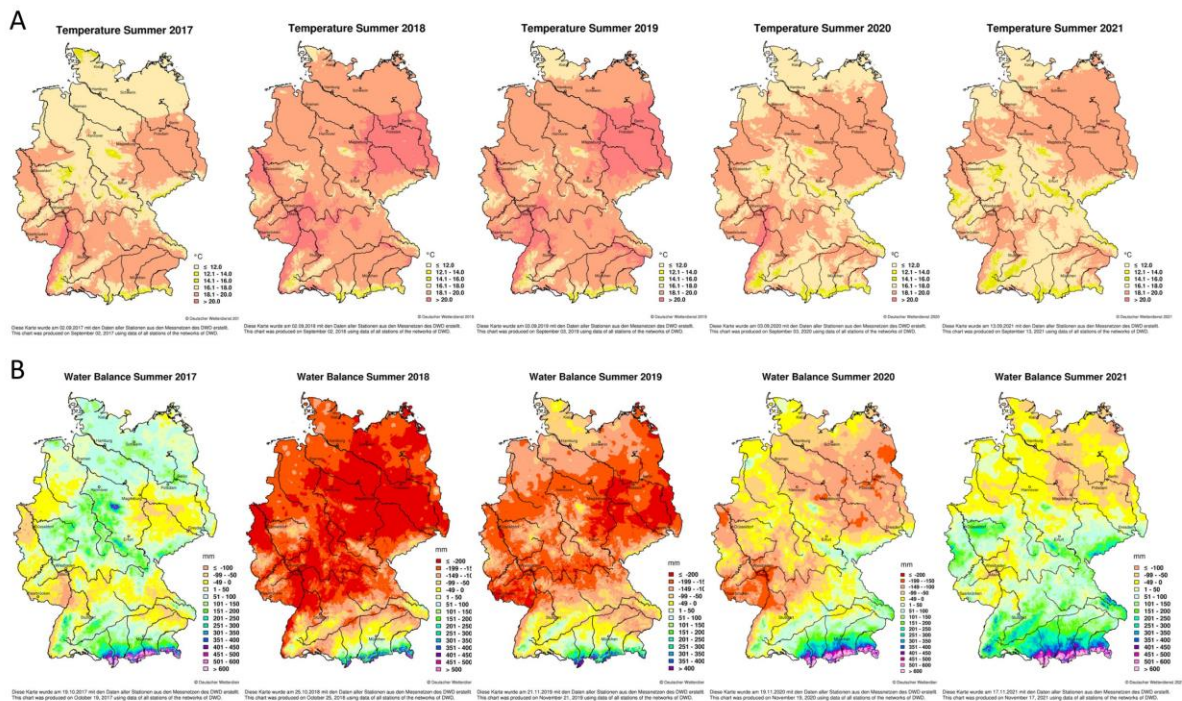
347 In Germany as well as in the Netherlands it, however, remains unclear which influence the absence of  
348 USUV surveillance programs had on the first detection of USUV in the two countries. In the case of  
349 Germany, the first mosquito monitoring study took place in 2009 at the same sampling site as in 2010,  
350 when the first infected mosquito pool was found in Weinheim in the Upper Rhine Valley (39). Prior to  
351 2009, first surveillance efforts were limited to serological investigations, restricted locally (37, 38) and

352 often not primarily focused on flaviviruses (at most testing for WNV (87)). Therefore, it is possible that  
353 individual USUV infections occurred before but as they were not accompanied by mass mortality of  
354 birds remained undetected. Serological studies prior to 2009 show neutralising antibodies against USUV  
355 in wild birds yet no molecular investigations were performed (37, 38). Furthermore, when working with  
356 BEAST-analyses one must always keep in mind that a TMRCA is only an estimation of an introduction  
357 event based strongly on the quantity and quality of the available data. Caution is always needed when  
358 interpreting these results as the inclusion of further samples can reveal different results. This was for  
359 example verified by the sequencing of an ancient hepatitis B virus which yielded new data on the  
360 evolution of hepatitis B, that was not apparent when only evaluating recent sequences (88).  
361 On a European scale, USUV was isolated for the first time in 2001 in Austria (“Strain Vienna”; USUV  
362 Europe 1) (13) and retrospectively in 1996 in Italy (also USUV Europe 1) (14). However, molecular  
363 clock analyses revealed that the first entry of USUV (classified as Europe 1) into central Europe was  
364 estimated to have occurred already in Spain in the late 20<sup>th</sup> century, with a virus closely related to USUV  
365 from Senegal. Furthermore, the lineages Europe 2 and 3 were calculated to have their origin in Austria  
366 in 1993 and Italy in 2007, respectively (15). Since then, USUV has spread throughout Europe with  
367 lineage Europe 4 detected in Italy from a sampling set from 2010–2014 with its estimated TMRCA  
368 dating back to 2003–2005 (34). Europe 5 was detected for the first time in Germany in 2016 (33) and  
369 so far, no TMRCA has been calculated for this lineage. The lineages Africa 2 and 3 descended from  
370 multiple independent introductions from sub-Saharan as indicated by analyses from Spain (15).  
371 A few publications have analysed the geographic flow of the USUV genome throughout Europe, with  
372 the majority of available whole genome sequences from Italy, the Netherlands, and Germany. Italy is  
373 considered an “USUV-donor” to neighbouring countries. Especially north-western Italy appears to have  
374 played a key role in the transfer of USUV to central Europe (from Switzerland to Germany to France  
375 and Belgium) and eastern Italy to central and eastern Europe (from Austria to Hungary to Serbia) (89).  
376 It must however be kept in mind that other publications have described an USUV spread in the other  
377 direction, i.e., from Austria to Italy and Germany (15, 34, 4). Analyses performed with USUV sequences  
378 from the Netherlands have confirmed an USUV-circulation between the Netherlands, Germany, and  
379 Belgium (36). USUV Europe 3 is less frequent in the Netherlands and is most likely periodically re-  
380 introduced from neighboring countries. By contrast the prevailing Africa 3 lineage was probably  
381 introduced into the Netherlands from Germany in 2016, overwintered there and has since then become  
382 enzootic.  
383 Flavivirus transmission dynamics are influenced by environmental and biological factors affecting the  
384 host as well as the vector of a virus. The population density of amplifying/reservoir species and the  
385 species immune fitness towards specific pathogens also has a significant influence on virus maintenance  
386 and spread in the environment (90, 91). Equally, population dynamics of mosquito vectors can play a  
387 role in virus transmission and are among others affected by population density, urbanisation, humidity,  
388 and temperature (92, 93). The spread of USUV may, therefore, have been favoured by the presence of  
389 beneficial environmental conditions for mosquitoes in recent years (33, 94, 95, 8). In 2016, for instance,  
390 an exceptionally high activity of USUV infections was observed in birds, correlating with temperature  
391 anomalies in September in Western Europe (33). Higher temperatures shorten the extrinsic incubation  
392 period (time required for virus replication in the mosquito) which in turn influences the population  
393 dynamics of mosquitoes and as a result the vector-host contact rate (92, 96, 90). A similar scenario of  
394 optimal weather conditions was observed again in Germany in 2018 with an early humid spring  
395 combined with a warm and dry summer (Fig 6) (45, 95). This might have also paved the way for the  
396 introduction of WNV into Germany in the same year (97). The extensive USUV outbreaks for example  
397 in 2016 and 2018 are in the literature often associated with optimal weather conditions for mosquito-  
398 borne virus transmission (98, 90). Especially the fulminant outbreak in 2018 led to a massive die-off  
399 of blackbirds, a species highly susceptible to USUV, and consequently a decline in the species  
400 population throughout Germany (45). The observed high seroprevalence of USUV antibodies in 2018  
401 compared to the prior years (45) lets one hypothesize that USUV thereafter faced a higher proportion of  
402 non-naïve hosts for its replication cycle. This could help explain the absence of USUV outbreaks of a  
403 comparable magnitude in the consecutive years even though the weather conditions continued to  
404 encourage arbovirus transmission. This phenomenon was also observed after the USUV outbreak in  
405 Austria in 2001, where herd immunity of the wild bird populations protected susceptible species from a  
406 severe USUV disease in subsequent years (99).

407 Arboviruses such as USUV or the closely-related flavivirus WNV can overwinter in a susceptible host  
 408 and/or vector species. In the past decade, the nationwide German wild bird surveillance network for  
 409 zoonotic arthropod-borne viruses has consistently provided evidence for the persistence of these  
 410 arboviruses in the German avifauna (38, 40, 43–45). Similarly, the endemicity of both arboviruses was  
 411 confirmed in findings from indigenous mosquito species, i.e., *Culex pipiens* (42, 41), that are known to  
 412 be vector competent for both viruses (92, 100). Increased mosquito breeding, facilitated by high summer  
 413 temperatures as well as a sufficiently high water balance (Fig 6), can lead to a longer mosquito and  
 414 virus-transmission season. Taken together the overwintering of mosquitoes infected with flaviviruses  
 415 (101) and the here discussed evolution of different USUV lineages within the country verify the  
 416 persistence of the virus in Germany. In addition to optimal weather conditions favouring the spread of  
 417 endemic USUV lineages in Germany, new introductions from neighbouring countries may have taken  
 418 place, such as from the Netherlands, Czech Republic or Italy, as well as from long-distance migrants.  
 419 Newly introduced strains in turn spread with ease under the favourable environmental conditions (Fig  
 420 1).

421  
 422

423 **Fig 6. Climatological maps of Germany displaying (A) temperature (in degrees Celsius) and (B)**  
 424 **water balance (in millimetre) based on data collected in the summers 2017–2021 (102).**  
 425 **Climatological maps were downloaded from the German Weather Service (103).**



Composed from climatological maps from the Deutscher Wetterdienst (German Weather Service) (Deutscher Wetterdienst (2021), 'Climatological maps of Germany', <<https://www.dwd.de/EN/ourservices/klimakartendeutschland/klimakartendeutschland.html?nn=495490>>, accessed 08.11.2022).

426

## 427 **Conclusion**

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

437

## 438 **Data availability**

439 All data generated or analyzed during this study are included in this published article and its  
440 Supporting Information files. Raw sequencing data have been submitted to the NCBI database  
441 (XXX) and will be freely accessible if the manuscript is accepted for publication.

442

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465

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467 Conceptualization, F.B., C.M.H., B.S., M.H.G., and U.Z.; methodology, F.B., C.M.H., and B.S.; data  
468 curation, F.B., C.M.H., U.Z. and B.S.; software, F.B., C.M.H., and B.S.; validation, F.B., C.M.H., B.S.,  
469 U.Z. and M.H.G.; formal analysis, F.B., C.M.H., U.Z. and B.S.; investigation, F.B., C.M.H., F.M., A.M.,  
470 S.B., N.S., G.P., S.K., T.S., J.S., C.S., L.H., M.P., and A.H.; resources, F.B., C.M.H., F.M., A.M., S.B.,  
471 N.S., G.P., S.K., T.S., J.S., C.S., L.H., M.P., A.H., U.Z., and M.H.G.; writing—original draft  
472 preparation, F.B., C.M.H., B.S., and U.Z.; writing—review and editing, F.B., C.M.H., B.S., F.M., A.M.,  
473 S.B., N.S., G.P., S.K., T.S., J.S., C.S., L.H., M.P., A.H., M.H.G., and U.Z.; visualization, F.B., C.M.H.  
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## 483 Literature Cited

- 484 1. Calisher CH, Gould EA. Taxonomy of the virus family Flaviviridae. In: Elsevier; 2003. p. 1–19  
485 (Advances in Virus Research).
- 486 2. Bakonyi T, Gould EA, Kolodziejek J, Weissenböck H, Nowotny N. Complete genome analysis and  
487 molecular characterization of Usutu virus that emerged in Austria in 2001: comparison with the  
488 South African strain SAAR-1776 and other flaviviruses. *Virology* 2004; 328(2):301–10.
- 489 3. Williams MC, Simpson DI, Haddow AJ, Knight EM. The Isolation of West Nile Virus From Man And  
490 of Usutu Virus From The Bird-Biting Mosquito *Mansonia Aurites* (Theobald) in The Entebbe Area of  
491 Uganda. *Ann Trop Med Parasitol* 1964; 58:367–74.
- 492 4. Roesch F, Fajardo A, Moratorio G, Vignuzzi M. Usutu Virus: An Arbovirus on the Rise. *Viruses* 2019;  
493 11(7).
- 494 5. Saiz J-C, Blazquez A-B. Usutu virus: current knowledge and future perspectives. *VAAT* 2017;  
495 Volume 9:27–40.
- 496 6. Bakonyi T, Erdélyi K, Ursu K, Ferenczi E, Csörgo T, Lussy H et al. Emergence of Usutu virus in  
497 Hungary. *J Clin Microbiol* 2007; 45(12):3870–4.
- 498 7. Weissenböck H, Kolodziejek J, Fragner K, Kuhn R, Pfeffer M, Nowotny N. Usutu virus activity in  
499 Austria, 2001–2002. *Microbes and Infection* 2003; 5(12):1132–6.
- 500 8. Becker N, Jöst H, Ziegler U, Eiden M, Höper D, Emmerich P et al. Epizootic emergence of Usutu  
501 virus in wild and captive birds in Germany. *PLoS One* 2012; 7(2):e32604.
- 502 9. Lühken R, Jöst H, Cadar D, Thomas SM, Bosch S, Tannich E et al. Distribution of Usutu Virus in  
503 Germany and Its Effect on Breeding Bird Populations. *Emerg Infect Dis* 2017; 23(12):1994–2001.
- 504 10. Steinmetz HW, Bakonyi T, Weissenböck H, Hatt J-M, Eulenberger U, Robert N et al. Emergence  
505 and establishment of Usutu virus infection in wild and captive avian species in and around Zurich,  
506 Switzerland--genomic and pathologic comparison to other central European outbreaks. *Vet Microbiol*  
507 2011; 148(2-4):207–12.
- 508 11. Manarolla G, Bakonyi T, Gallazzi D, Crosta L, Weissenböck H, Dorrestein GM et al. Usutu virus in  
509 wild birds in northern Italy. *Vet Microbiol* 2010; 141(1-2):159–63.
- 510 12. Hubálek Z, Rudolf I, Čapek M, Bakonyi T, Betášová L, Nowotny N. Usutu virus in blackbirds (*Turdus*  
511 *merula*), Czech Republic, 2011-2012. *Transbound Emerg Dis* 2014; 61(3):273–6.
- 512 13. Weissenböck H, Kolodziejek J, Url A, Lussy H, Rebel-Bauder B, Nowotny N. Emergence of Usutu  
513 virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe.  
514 *Emerg Infect Dis* 2002; 8(7):652–6.
- 515 14. Weissenböck H, Bakonyi T, Rossi G, Mani P, Nowotny N. Usutu virus, Italy, 1996. *Emerg Infect Dis*  
516 2013; 19(2):274–7.
- 517 15. Engel D, Jöst H, Wink M, Börstler J, Bosch S, Garigliany M-M et al. Reconstruction of the  
518 Evolutionary History and Dispersal of Usutu Virus, a Neglected Emerging Arbovirus in Europe and  
519 Africa. *mBio* 2016; 7(1):e01938-15.

- 520 16. Pecorari M, Longo G, Gennari W, Grottola A, am Sabbatini, Tagliazucchi S et al. First human case  
521 of Usutu virus neuroinvasive infection, Italy, August-September 2009. *Euro Surveill* 2009; 14(50).
- 522 17. Santini M, Vilibic-Cavlek T, Barsic B, Barbic L, Savic V, Stevanovic V et al. First cases of human  
523 Usutu virus neuroinvasive infection in Croatia, August-September 2013: clinical and laboratory  
524 features. *J Neurovirol* 2015; 21(1):92–7.
- 525 18. Grottola A, Marcacci M, Tagliazucchi S, Gennari W, Di Gennaro A, Orsini M et al. Usutu virus  
526 infections in humans: a retrospective analysis in the municipality of Modena, Italy. *Clin Microbiol*  
527 *Infect* 2017; 23(1):33–7.
- 528 19. Nagy A, Mezei E, Nagy O, Bakonyi T, Csonka N, Kaposi M et al. Extraordinary increase in West Nile  
529 virus cases and first confirmed human Usutu virus infection in Hungary, 2018. *Euro Surveill* 2019;  
530 24(28).
- 531 20. Pacenti M, Sinigaglia A, Martello T, Rui ME de, Franchin E, Pagni S et al. Clinical and virological  
532 findings in patients with Usutu virus infection, northern Italy, 2018. *Euro Surveill* 2019; 24(47).
- 533 21. Simonin Y, Sillam O, Carles MJ, Gutierrez S, Gil P, Constant O et al. Human Usutu Virus Infection  
534 with Atypical Neurologic Presentation, Montpellier, France, 2016. *Emerg Infect Dis* 2018; 24(5):875–  
535 8.
- 536 22. Vilibic-Cavlek T, Kaic B, Barbic L, Pem-Novosel I, Slavic-Vrzic V, Lesnikar V et al. First evidence of  
537 simultaneous occurrence of West Nile virus and Usutu virus neuroinvasive disease in humans in  
538 Croatia during the 2013 outbreak. *Infection* 2014; 42(4):689–95.
- 539 23. Cadar D, Simonin Y. Human Usutu Virus Infections in Europe: A New Risk on Horizon? *Viruses*  
540 2023; 15(1):77.
- 541 24. Bergmann F, Trachsel DS, Stoeckle SD, Bernis Sierra J, Lübke S, Groschup MH et al.  
542 Seroepidemiological Survey of West Nile Virus Infections in Horses from Berlin/Brandenburg and  
543 North Rhine-Westphalia, Germany. *Viruses* 2022; 14(2).
- 544 25. Ganzenberg S, Sieg M, Ziegler U, Pfeffer M, Vahlenkamp TW, Hörügel U et al. Seroprevalence and  
545 Risk Factors for Equine West Nile Virus Infections in Eastern Germany, 2020. *Viruses* 2022; 14(6).
- 546 26. Montagnaro S, Piantedosi D, Ciarcia R, Loponte R, Veneziano V, Fusco G et al. Serological  
547 Evidence of Mosquito-Borne Flaviviruses Circulation in Hunting Dogs in Campania Region, Italy.  
548 *Vector Borne Zoonotic Dis* 2019; 19(2):142–7.
- 549 27. García-Bocanegra I, Paniagua J, Gutiérrez-Guzmán AV, Lecollinet S, Boadella M, Arenas-Montes A  
550 et al. Spatio-temporal trends and risk factors affecting West Nile virus and related flavivirus exposure  
551 in Spanish wild ruminants. *BMC Vet Res* 2016; 12(1):249.
- 552 28. Escribano-Romero E, Lupulović D, Merino-Ramos T, Blázquez A-B, Lazić G, Lazić S et al. West Nile  
553 virus serosurveillance in pigs, wild boars, and roe deer in Serbia. *Vet Microbiol* 2015; 176(3-4):365–9.
- 554 29. Cadar D, Becker N, Campos RdM, Börstler J, Jöst H, Schmidt-Chanasit J. Usutu virus in bats,  
555 Germany, 2013. *Emerg Infect Dis* 2014; 20(10):1771–3.
- 556 30. Romeo C, Lecollinet S, Caballero J, Isla J, Luzzago C, Ferrari N et al. Are tree squirrels involved in  
557 the circulation of flaviviruses in Italy? *Transbound Emerg Dis* 2018; 65(5):1372–6.
- 558 31. Diagne MM, Ndione MHD, Di Paola N, Fall G, Bedekelabou AP, Sembène PM et al. Usutu Virus  
559 Isolated from Rodents in Senegal. *Viruses* 2019; 11(2).



- 560 32. Bakonyi T, Jungbauer C, Aberle SW, Kolodziejek J, Dimmel K, Stiasny K et al. Usutu virus infections  
561 among blood donors, Austria, July and August 2017 - Raising awareness for diagnostic challenges.  
562 *Euro Surveill* 2017; 22(41).
- 563 33. Cadar D, Lühken R, van der Jeugd H, Garigliany M, Ziegler U, Keller M et al. Widespread activity of  
564 multiple lineages of Usutu virus, western Europe, 2016. *Euro Surveill* 2017; 22(4).
- 565 34. Calzolari M, Chiapponi C, Bonilauri P, Lelli D, Baioni L, Barbieri I et al. Co-circulation of two Usutu  
566 virus strains in Northern Italy between 2009 and 2014. *Infect Genet Evol* 2017; 51:255–62.
- 567 35. Kemenesi G, Buzás D, Zana B, Kurucz K, Krtinic B, Kepner A et al. First genetic characterization of  
568 Usutu virus from *Culex pipiens* mosquitoes Serbia, 2014. *Infect Genet Evol* 2018; 63:58–61.
- 569 36. Oude Munnink BB, Mürger E, Nieuwenhuijse DF, Kohl R, van der Linden A, Schapendonk CME et  
570 al. Genomic monitoring to understand the emergence and spread of Usutu virus in the Netherlands,  
571 2016–2018. *Sci Rep* 2020; 10(1):2798.
- 572 37. Linke S, Niedrig M, Kaiser A, Ellerbrok H, Müller K, Müller T et al. Serologic evidence of West Nile  
573 virus infections in wild birds captured in Germany. *Am J Trop Med Hyg* 2007; 77(2):358–64.
- 574 38. Seidowski D, Ziegler U, Rönn JAC von, Müller K, Hüppop K, Müller T et al. West Nile virus  
575 monitoring of migratory and resident birds in Germany. *Vector Borne Zoonotic Dis* 2010; 10(7):639–  
576 47.
- 577 39. Jöst H, Bialonski A, Maus D, Sambri V, Eiden M, Groschup MH et al. Isolation of usutu virus in  
578 Germany. *Am J Trop Med Hyg* 2011; 85(3):551–3.
- 579 40. Ziegler U, Seidowski D, Globig A, Fereidouni SR, Ulrich RG, Groschup MH. Sentinel birds in wild-  
580 bird resting sites as potential indicators for West Nile virus infections in Germany. *Arch Virol* 2010;  
581 155(6):965–9.
- 582 41. Kampen H, Holicki CM, Ziegler U, Groschup MH, Tews BA, Werner D. West Nile Virus Mosquito  
583 Vectors (Diptera: Culicidae) in Germany. *Viruses* 2020; 12(5).
- 584 42. Scheuch DE, Schäfer M, Eiden M, Heym EC, Ziegler U, Walther D et al. Detection of Usutu, Sindbis,  
585 and Batai Viruses in Mosquitoes (Diptera: Culicidae) Collected in Germany, 2011–2016. *Viruses* 2018;  
586 10(7).
- 587 43. Ziegler U, Jöst H, Müller K, Fischer D, Rinder M, Tietze DT et al. Epidemic Spread of Usutu Virus in  
588 Southwest Germany in 2011 to 2013 and Monitoring of Wild Birds for Usutu and West Nile Viruses.  
589 *Vector Borne Zoonotic Dis* 2015; 15(8):481–8.
- 590 44. Michel F, Fischer D, Eiden M, Fast C, Reuschel M, Müller K et al. West Nile Virus and Usutu Virus  
591 Monitoring of Wild Birds in Germany. *Int J Environ Res Public Health* 2018; 15(1).
- 592 45. Michel F, Sieg M, Fischer D, Keller M, Eiden M, Reuschel M et al. Evidence for West Nile Virus and  
593 Usutu Virus Infections in Wild and Resident Birds in Germany, 2017 and 2018. *Viruses* 2019; 11(7).
- 594 46. Ziegler U, Bergmann F, Fischer D, Müller K, Holicki CM, Sadeghi B et al. Spread of West Nile Virus  
595 and Usutu Virus in the German Bird Population, 2019–2020. *Microorganisms* 2022; 10(4).
- 596 47. Ziegler U, Fast C, Eiden M, Bock S, Schulze C, Hoepfer D et al. Evidence for an independent third  
597 Usutu virus introduction into Germany. *Vet Microbiol* 2016; 192:60–6.
- 598 48. Cadar D, Bosch S, Jöst H, Börstler J, Garigliany M-M, Becker N et al. Putative Lineage of Novel  
599 African Usutu Virus, Central Europe. *Emerg Infect Dis* 2015; 21(9):1647–50.

- 600 49. Sieg M, Schmidt V, Ziegler U, Keller M, Höper D, Heenemann K et al. Outbreak and Cocirculation  
601 of Three Different Usutu Virus Strains in Eastern Germany. *Vector Borne Zoonotic Dis* 2017;  
602 17(9):662–4.
- 603 50. Ben Hassine T, Massis F de, Calistri P, Savini G, BelHaj Mohamed B, Ranen A et al. First detection  
604 of co-circulation of West Nile and Usutu viruses in equids in the south-west of Tunisia. *Transbound*  
605 *Emerg Dis* 2014; 61(5):385–9.
- 606 51. Durand B, Haskouri H, Lowenski S, Vachiere N, Beck C, Lecollinet S. Seroprevalence of West Nile  
607 and Usutu viruses in military working horses and dogs, Morocco, 2012: dog as an alternative WNV  
608 sentinel species? *Epidemiol Infect* 2016; 144(9):1857–64.
- 609 52. Mannasse B, Mendelson E, Orshan L, Mor O, Shalom U, Yeger T et al. Usutu Virus RNA in  
610 Mosquitoes, Israel, 2014-2015. *Emerg Infect Dis* 2017; 23(10):1699–702.
- 611 53. Chaintoutis SC, Dovas CI, Papanastassopoulou M, Gewehr S, Danis K, Beck C et al. Evaluation of a  
612 West Nile virus surveillance and early warning system in Greece, based on domestic pigeons. *Comp*  
613 *Immunol Microbiol Infect Dis* 2014; 37(2):131–41.
- 614 54. Eiden M, Gil P, Ziegler U, Rakotoarivony I, Marie A, Frances B et al. Emergence of two Usutu virus  
615 lineages in *Culex pipiens* mosquitoes in the Camargue, France, 2015. *Infect Genet Evol* 2018; 61:151–  
616 4.
- 617 55. Bakonyi T, Busquets N, Nowotny N. Comparison of complete genome sequences of Usutu virus  
618 strains detected in Spain, Central Europe, and Africa. *Vector Borne Zoonotic Dis* 2014; 14(5):324–9.
- 619 56. Bażanów B, van Jansen Vuren P, Szymański P, Stygar D, Frącka A, Twardoń J et al. A Survey on  
620 West Nile and Usutu Viruses in Horses and Birds in Poland. *Viruses* 2018; 10(2).
- 621 57. Rudolf I, Bakonyi T, Šebesta O, Mendel J, Peško J, Betášová L et al. Co-circulation of Usutu virus  
622 and West Nile virus in a reed bed ecosystem. *Parasit Vectors* 2015; 8:520.
- 623 58. Buckley A, Dawson A, Moss SR, Hinsley SA, Bellamy PE, Gould EA. Serological evidence of West  
624 Nile virus, Usutu virus and Sindbis virus infection of birds in the UK. *J Gen Virol* 2003; 84(Pt 10):2807–  
625 17. Available from: URL:  
626 <https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.19341-0>.
- 627 59. Barbic L, Vilibic-Cavlek T, Listes E, Stevanovic V, Gjenero-Margan I, Ljubin-Sternak S et al.  
628 Demonstration of Usutu virus antibodies in horses, Croatia. *Vector Borne Zoonotic Dis* 2013;  
629 13(10):772–4.
- 630 60. Rijks JM, Kik ML, Slaterus R, Foppen R, Stroo A, IJzer J et al. Widespread Usutu virus outbreak in  
631 birds in the Netherlands, 2016. *Euro Surveill* 2016; 21(45).
- 632 61. Hönig V, Palus M, Kaspar T, Zemanova M, Majerova K, Hofmannova L et al. Multiple Lineages of  
633 Usutu Virus (Flaviviridae, Flavivirus) in Blackbirds (*Turdus merula*) and Mosquitoes (*Culex pipiens*, *Cx.*  
634 *modestus*) in the Czech Republic (2016-2019). *Microorganisms* 2019; 7(11).
- 635 62. Faria NR, Kraemer MUG, Hill SC, Goes de Jesus J, Aguiar RS, Iani FCM et al. Genomic and  
636 epidemiological monitoring of yellow fever virus transmission potential. *Science* 2018;  
637 361(6405):894–9.
- 638 63. Faria NR, Quick J, Claro IM, Théze J, Jesus JG de, Giovanetti M et al. Establishment and cryptic  
639 transmission of Zika virus in Brazil and the Americas. *Nature* 2017; 546(7658):406–10.
- 640 64. Faria NR, Da Azevedo RdSS, Kraemer MUG, Souza R, Cunha MS, Hill SC et al. Zika virus in the  
641 Americas: Early epidemiological and genetic findings. *Science* 2016; 352(6283):345–9.

- 642 65. Hill SC, Neto de Vasconcelos J, Granja BG, Thézé J, Jandondo D, Neto Z et al. Early Genomic  
643 Detection of Cosmopolitan Genotype of Dengue Virus Serotype 2, Angola, 2018. *Emerg Infect Dis*  
644 2019; 25(4):784–7.
- 645 66. Naveca FG, Claro I, Giovanetti M, Jesus JG de, Xavier J, Iani FCdM et al. Genomic, epidemiological  
646 and digital surveillance of Chikungunya virus in the Brazilian Amazon. *PLoS Negl Trop Dis* 2019;  
647 13(3):e0007065.
- 648 67. Holicki CM, Bergmann F, Stoek F, Schulz A, Groschup MH, Ziegler U et al. Expedited retrieval of  
649 high-quality Usutu virus genomes via Nanopore sequencing with and without target enrichment.  
650 *Front. Microbiol.* 2022; 13. Available from: URL:  
651 <https://www.frontiersin.org/articles/10.3389/fmicb.2022.1044316>.
- 652 68. Ziegler U, Eiden M, Fast C, Keller M, Groschup MH. Tiergesundheitsjahresbericht 2017: Usutu-  
653 Virus-Infektion (USUV) – Usutu virus infection: Friedrich-Loeffler-Institut; 2017 [cited 2022 Nov 10].  
654 Available from: URL:  
655 [https://www.openagrar.de/rsc/viewer/openagrar\\_derivate\\_00019140/SD201845870.pdf?page=1](https://www.openagrar.de/rsc/viewer/openagrar_derivate_00019140/SD201845870.pdf?page=1).
- 656 69. Ziegler U, Eiden M, Fast C, Keller M, Groschup MH. Tiergesundheitsjahresbericht 2018: Usutu-  
657 Virus-Infektion (USUV) – Usutu virus infection: Friedrich-Loeffler-Institut; 2018. Available from: URL:  
658 [https://www.openagrar.de/rsc/viewer/openagrar\\_derivate\\_00025589/SD201954064.pdf?page=1](https://www.openagrar.de/rsc/viewer/openagrar_derivate_00025589/SD201954064.pdf?page=1).
- 659 70. Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit. Datensammlung West-  
660 Nil-Fieber zur Virusinfektion bei Vögeln [cited 2022 Dec 14]. Available from: URL: [https://westnil-  
661 fieber.fli.de/anmeldung/?returnurl=%2fgib-daten-ein%2f](https://westnil-fieber.fli.de/anmeldung/?returnurl=%2fgib-daten-ein%2f).
- 662 71. Quick J, Grubaugh ND, Pullan ST, Claro IM, Smith AD, Gangavarapu K et al. Multiplex PCR method  
663 for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples.  
664 *Nat Protoc* 2017; 12(6):1261–76.
- 665 72. Wick RR, Judd LM, Holt KE. Performance of neural network basecalling tools for Oxford Nanopore  
666 sequencing. *Genome Biol* 2019; 20(1):129.
- 667 73. Goodacre N, Aljanahi A, Nandakumar S, Mikailov M, Khan AS. A Reference Viral Database (RVDB)  
668 To Enhance Bioinformatics Analysis of High-Throughput Sequencing for Novel Virus Detection.  
669 *mSphere* 2018; 3(2).
- 670 74. Clausen PTLC, Aarestrup FM, Lund O. Rapid and precise alignment of raw reads against redundant  
671 databases with KMA. *BMC Bioinformatics* 2018; 19(1):307.
- 672 75. Li H. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* 2018; 34(18):3094–  
673 100.
- 674 76. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. *Nucleic Acids Res* 2010;  
675 38(Database issue):D46-51.
- 676 77. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic*  
677 *Acids Res* 2004; 32(5):1792–7.
- 678 78. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11.  
679 *Mol Biol Evol* 2021; 38(7):3022–7.
- 680 79. FigTree version 1.4.3.; 2012. Available from: URL: <http://tree.bio.ed.ac.uk/software/figtree/>.
- 681 80. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol*  
682 *Biol* 2007; 7:214.

- 683 81. Drummond AJ, Rambaut A, Shapiro B, Pybus OG. Bayesian coalescent inference of past  
684 population dynamics from molecular sequences. *Mol Biol Evol* 2005; 22(5):1185–92.
- 685 82. GeoBasis-DE. Available from: URL: <https://gdz.bkg.bund.de/index.php/default/open-data.html>.
- 686 83. Störk T, Le Roi M de, Haverkamp A-K, Jesse ST, Peters M, Fast C et al. Analysis of avian Usutu virus  
687 infections in Germany from 2011 to 2018 with focus on dsRNA detection to demonstrate viral  
688 infections. *Sci Rep* 2021; 11(1):24191.
- 689 84. Oude Munnink BB, Kik M, Bruijn ND de, Kohl R, van der Linden A, Reusken CBEM et al. Towards  
690 high quality real-time whole genome sequencing during outbreaks using Usutu virus as example.  
691 *Infect Genet Evol* 2019; 73:49–54.
- 692 85. Zehender G, Veo C, Ebranati E, Carta V, Rovida F, Percivalle E et al. Reconstructing the recent  
693 West Nile virus lineage 2 epidemic in Europe and Italy using discrete and continuous phylogeography.  
694 *PLoS One* 2017; 12(7):e0179679.
- 695 86. Chaintoutis SC, Papa A, Pervanidou D, Dovas CI. Evolutionary dynamics of lineage 2 West Nile  
696 virus in Europe, 2004-2018: Phylogeny, selection pressure and phylogeography. *Molecular*  
697 *Phylogenetics and Evolution* 2019; 141:106617. Available from: URL:  
698 <https://www.sciencedirect.com/science/article/pii/S1055790319303288>.
- 699 87. Hlinak A, Mühle RU, Werner O, Globig A, Starick E, Schirrneier H et al. A virological survey in  
700 migrating waders and other waterfowl in one of the most important resting sites of Germany. *J Vet*  
701 *Med B Infect Dis Vet Public Health* 2006; 53(3):105–10.
- 702 88. Mühlemann B, Jones TC, Damgaard PdB, Allentoft ME, Shevnina I, Logvin A et al. Ancient hepatitis  
703 B viruses from the Bronze Age to the Medieval period. *Nature* 2018; 557(7705):418–23.
- 704 89. Zecchin B, Fusaro A, Milani A, Schivo A, Ravagnan S, Ormelli S et al. The central role of Italy in the  
705 spatial spread of USUTU virus in Europe. *Virus Evol* 2021; 7(1):veab048.
- 706 90. Rubel F, Brugger K, Hantel M, Chvala-Mannsberger S, Bakonyi T, Weissenböck H et al. Explaining  
707 Usutu virus dynamics in Austria: model development and calibration. *Prev Vet Med* 2008; 85(3-  
708 4):166–86.
- 709 91. Durand B, Balança G, Baldet T, Chevalier V. A metapopulation model to simulate West Nile virus  
710 circulation in Western Africa, Southern Europe and the Mediterranean basin. *Vet Res* 2010; 41(3):32.
- 711 92. Holicki CM, Scheuch DE, Ziegler U, Lettow J, Kampen H, Werner D et al. German *Culex pipiens*  
712 biotype *molestus* and *Culex torrentium* are vector-competent for Usutu virus. *Parasit Vectors* 2020;  
713 13(1):625.
- 714 93. Holicki CM. Mosquito-borne Flaviviruses: Vector and Avian Host Susceptibility for West Nile Virus  
715 and Usutu Virus in Germany. Hannover: Tierärztliche Hochschule Hannover; 2020.
- 716 94. Brugger K, Rubel F. Simulation of climate-change scenarios to explain Usutu-virus dynamics in  
717 Austria. *Prev Vet Med* 2009; 88(1):24–31.
- 718 95. Semenza JC, Suk JE. Vector-borne diseases and climate change: a European perspective. *FEMS*  
719 *Microbiol Lett* 2018; 365(2).
- 720 96. Chvala S, Bakonyi T, Bukovsky C, Meister T, Brugger K, Rubel F et al. Monitoring of Usutu virus  
721 activity and spread by using dead bird surveillance in Austria, 2003-2005. *Vet Microbiol* 2007; 122(3-  
722 4):237–45.
- 723 97. Ziegler U, Lühken R, Keller M, Cadar D, van der Grinten E, Michel F et al. West Nile virus epizootic  
724 in Germany, 2018. *Antiviral Res* 2019; 162:39–43.

- 725 98. Weidinger P, Kolodziejek J, Bakonyi T, Brunthaler R, Erdélyi K, Weissenböck H et al. Different  
726 dynamics of Usutu virus infections in Austria and Hungary, 2017-2018. *Transbound Emerg Dis* 2020;  
727 67(1):298–307.
- 728 99. Meister T, Lussy H, Bakonyi T, Sikutová S, Rudolf I, Vogl W et al. Serological evidence of  
729 continuing high Usutu virus (Flaviviridae) activity and establishment of herd immunity in wild birds in  
730 Austria. *Vet Microbiol* 2008; 127(3-4):237–48.
- 731 100. Holicki CM, Ziegler U, Răileanu C, Kampen H, Werner D, Schulz J et al. West Nile Virus Lineage 2  
732 Vector Competence of Indigenous Culex and Aedes Mosquitoes from Germany at Temperate Climate  
733 Conditions. *Viruses* 2020; 12(5).
- 734 101. Kampen H, Tews BA, Werner D. First Evidence of West Nile Virus Overwintering in Mosquitoes in  
735 Germany. *Viruses* 2021; 13(12).
- 736 102. Constant O, Bollore K, Clé M, Barthelemy J, Foulongne V, Chenet B et al. Evidence of Exposure to  
737 USUV and WNV in Zoo Animals in France. *Pathogens* 2020; 9(12).
- 738 103. Deutscher Wetterdienst. Climatological maps of Germany: Federal Ministry for Digital and  
739 Transport; 2022 [cited 2022 Nov 8]. Available from: URL:  
740 <https://www.dwd.de/EN/ourservices/klimakartendeutschland/klimakartendeutschland.html?nn=495>  
741 490.

742

## 743 **Supporting information**

744 **S1 Table. Detailed information on the origin of phylogenetically analyzed USUV from wild and**  
745 **captive birds in 2017 and 2021. Sample numbers are used in Fig 1.**

746

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

750

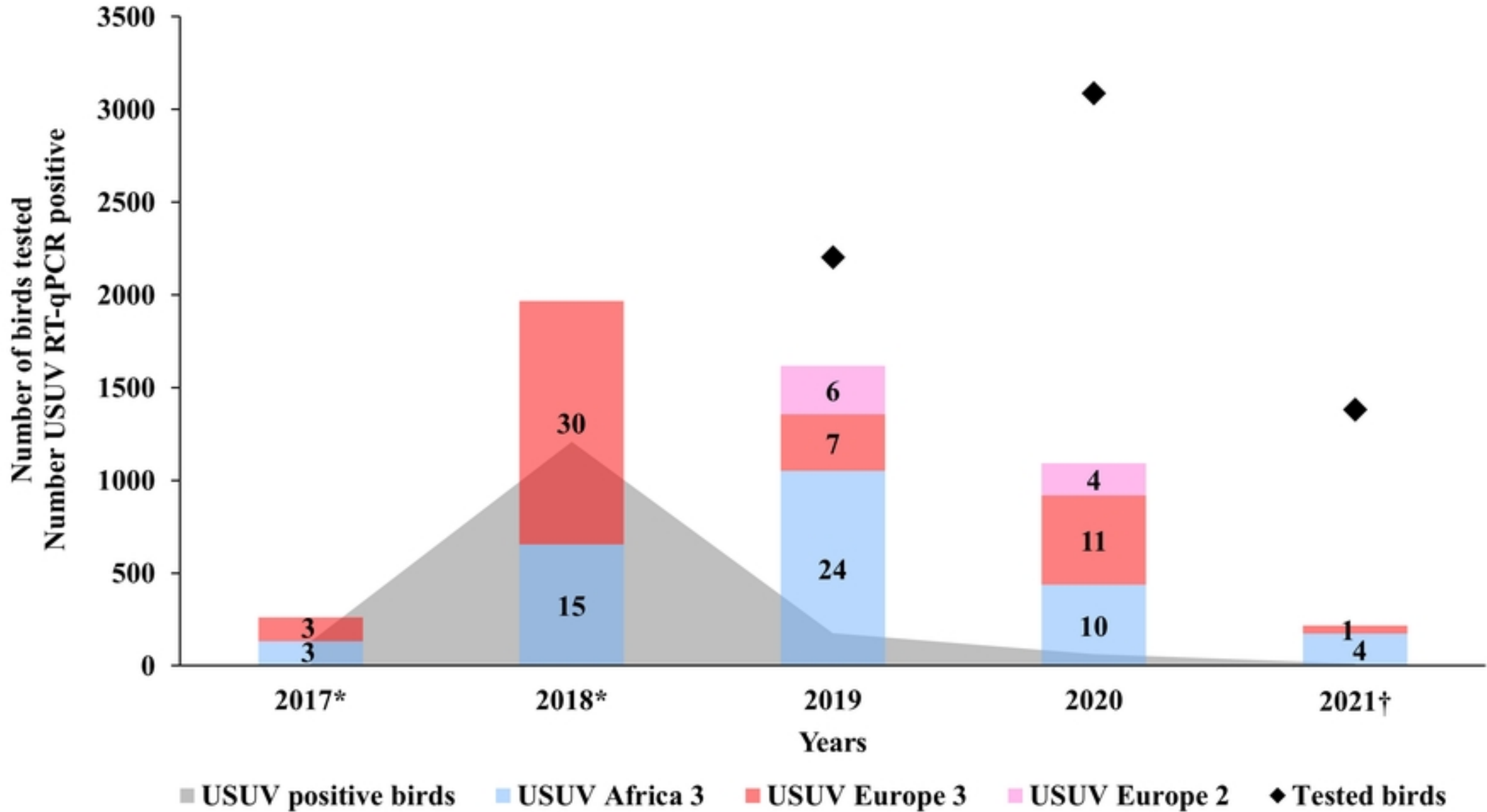
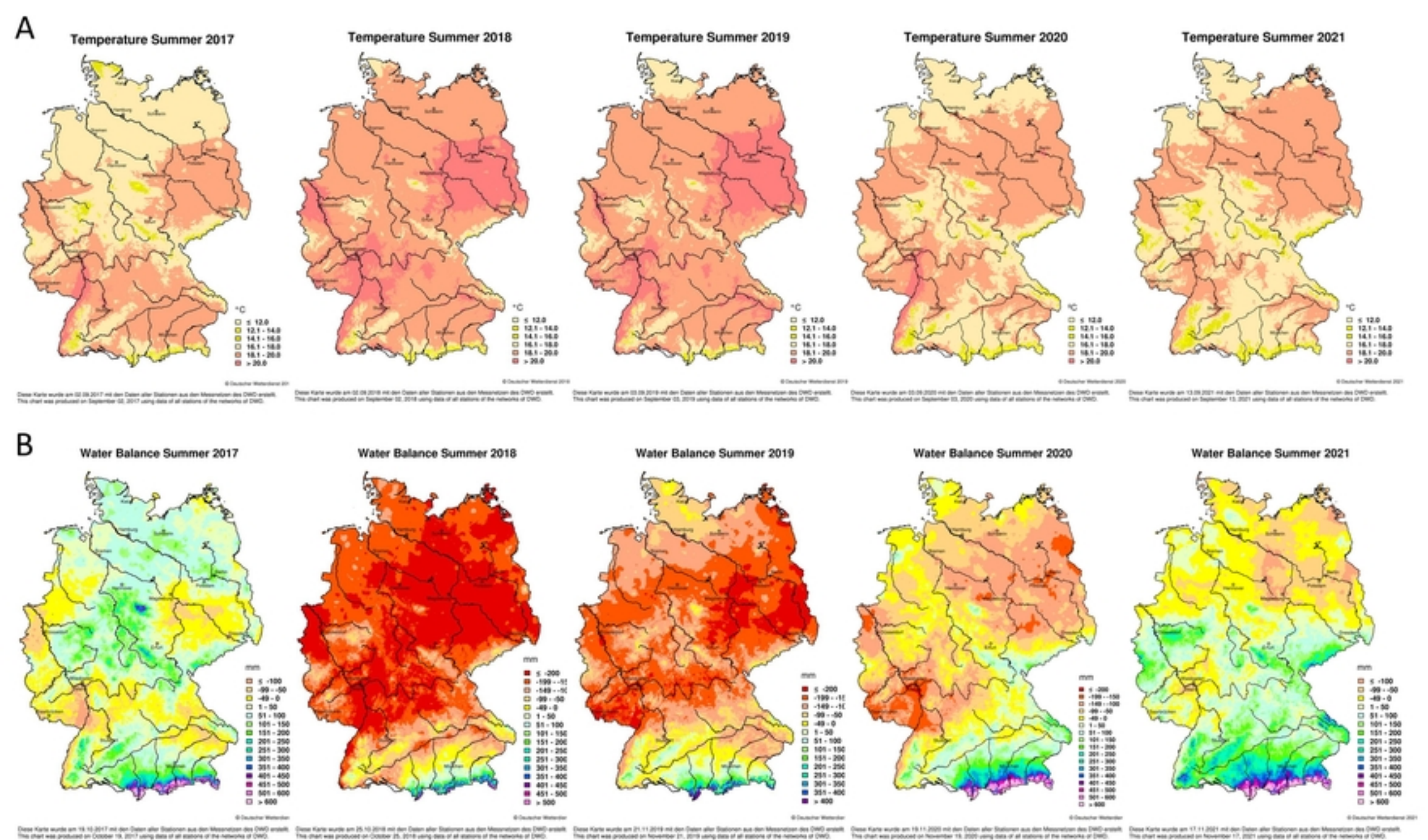


Figure 4



Composed from climatological maps from the Deutscher Wetterdienst (German Weather Service) (Deutscher Wetterdienst (2021), 'Climatological maps of Germany', <<https://www.dwd.de/EN/ourservices/klimakartendeutschland/klimakartendeutschland.html?nn=495490>>, accessed 08.11.2022.)

Figure 6

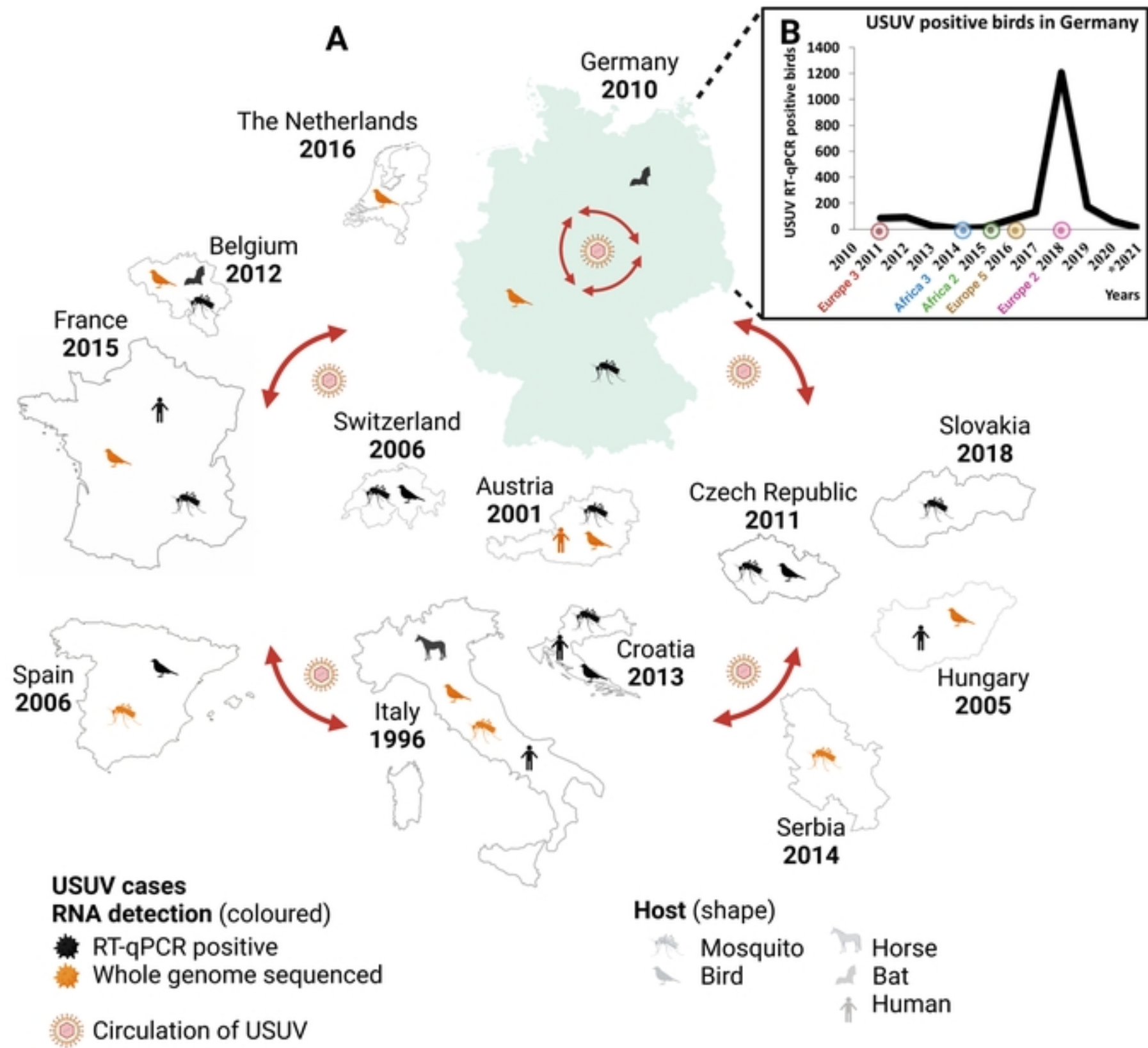


Figure 1





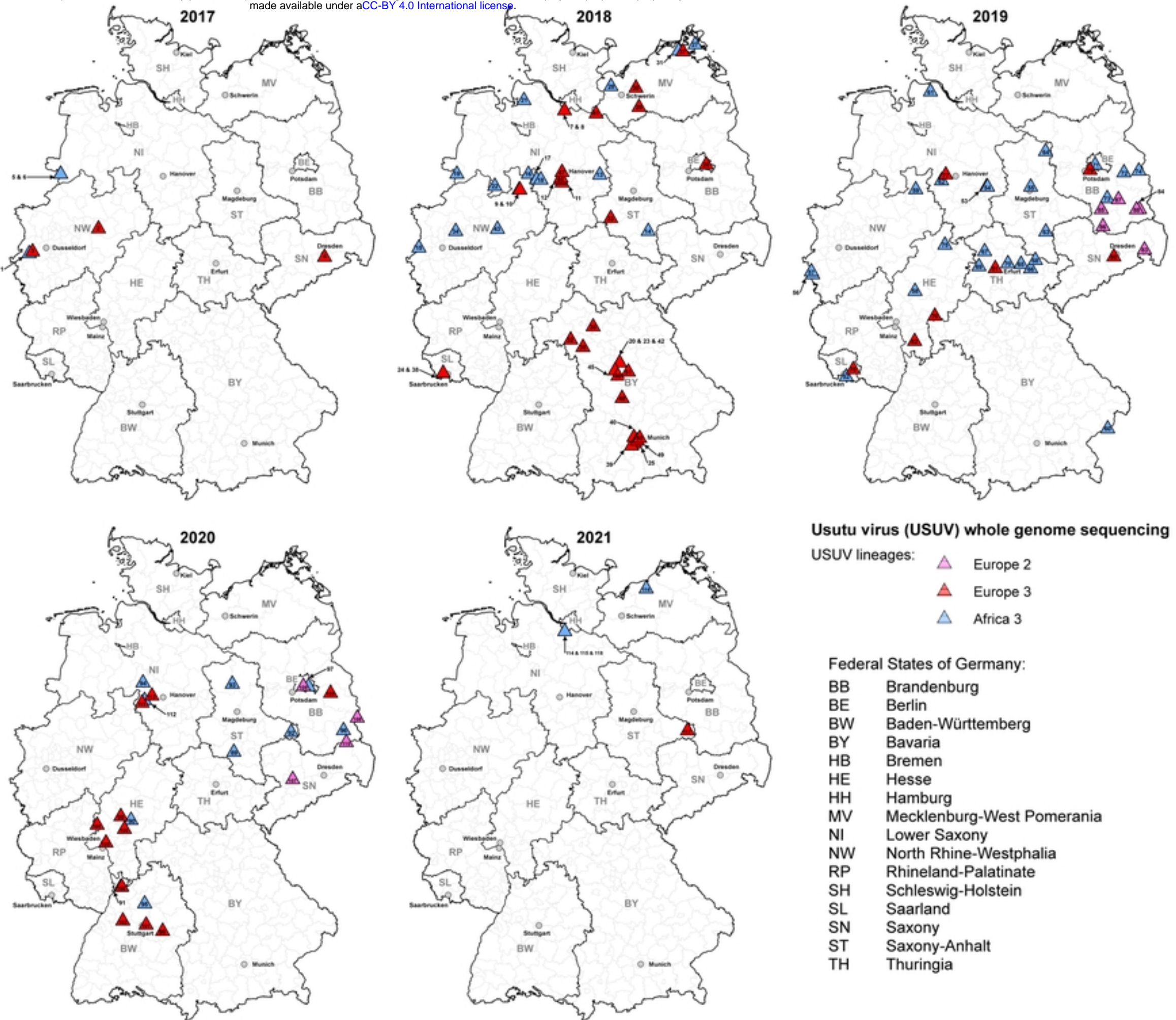


Figure 3

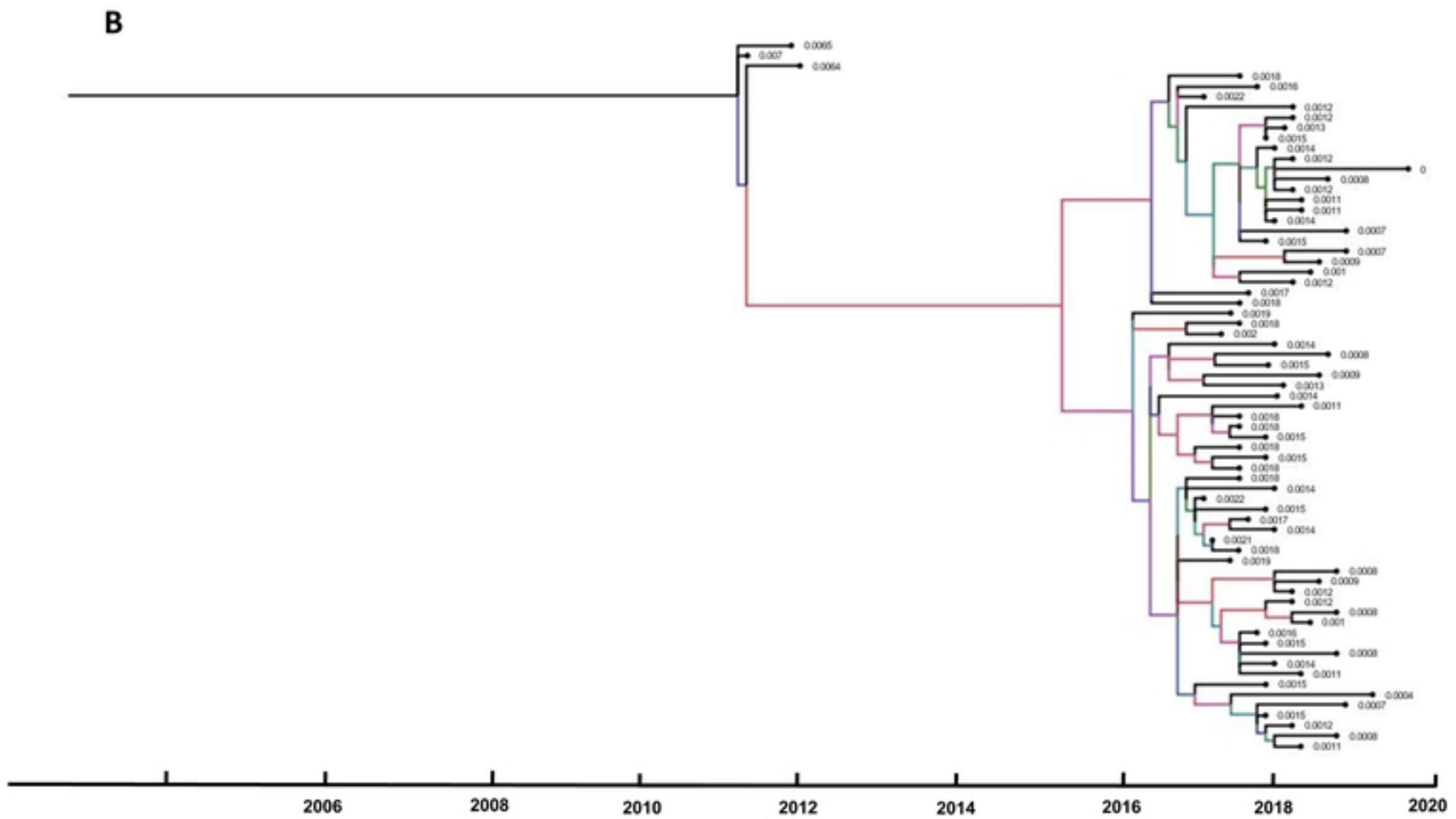
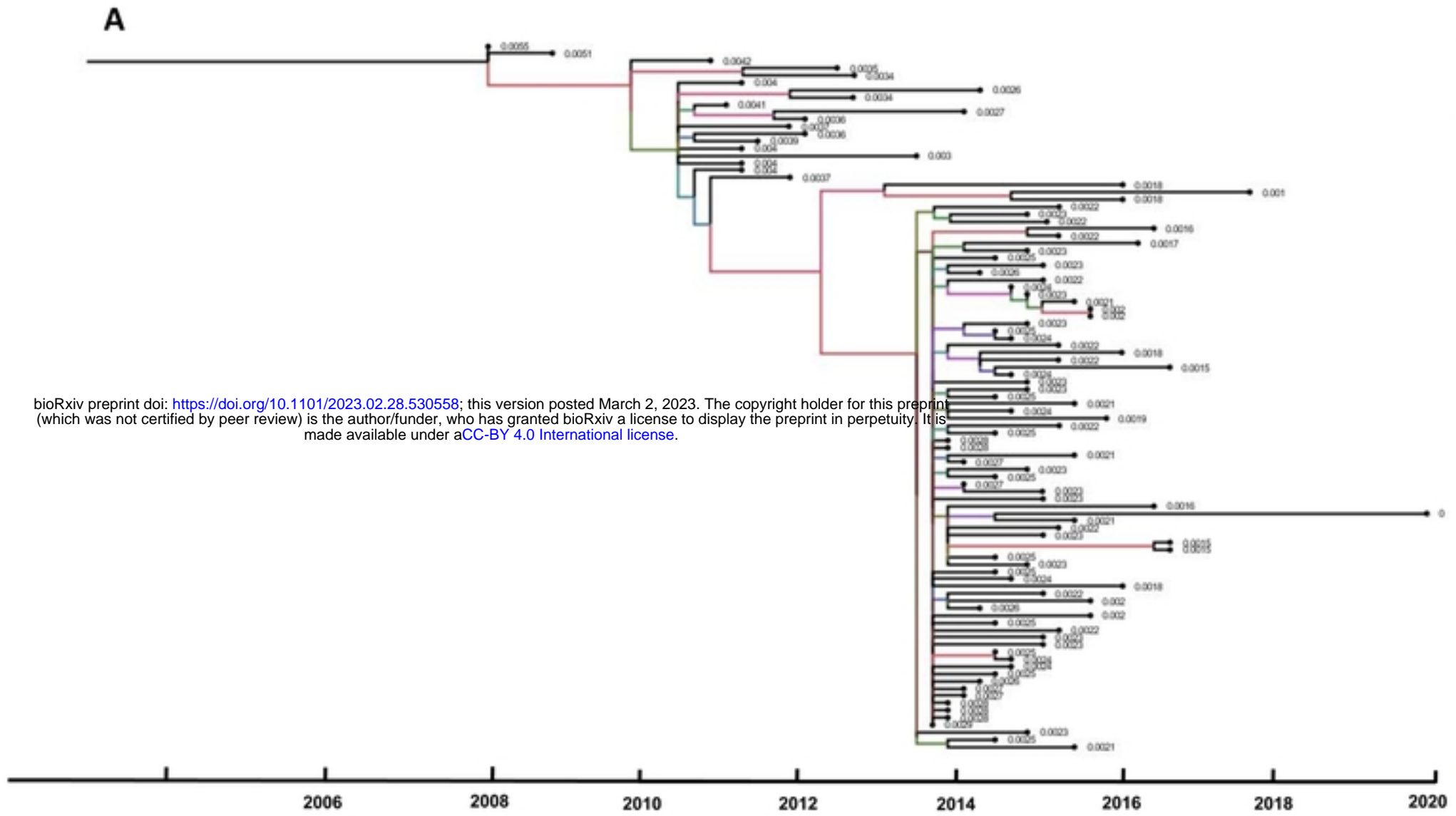


Figure 5