Report

Current Biology

Emergence, continuity, and evolution of *Yersinia pestis* throughout medieval and early modern Denmark

Highlights

- Detected *Yersinia pestis* in time frames congruent with the sparse historical record
- Detected plague at a crude rate of 8.3% in Danish individuals sampled
- Observed continual re-seeding of plague strains, causing localized epidemics

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In brief

Eaton et al. report 13 new ancient *Yersinia pestis* genomes from regions throughout medieval Denmark, demonstrating the continual evolution and reintroduction of *Y. pestis* strains throughout the second plague pandemic and highlighting how population-scale genomic evidence can be used to test hypotheses on disease mortality and epidemiology.

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Emergence, continuity, and evolution of *Yersinia pestis* throughout medieval and early modern Denmark

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SUMMARY

The historical epidemiology of plague is controversial due to the scarcity and ambiguity of available data.[1,2] A common source of debate is the extent and pattern of plague re-emergence and local continuity in Europe during the 14th–18th century CE.[3] Despite having a uniquely long history of plague (~5,000 years), Scandinavia is relatively underrepresented in the historical archives.[4,5] To better understand the historical epidemiology and evolutionary history of plague in this region, we performed in-depth (n = 298) longitudinal screening (800 years) for the plague bacterium *Yersinia pestis* (*Y. pestis*) across 13 archaeological sites in Denmark from 1000 to 1800 CE. Our genomic and phylogenetic data captured the emergence, continuity, and evolution of *Y. pestis* in this region over a period of 300 years (14th–17th century CE), for which the plague-positivity rate was 8.3% (3.3%–14.3% by site). Our phylogenetic analysis revealed that the Danish *Y. pestis* sequences were interspersed with those from other European countries, rather than forming a single cluster, indicative of the generation, spread, and replacement of bacterial variants through communities rather than their long-term local persistence. These results provide an epidemiological link between *Y. pestis* and the unknown pestilence that afflicted medieval and early modern Europe. They also demonstrate how population-scale genomic evidence can be used to test hypotheses on disease mortality and epidemiology and help pave the way for the next generation of historical disease research.

RESULTS

Afro-Eurasia endured a 500-year pandemic of plague between the 14th and 19th centuries CE,[1,2] with evidence of an on-going presence in the Middle East.[3–5] In Europe, outbreaks of this "second pandemic" of plague reoccurred approximately every 10 years, with decreasing mortality rates when compared with the first wave, the Black Death (1346–1353), where rates reached 65%.[2] Paleogeneticists have identified the plague bacterium *Yersinia pestis* (*Y. pestis*) as the most likely agent, although the epidemiology of this pandemic remains controversial, with major debates over levels of mortality[6] and patterns of spread.[1,2] The ecology of *Y. pestis* involves both persistence in rodent reservoirs with occasional zoonotic "spillovers" to humans, followed by larger scale human-mediated transmission that results in major pandemics.[7] As a result, both *Y. pestis* exposure and spread are known to vary between regions and over time.[8,9] However, these differences are challenging to reveal for past occurrences and have

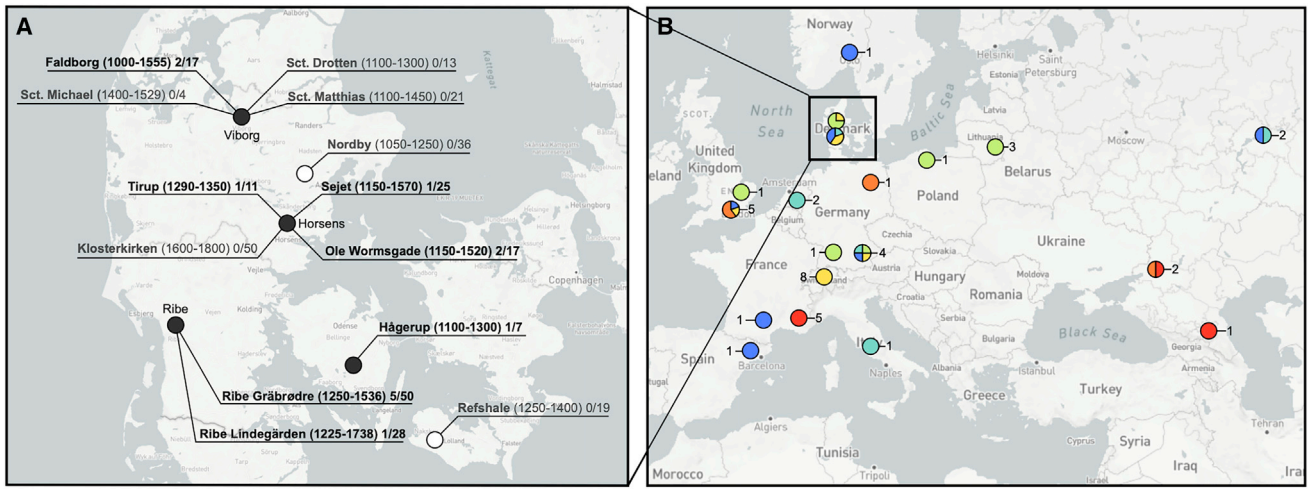

Figure 1. Geographic distribution of 298 archaeological samples used in this study

(A) Map of 6 municipalities sampled in Denmark encompassing 13 archaeological sites. Site labels indicate: archaeological site (earliest date sampled–latest date sampled) *Y. pestis*-positive individuals/total individuals. *Y. pestis*-positive sites are bolded.

(B) Map of 49 *Y. pestis* genomes used for phylogenetic analysis. The sampling locations were standardized to the centroid of the associated province/state. Colors indicate the sampling dates as estimated from the Bayesian molecular clock analysis. Numbered labels indicate the number of genomes sampled from each location.

See also STAR Methods and Data S1.

led to controversy concerning the presence, location, and role of rodent and sciurid plague reservoirs in Europe. Questions about the importance of human dissemination in the re-emergence of plague epidemics during the second pandemic also linger.[10]

Recent studies have attempted to address persistence by synthesizing genetic evidence[1] and historical records[10–12] across Europe. These sources have significant geographic gaps, such as the complete lack of evidence from Scandinavia in digitized databases,[13,14] reflecting the sparseness of historical sources and ambiguity with regard to disease terminology during the medieval period.[2] Recent ancient DNA research[15] has established the presence of a sister lineage of pandemic *Y. pestis* in Sweden some 5,000 years ago, sometimes termed the Neolithic plague, raising the possibility of long-term persistence of plague in Scandinavia, with pandemic *Y. pestis* re-emerging as a local, enzootic disease.

We detected the presence of *Y. pestis* DNA in 13 individuals from 7 archaeological sites, using PCR and targeted capture coupled sequencing (Figure 1A). Across these sites, 8.3% of individuals (13/157) tested positive (PCR and sequencing), ranging from 3.3% at Ribe Lindegården to 14.3% at Hågerup (Figure 1). Given the caveats of taphonomic and other ancient DNA biases, the positivity rate could either be an underestimate of the "true" prevalence of *Y. pestis* in Danish populations at these times or instead an overestimate due to selective mortality, presenting an osteological paradox (i.e., disproportionate number of deaths of frail individuals) that makes assemblages of deceased individuals unrepresentative of those living at the time.[6] Interestingly, however, while the exact extrapolation is unclear, our *Y. pestis* positivity rate (3.3%–14.3%) aligns with rough mortality estimates (5%–20%) during later epidemics of the medieval and early modern period in some contexts.[7–9]

Of the 13 plague-positive individuals, 9 had sufficient sequencing depth (>3×) for genome-wide SNP calling and thus phylogenetic analysis (Figure 2C). To estimate a time-scaled phylogeny and tip dates for these 9 samples, we fitted a relaxed molecular clock to an alignment of *Y. pestis* genomes that included 40 other isolates (Figure 1B). We found that the Danish sequences did not cluster together but rather were interspersed among the diversity of medieval and early modern *Y. pestis* from across Europe with strong support (posterior probability = 1.0) (Figure 3). We also found no evidence that these sequences were the descendant of the Neolithic lineages of *Y. pestis* found in Scandinavia (5,000 YBP)[10]. Similarly, there was no evidence of *Y. pestis* DNA in Danish samples between 1000 and 1300 CE, despite sampling a minimum of 85 individuals and potentially a maximum of 165 individuals that pre-date the 14th century (Figure 2A, see STAR Methods for clarification).

The factors influencing the preservation of ancient DNA are wide-ranging, such that the absence of *Y. pestis* DNA, while in alignment with historical documentation, must be carefully considered. However, given our mean positivity rate of 8.3%, and assuming a similar range in DNA preservation, we expected to detect *Y. pestis* DNA in 7–13 individuals from this time frame had it been present. Accordingly, these results suggest that lineages of *Y. pestis* causing pandemic plague were new to medieval Denmark and were not widespread until at least the 14th century.

We found the earliest evidence of *Y. pestis* in Denmark in the town of Ribe from two individuals associated from the first half of the 14th century, dated to 1333 (1301–1366) and 1350 (1319–1383). These estimates are highly congruent with the historical record, which first documented the appearance of plague in Denmark at Ribe in 1349.[4] Furthermore, these sequences fell within an unresolved cluster (posterior probability = 0.15) of samples from Northern and Western Europe (Figures 3 and S1), which has been linked to the rapid spread of *Y. pestis* during the Black Death (1343–1356).[11] One sequence (G.25A) falls

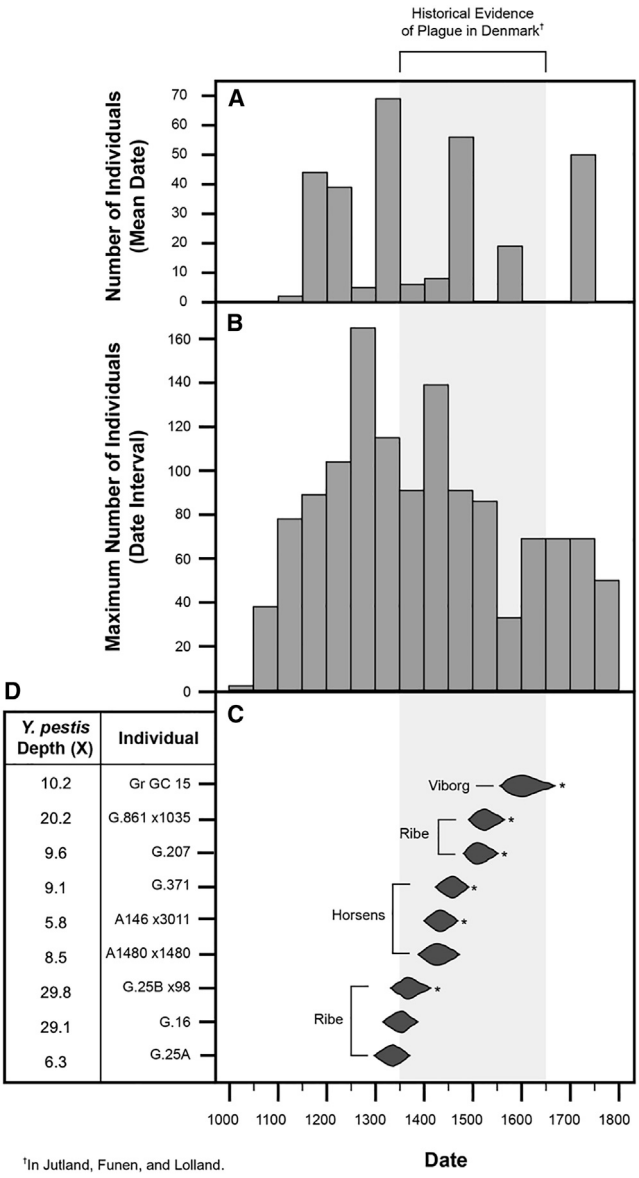

Figure 2. Temporal distribution of archaeological samples used in this study

(A) Mean skeletal dates for all individuals (n = 298).

(B) Skeletal date intervals for all individuals (n = 298) using a bin width of 50 years.

(C) Violin plots of the posterior distribution of Y. pestis tip dates for plague-positive individuals (n = 9) from the Bayesian molecular clock analysis.

(D) Mean sequencing depth of the *Y. pestis* chromosome.

See also STAR Methods and Data S1.

close to the root of second pandemic strains with samples from Norway, England, and Spain, suggesting rapid dissemination of the plague bacterium in the first years of the Black Death pandemic. Our molecular dates support this historical association, albeit weakly. The precise epidemic period cannot be resolved due to the wide uncertainty of our date estimates (>50 years). The second sequence (G.16) within this initial cluster appears to be a reintroduction of a slightly later circulating strain, not a re-emergence from the earlier outbreak represented by the sequence from G25A.

We next identified *Y. pestis* in the latter half of the 14th century from a third individual from Ribe (G.25B), dated to 1370 (1336–1408), which clustered strongly (posterior probability = 0.99) with post-Black Death samples from the Netherlands and Russia. These samples have previously been attributed to what has been termed the *pestis secunda* (1357–1366),[2] although *pestis tertia* (1364–1376)[8] is an equally likely candidate. This clade currently contains samples from Northern European countries (Russia, the Netherlands, and now Denmark) and has broader epidemiological significance as it is directly ancestral to the third or modern pandemic of plague (19th–20th century).[12] Our results therefore reveal new global connections between medieval Danish populations and modern epidemics of plague, including recent outbreaks in Madagascar.[13]

We found no evidence of plague at Ribe between 1408 and 1484. This was surprising, as 86% of individuals (43/50) from this site were archaeologically dated to between 1400 and 1536. Instead, the distribution of *Y. pestis* DNA shifts during this period from the western coast of Jutland to the eastern coast at Horsens. We recovered three distinct, yet possibly contemporaneous, *Y. pestis* sequences from three sites near Horsens, dated to 1429 (1392–1467), 1433 (1403–1464), and 1457 (1427–1487), for which there is no written evidence (i.e., Tirup, Sejet, and Ole Wormsgade). These genomes were most closely related to *Y. pestis* sequences isolated from individuals sampled in Germany, Lithuania, Poland, and England and likely represent a separate introduction of plague to Denmark from a circulating epidemic in northern European countries at the time (Figure 3). This geographic association parallels the historical record, in which outbreaks in Denmark appear to coincide with those in the Baltic region.[8] Recent studies have demonstrated that the directionality and spread of zoonotic diseases cannot be robustly inferred from genomic data alone.[12,14] Instead, our results establish an epidemiological link between *Y. pestis* and historical case records in Denmark, which could be jointly modeled with greater resolving power[15] in future work.

After a long absence, plague returned to Ribe in the 16th century, with *Y. pestis* isolated from two genomes dated to 1513 (1484–1546) and 1525 (1494–1560), which are closely related and could represent local persistence or continued circulation within Denmark post introduction. Our last evidence of plague in Denmark comes from the northern site of Faldborg (Viborg), dated to 1594 (1550–1649). This places a final bound on our estimate of plague's disappearance from Denmark at 1649, which is congruent with the historical record, with the last recorded outbreak of plague in Jutland in the years 1654–1657.[4] Accordingly, we found no evidence of *Y. pestis* in Denmark between 1649 and 1800 CE in the DNA isolated from 70 individual remains (Figure 2A), a result consistent with Benedictow's claim from written documentation.[4]

DISCUSSION

This is the first population-level analysis of ancient *Y. pestis* from an in-depth (n = 298) longitudinal sampling from 1000 to 1800 CE

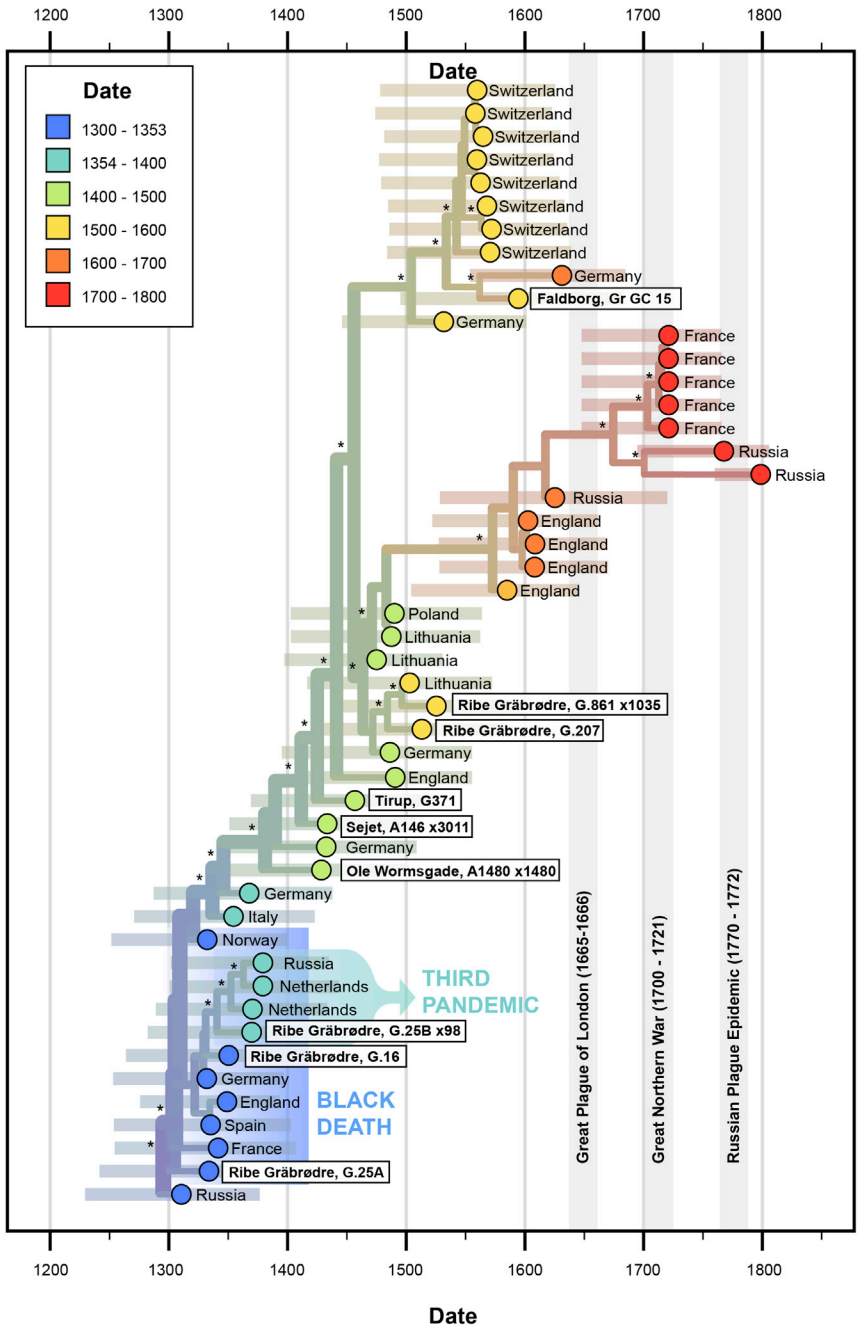

Figure 3. Maximum clade credibility (MCC) tree depicting a time-scaled phylogeny of 49 European *Y. pestis* genomes

Asterisks indicate clades with strong posterior support (≥0.95). Colors indicate the mean sampling dates as estimated from the Bayesian molecular clock analysis. Error bars indicate tip-dating uncertainty, as represented by the 95% highest posterior density (HPD) interval.

See also STAR Methods, Data S1, and Figure S1.

(800 years) within a single country (Denmark). We describe the earliest known appearance of *Y. pestis* in Denmark (14th century) and document its continuity over a period of 300 years (up to the 17th century), in what appears to be three stages, and its apparent disappearance from Denmark by 1649. We observed an early (~1350 CE) introduction of plague to the western coast of Denmark (Ribe) during the Black Death, with its rapid spread across Northern Europe indicative of human-mediated movement and transmission. Following a short period of absence in sampled locations, plague re-appeared (~1430 CE) on the eastern side of Denmark (Horsens) as a reintroduction from other Baltic countries, returning again approximately 50 years later, this time on the western side (Ribe). Until this point in the pandemic, the structure of the phylogeny and position of Danish *Y. pestis* sequences suggest a "progressive" (i.e., "ladder-like") evolution, likely representing its continuing evolution and reintroduction into populations with a sufficient number of susceptible hosts rather than its long-term persistence within individual communities. The final stage of the pandemic is represented by a single sequence from Northern Denmark (Viborg region) within a cluster that appears to have spread across a Northern-Southern European transect. This sequence sits close to the final recorded date of plague's presence in Denmark in ~1659 CE. Thus, the Viborg and Hågerup recoveries likely reflect a spread of plague from north central German regions, with the port city epidemics (Horsens-related sites of Sejet and Ole Wormsgade) likely seeded by maritime plague dispersals[16] The picture painted is one of continual seeding of plague strains into Denmark—which caused localized epidemics, subsequently died out, only to be re-seeded at a later date from another location (i.e., country or locality)—through what appears to be human-mediated transmission.

Here, we provide preliminary, crude estimates of the positivity rates of historical plague from molecular evidence as we detected *Y. pestis* in an average of 8.3% of Danish individuals sampled. Given the vagaries of detecting plague in skeletal remains (stage of infection, bacteremia load, and overall molecular taphonomy), one should take this number with caution, but it may be a useful benchmark, when comparing other positivity rates from other remains sampled across the second pandemic. Our phylogenetic analysis was highly congruent with the sparse textual evidence of pestilence in Denmark with respect to the timing of outbreaks and geographic ties to the Baltic region. Our population, geographic, and temporal analyses highlight the complex epidemiology and ecology of plague in the Danish outbreaks that were possibly reflecting the persistence of *Y. pestis* strains (G16 and G25B, G861 and 207) within Denmark, as well as those caused

by the repeated reintroduction and movement of bacteria from elsewhere during epidemic periods (G25A, A1480, A146, G371, and GR GC 15). We also provide new evidence of plague exposure among Danish populations, both in urban centers such as Ribe and importantly at rural sites such as Tirup, where there is no surviving historical evidence. These results confront silences in the historical record, reveal new connections between our past and present experience of plague, and broaden our understanding of the epidemiology of re-emerging diseases.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.01.064.

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AUTHOR CONTRIBUTIONS

K.E., R.K.S., J.K., E.C.H., and H.N.P. designed the study. J.A.G. identified and collected the Danish samples and contributed to the archaeological dating. J.L.B., and S.N.D. provided access to archaeological sites and materials, and J.L.B. reviewed the archaeological dating. V.G. performed radiocarbon dating. K.E., R.K.S., and J.K. performed laboratory analysis. A.G.C. and N.V. provided historical sources and interpretation. S.D. and L.F. critiqued and revised the computational methods and discussion. G.B.G. provided access to computational resources and data storage. H.N.P., E.C.H., and G.B.G. supervised the study. K.E. wrote the manuscript with contributions from all authors.

DECLARATION OF INTERESTS

J.K. declares financial interest in Daicel Arbor Biosciences, which provided the myBaits probes for targeted capture.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human skeletal material (tooth)	This study	ID no. A19 x21
Human skeletal material (tooth)	This study	ID no. Gr GCx15
Human skeletal material (tooth)	This study	ID no. G.25A
Human skeletal material (tooth)	This study	ID no. G. 16
Human skeletal material (tooth)	This study	ID no. G207
Human skeletal material (tooth)	This study	ID no. G. 861 x1035
Human skeletal material (tooth)	This study	ID no. ID 319
Human skeletal material (tooth)	This study	ID no. G. 25B x98
Human skeletal material (tooth)	This study	ID no. A146x3011
Human skeletal material (tooth)	This study	ID no. G371
Human skeletal material (tooth)	This study	ID no. x1265
Human skeletal material (tooth)	This study	ID no. x1155 A1155
Human skeletal material (tooth)	This study	ID no. A1480 x1480
285 additional human skeletal remains were screened in this study, but they did not contain *Y.pestis*	This study, Data S1A	See Data S1A
Chemicals, peptides, and recombinant proteins		
Proteinase K	Roche	Catalog no. 05114403001
0.5M EDTA	VWR	Catalog no. 97062-656
Tris-Cl (1M)	Fisher	Catalog no. BP1758-500
Calcium chloride dihydrate	Sigma-Aldrich	Catalog no. 208291
DTT	Fisher	Catalog no. PRV3151
Polyvinylpyrrolidone	Fisher	Catalog no. BP431100
N-phenacylthiazolium bromide	Oakwood Chemical	Catalog no. 080244
Guanidine Hydochloride	Fisher	Catalog no. BP1781
Isopropanol (100%)	Fisher	Catalog no. BP26181
Tween-20	Sigma-Aldrich	Catalog no. P9416
3M Sodium Acetate (pH 5.2)	VWR	Catalog no. 97062-834
AmpliTaq Gold, 10X PCR Buffer II, $MgCl_2$	ThermoFisher Scientific	Catalog no. N8080245
dNTP mix	New England Biolabs	Catalog no. N0447L
BSA	New England Biolabs	Catalog no. B9000S
EvaGreen	VWR	Catalog no. 89138-982
Bst polymerase & Thermopol Reaction Buffer	New England Biolabs	Catalog no. M0275S
PEG-4000	Qiagen	Catalog no. 133085
T4 DNA Ligase & Reaction Buffer	New England Biolabs	Catalog no. M0202L
T4 Polynucleotide Kinase	New England Biolabs	Catalog no. M0201L
T4 DNA polymerase & BE Buffer 2.1	New England Biolabs	Catalog no. M0273L
ATP	Fisher Scientific	Catalog no. FERR0441
KAPA SYBR FAST	Millipore Sigma	Catalog no. KK4608
PhiX CONTROL V3 KIT	Illumina	Catalog no. FC-110-3001
NuSieve GTG Aagarose	Lonza	Catalog no. 50081
GeneRuler 50bp DNA Ladder	Fisher	Catalog no. SM1212
6X TriTrack Loading Dye	Thermo	Catalog no. R1161
SYBR Safe DNA Gel Stain	Invitrogen	Catalog no. S33102

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human Cot-1 DNA	Invitrogen	Catalog no. 15279011
UltraPure Salmon Sperm DNA Solution	Invitrogen	Catalog no. 15632011
RNAsecure™ RNase Inactivation Reagent	Thermo	Catalog no. AM7005
Critical commercial assays		
QIAquick Nucleotide Removal Kit	Qiagen	Catalog no. 28306
MinElute PCR Purification Kit	Qiagen	Catalog no. 28006
High Pure Viral Nucleic Acid Large Volume Kit	Roche	Catalog no. 05114403001
myBaits Custom	Arbor Biosciences	N/A
Deposited data		
Y. pestis genomes (Denmark)	This Study	NCBI BioProject PRJNA926136
Previously published *Y. pestis* genomes	See Data S1E	See Data S1E
Oligonucleotides		
Pla Screen Forward Primer: ATGCCCTGAAAGACGTGGAGAA	IDT	N/A
Pla Screen Reverse Primer: GGGCGCTCATTCTGTTGTTT	IDT	N/A
ILPr_reampP5F_MeyerIS5: AATGATACGGCGACCACCGA	IDT	N/A
ILPr_reampP7R_MeyerIS6: CAAGCAGAAGACGGCATACGA	IDT	N/A
Enrichments Probes	Arbor Biosciences	N/A
Software and algorithms		
FastQC	Andrews[17]	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
leeHom	Renaud et al.[18]	https://github.com/grenaud/leeHom
Burrow-Wheeler Aligner (BWA)	Li and Durbin[19]	https://doi.org/10.1093/bioinformatics/btp324
Bam-rmdup (Biohazard)	https://hackage.haskell.org/package/biohazard-2.1/docs/Bio-Bam-Rmdup.html[20]	https://hackage.haskell.org/package/biohazard-2.1/docs/Bio-Bam-Rmdup.html
mapDamage 2.0	Jónsson et al.[21] Data S2	https://github.com/ginolhac/mapDamage
Samtools	Li et al.[22]	https://academic.oup.com/bioinformatics/article/25/16/2078/204688?login=false
BCL2BAM2FASTQ	N/A	https://github.com/grenaud/BCL2BAM2FASTQ
Krona	Ondov et al.[23]	https://github.com/marbl/Krona
Snippy	Seeman[18]	https://github.com/tseemann/snippy
BEAST2.5	Bouckaert al.[22]	https://journals-plos-org.libaccess.lib.mcmaster.ca/ploscompbiol/article?id=10.1371/journal.pcbi.1006650
Seaborn	Waskom[20]	https://seaborn.pydata.org/
Nextstrain	Hadfield et al.[21]	https://docs.nextstrain.org/en/latest/index.html
Libbam	N/A	https://github.com/grenaud/libbam
nf-core/eager	Yates et al.[17]	https://nf-co.re/eager
sra-toolkit	N/A	https://github.com/ncbi/sra-tools

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hendrik Poinar (poinarh@mcmaster.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Raw sequencing data generated used from this study has been deposited at Consensus sequences for each genome have been deposited in NCBI BioProject PRJNA926136 and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Archaeological Information

The remains used in this study were provided by ADBOU which currently curates skeletal remains from ~ 17,000 Danish individuals, dated from the Viking Age to the early modern period (Data S1A and S1B). While not a comprehensive sampling of the entire country, we attempted to include a geographically wide expanse with a variety of locations and time periods represented, the samples stemmed from 15 archaeological sites from the mainland (Jutland), as well as two islands (Funen and Lolland). Based on the skeletal,[24] site and radiocarbon dates, these individuals represent ~800 years of human settlement (1000-1800 CE). We include the historically associated pandemic period in Denmark (1350-1657), the so-called quiescent periods before 1348 (1000-1350 CE) and the long tail end of the second pandemic (1658-1800) for which no outbreaks of plague were historically documented in Denmark[1,4] (see Data S1A and S1B).

The following sites were previously described by Klunk et al.[25]: Ribe Gräbrødre/Grey Friar (ASR 1015), Ribe Lindegärden (ASR 13 II/ASR 2391), Nordby (FHM 3970), Klosterkirken (HOM 1272), Sejet (HOM 1046), Ole Wormsgade (HOM 1649), Tirup (VKH 1201), Hågerup (ØHM 1247), Refshale (Refshale), Sct. Drotten (VSM 09264/VSM 902F), Sct. Mathias (VSM 855F), Faldborg (VSM 29F).

Two additional sites not previously described by Klunk et al. [25] were used in this study:

Sct. Mathias (VSM 906F)

This cemetery was connected to Sct. Mathias church[26] in the city of Viborg, where previous excavations had been done at VSM 855F, VSM 209F, and VSM 09.264. During the excavation of this site 23 burials were recorded, many were poorly preserved, and the graves were dissolved. In this study only 3 individuals were used from this VSM 906F. The graves have been dated from before 1250 and until 1350.

Sct. Mikkel (JAH 1-77)

Skt. Mikkel cemetery[27] was in the city of Viborg, its usage dates from 1100 to the early 1500s, it was associated with the Sct. Mikkel church stopped being in use around 1529 when all churches associate with the city were demolished. However, the burials in this cemetery are shown to have been present before the construction of the church. Only 4 samples from tish site were used for this study but they dated form 1350-1500.

Permits

We obtained permission from museums curating the remains of all samples we used. These are modifications/truncations of the letters we received from each site and they can be requested directly from the lead contact should they wished to be viewed.

Sydvestjyske Museer Morten Søvsø gives the permission to use the skeletons: Ribe Gräbrødre G. 861 x1035, Ribe Gräbrødre G. 207, Ribe Gräbrødre G. 25B x98, Ribe Gräbrødre G. 16, Ribe Gräbrødre G. 25A in the aDNA study

The Viborg Musuem and Lars Agersnap Larsen gives his permission to use the two Faldborg skeletons, but remarks that their numbers should be given using the updated numbering by Viborg Museum: Faldborg Gr. GC 15 should be **VSM F29 Faldborg, grav C, skelet X15** *or just* **VSM F29x15** Faldborg Gr. ID 319 should be **VSM F29 Faldborg, grav ID, skelet X319** *or just* **VSM F29x319**

The Øhavsmuseet and Poul Baltzer Heide give permission to use Hågerup A19 X21 (properly named: ØHM 1247 A19/X21)in the aDNA study.

Lone Seeberg from Horsens museum gives the permission to use:

Sejet A146 x3011, Ole Wormsgade A1480 X1480, Tirup G. 371 in the aDNA study.

METHOD DETAILS

All laboratory work was performed in designated clean room facilities at the McMaster Ancient DNA Centre at McMaster University, Hamilton, Canada.

Sampling, extraction, and screening

From each individual we subsampled between ~10-100mg from a single tooth pulp cavity or tooth root. In cases where the entire root was subsampled, a Dremel attached to a diamond cutting wheel was used to cut off the root of tooth. Roots were then placed between two weigh boats and crushed with a hammer; this method generated ~50-100mg subsamples. When the pulp cavity alone was subsampled, the exact method varied based on the tooth type.[25] For multi-rooted teeth a Dremel attached to a diamond cutting wheel was used to separate the crown from the root. Once separated a diamond wheel point drill bit was used to subsample inside the crown and down each root to collect the tissues surrounding the pulp cavity. For single-rooted teeth the tip of the root was cut off with a diamond cutting wheel, and a drill bit was used to reach up the root and collect the tissues. This method, used for pulp cavity subsampling, generated subsamples weighing between ~10-50 mg. Between each sample tools were placed in a beaker filled with 5% bleach for 5 minutes, rinsed with ultrapure water, and dried at 250°*C*. All surfaces were also cleaned with 5% bleach and ultrapure water. At the end of each subsampling session tools were heated for 4 hours at 450°*C* and subsampling surfaces were UV irradiated.

Subsamples and associated blanks then underwent two rounds of demineralization and digestion, where they were incubated on a heater/shaker plate with 500μL of 0.5M EDTA (pH 8) at 22°*C* for 24-hours. Followed by a 24-hour incubation at 25°*C* with 500μL of a proteinase-K based digestion buffer.[28] After each 24-hour incubation, tubes were spun at maximum speed for 3 minutes allowing for the supernatant to be removed and stored at -20°*C*. The resulting 2 mL supernatant was then combined with a high-volume guanidinium hydrochloride-based binding buffer and added to a silica-based Roche collection column for DNA purification. DNA was purified using a Roche wash buffer, eluted in 50μL of EBT, and stored at -20°*C*.[29]

Purified DNA extracts were then diluted and a preliminary qPCR assay to screen for the presence or absence of *pla* was performed on each replicate, using previously described primers.[30] The dilutions and straight extracts were then tested in replicate one final time for samples which initially screened positive. For a sample to classify as positive and be carried forward to sequencing 4-6 of their replicates would display the following features: Cq values less than 40, an exponential amplification curve, and a melt peak of 78°*C* (or within 1° of the standards). Standards were generated from previously known plague positive samples from which *pla* had been amplified through qPCR (see Data S1C for plague positive samples).

Library preparation and Sequencing

Double-stranded DNA libraries (40μL) were prepped using 10μL of straight extract from each plague positive sample and their corresponding blanks. From these libraries 10μL were indexed, purified into 12μL indexed libraries and then quantified via qPCR using serially diluted 10 nM PhiX Illumina libraries as standards.[31,32] For shotgun sequencing Indexed libraries were then pooled to equivalent concentrations and size selected for molecules ranging from 150bp to 500bp on an agarose gel to remove adapter dimers and modern contaminants. The final purified pool was given to the McMaster Genomics Facility for 2x100 paired-end sequencing on an Illumina HiSeq machine. Indexed and non-indexed libraries were stored at -20°*C* for future sequencing and *Y. pestis* enrichment. Libraries which continued to appear as plague positive after shotgun screening were then enriched (see below). Samples A146x3011, G371, x1265, x115 A115, and A1480 x1480 were the first set of positive samples identified and they underwent multiple rounds of enrichment and sequencing to obtain the desired amount of coverage. Each enriched library from these initial plague positive samples were sequenced to a depth of ~1M-5M in each round. The indexed libraries for samples A19 x21, Gr GCx15, G. 16, G207, G. 861 x1035, ID 319, and G.25B x98 were sequenced 2-3 multiple times to a depth of 5M-20M each time, until a desired coverage[12] was reached or until the library's complexity had been exhausted (Data S1D).

Y. pestis Enrichment

Shotgun analysis was performed for each sample (see below), samples which continued to show signs of authentic ancient *Y. pestis* DNA following all screening steps were selected for targeted capture of the *Y. pestis* genome using previously designed probes[30] (Data S1C). Samples A19 x21, Gr GCx15, G. 16, G207, G. 861 x1035, ID 319, and G.25B x98, underwent an initial round of enrichment to confirm the plague positive assignment from shotgun analyses. Following this, libraries were indexed again or in some cases new libraries were made from additional subsamples because previous libraries had been exhausted. This resulted in each sample undergoing 3-4 rounds of enrichment, often indexed libraries were enriched in duplicate, and each enriched fraction was sequenced to a depth of ~1M-5M, until desired coverage was achieved (see below). Indexed libraries from A19 x21, Gr GCx15, G. 16, G207, G. 861 x1035, ID 319, and G.25B x98 underwent a single round of enrichment in duplicate, and they were sequenced 2-3 times (as stated above). All corresponding blanks were combined 2-per tube, enriched together, quantitation of these blanks showed that their concentrations ranged between 100-1000pM, to maximize the amount of information collected from these blanks 6μL of each combined library was pooled for sequencing.

QUANTIFICATION AND STATISTICAL ANALYSIS

Shotgun sequencing analysis

FastQC was used to perform quality control analysis on fastq files generated after each sequencing run.[33] Sequenced molecules were trimmed and merged using leeHom, with a flag specific for ancient DNA.[34] Molecules were then mapped to the *Y. pestis* CO92 reference genome using network-aware-bwa with an edit distant of 0.01 (-n 0.01), with seeding disabled (-l 16500), and gap opens set to 2 (-o 2).[35] Mapped molecules were sorted using samtools sort,[36] files from L001 and L002 were merged using samtools merge,[36] duplicates were removed with biohazard's bam-rmdup.[37] Samtools view was used to filter mapped molecules to a minimum length of 35 (-m 35) and a map quality of 30 (-q 30).[36] Samtools view (-c) was used to count the number of molecules mapping to the hard-masked *Y. pestis* reference genome, average coverage depth was calculated using samtools depth (-a) and dividing by the total number of sites[36] (see Data S1D for sequencing and mapping statisitcs). The same steps were taken when mapping data to the Human reference genome (hg38). Molecules were authenticated as ancient after analyses using mapDamage which provided length distributions and damage patterns for all mapped molecules.[38] Fasta files containing deduplicated molecules of length greater than 35 base pairs were metagenomically filtered using blastn. We used the nt_v5 database, specified an e-value of 0.00001, and the task was set to megablast.[39] To simplify visualization of the metagenomic data we used Krona plot and looked specifically at the number of *Y. pestis* and *Y. pseudotuberculosis* specific molecules in comparison to the total number of molecules in the *Y. pseudotuberculosis* complex.[23] Samples chosen for targeted capture (see above) had more reads mapping to hg38 than CO92, had a higher proportion of *Y. pestis* molecules than *Y. pseudotuberculosis*, had average fragment length distributions of ~55bp, and had damage patterns characteristic of ancient DNA[40] (Data S1D).

Bioinformatic processing and phylogenetic analyses

Following enrichment (see above), sequenced molecules were aligned to the *Y. pestis* CO92 reference genome using the *nf-core/eager* pipeline set to default parameters, with a minimum read length of 35, an edit distance of 0.01, and a 16bp seed length.[17] Unmerged reads were not retained for downstream steps, and samples with less than 70% of the reference genome covered at 3X were not included in downstream phylogenetic steps. For phylogenetic analyses fastq files previously existing *Y. pestis* genomes were downloaded using the sra-toolkit. Samples were then processed using the nf-core/eager pipeline with the same parameters mentioned above. Following this initial processing, 40 high-coverage *Y. pestis* genomes (>3X) dated between the 14th and 18th centuries (Data S1E) were carried forward to phylogenetic analyses. Pairwise alignments were made using snippy, with the following flags: –mapqual 30, –mincov 30, –minfrac 0.9, and –basequal 20.[18] Next, multiple alignments were made using snippy with the following flags: –mask auto, –mask-char X.

For phylogenetic analyses, we performed a Bayesian Evaluation of Temporal Signal (BETS)[19] with BEAST2[22] to assess whether these data had a temporal signal and the optimal molecular clock model for our analyses.[12] We assumed a constant population size coalescent tree prior for statistical convenience and compared the use of a strict clock and an uncorrelated lognormal (UCLN) relaxed clock. The tip-dates of previously-published genomes (N=40), were assigned parametric priors in the form of normal distributions using the mean radiocarbon/mortuary date and half the uncertainty as the standard deviation. All Danish samples were assigned priors according to their date range from archaeological information with a mean date of 1330 CE and a standard deviation of 115 years, to account for the uncertainty in collection dates. Bayes factors were calculated by taking the difference in marginal likelihoods of each candidate model, as estimated with a generalized stepping stone (GSS). For the model with the highest marginal likelihood, we sampled from the posterior for 100,000,000 generations and verified that the effective sample size (ESS) of all relevant parameters was greater than 200. Data visualization was performed using the python package *seaborn*[20] and *auspice*, a component of the Nextstrain visualization suite[21] (Figure S1).