Integrative approach using novel *Yersinia pestis* genomes to revisit the historical landscape of plague during the Medieval Period

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- SUPPLEMENTARY INFORMATION -

History of plague in the abbey of San Salvatore

The abbey of San Salvatore is located ca. 75 km south of Siena, in the mountainous territory of the Monte Amiata. Traditionally, the founding of the abbey was attributed to the Lombard King Ratchis in the second half of the 8th century CE in order to protect and control trade and pilgrim traffic. Upon its establishment, the monastery was an important staging point along the Via Francigena, a complex system of routes, connecting centers of Christianity with Northern and Western Europe along with local and regional traffic (1). The fortified settlement of Abbadia San Salvatore (Castel di Badia) soon emerged around the abbey and established itself as the region's principal market town. Various signorial lords gained control of the abbey and its territory from the 12th century until 1347, shortly before the start of the Black Death, when it officially became part of the Republic of Siena.

Archaeology and osteology of the abbey of San Salvatore

Between 1997 and 2007, excavations were carried out in the abbey of San Salvatore and in its surrounding perimeter (2). In the north-eastern side of the monastery (Fig. S1), a burial area was excavated on three occasions between 2003 and 2007, resulting in the identification of several main trenches (Fig. S2) and many intersecting standard single graves. No radiocarbon dating was carried out, but, based on the site stratigraphy and relative dating of the ceramic artefacts, the long and parallel trenches date back to the middle or second half of the 14th century, which coincides with the Black Death (1347-1353 CE) and documented plague outbreaks in Siena (1363, 1374, 1375, 1383, and 1390) (3, 4).

The individuals recovered from these structures had been arranged in an imbricated way, were buried without personal belongings, and wrapped in shrouds. A few small rings and coins were sporadically found within the grave, but none could be associated with a particular individual.

In total, 64 individuals were excavated from the north-eastern area of the monastery including seven skeletons belonging to later burials, intersecting the trench-like mass graves (Fig. S2). Osteological analysis identified 40 adults and 24 subadults (2)**.** The intersections of the trenches with more recent burials and the later constructions of interfering walls have hindered subsequent archaeological excavations, but on the basis of the archaeological analysis (5), the total number of individuals interred in the trenches should be considerably higher than 57, suggesting that a major epidemic event had occurred.

Parchments of Monte Amiata

The gathered data sheds light on the demographic impact of the Black Death on the abbey's region in at least two aspects: (i) it demonstrates the high percentage of people killed by the plague within four months from late June to early September 1348; (ii) it clearly illustrates the long-term consequences of plague, and in particular, the collapse of local populations, which could have been caused by repeated waves of plague. 1348 clearly stands out as the year with the highest impact according to the data at hand. We cannot exclude that the collapse of local populations might have been facilitated by lower levels of fertility, or the relocation of survivors into larger towns and cities after the Black Death. Nevertheless, the large number of victims recovered during the archaeological excavations can only be attributed to a major event, like the one observed in 1348. Within four months (June to early September 1348), the number of death-bed testaments and inferred number of deaths increased dramatically (Fig. S3, Table S2). Non-testamentary contracts, such as land transactions, comprised close to 100% of the documents in most normal years, but are completely absent during the four months of these testaments in 1348. Following these events, the total number of contracts dropped considerably. Moreover, people living in Abbadia San Salvatore were exempt from paying their taxes for the two years after 1348 (6).

Thus, we can conclude that in 1348 a major event took place in the region and that the individuals retrieved in Abbadia San Salvatore and analyzed in this study were the victims of the plague, which ravaged the region between June and September 1348.

Archaeological information on the churches of St Nicolay and St Clement in Oslo

The sampled skeleton SZ14604 (Fig. S4) was discovered during the excavation (7) of a graveyard undertaken as part of the Follo Line Project, a railway construction running through the heart of medieval Oslo. The churches of St Nicolay and St Clement were located immediately to the north and northeast of the excavation area, respectively. The excavated graveyard was situated close to St Nicolay's Church, which had been removed during construction of the Smaalensbanen railway line in the late 1870s (8). It is unknown whether a physical boundary ever separated the churches' graveyards. However, during the archaeological excavation it became apparent that both churches occupied a single plot of land enclosed by a wall and ditch (7).

According to stratigraphic evidence and radiocarbon dating, the first internment took place during the late 11th century. The graveyard expanded throughout the 13–14th century, before contracting back towards its original extent around the first quarter of the 15th century, suggesting a decrease in burial prior to the abandonment of the graveyard. St Nicolay's Church is not mentioned in any preserved written sources after 1461, and may have gone out of use sometime in the mid-15th century, shortly after the burial ceased in the area. This abandonment may have been an after effect of the church's reduced income caused by the Black Death in the mid-1300s (9, 10).

Skeleton OSL1/SZ14604 probably belonged to a male in his 30s or 40s. The palaeopathological examination of the skeletal remains documented discrete bone lesions, which could indicate that the studied individual suffered from arthritis (11). The skeleton was recovered from a shallow grave, that was part of a small concentration of burials, which lay

on the southern extent of the graveyard which also included a foetal burial. Unbaptised foeti are known to have been frequently buried outside the periphery of graveyards and churches in what is often considered unconsecrated ground. The location of skeleton OSL1/SZ14604 may, therefore, reflect the undesirable status of the deceased.

A phalangeal bone (*Pha. Int. manus sin.*) from skeleton OSL1/SZ14604 was radiocarbon dated to AD 1270–1320 AD (60%) / AD 1350–1390 AD (35.4%) (2-sigma, lab. ref. Ua-52762, calibrated using OxCal v3.10) (Table S7). The individual was one of 24 burials which were scattered throughout the graveyard, two of which were double burials, dating from the mid to late 14th century. Thirteen of these burials also produced a date, which could correlate to the first wave of Black Death in Norway. However, of these analysed skeletons, only OSL1/SZ14604 contained evidence for the presence of *Y. pestis*.

At first glance, it would appear that the individual died within the period AD 1270–1320, as there is a 60 % probability of the date being correct, as compared to a 35.4 % chance of the individual dying between AD 1350 and 1390. However, the phylogeny of *Yersinia pestis* identified in the skeleton indicates that the individual died during the first outbreak of the Black Death in Oslo. The precise year of the outbreak is debated (12–14), but it is safe to say, though, that it occurred sometime between 1348 and 1350 (15–17).

The interpretation of radiocarbon dates from human bone is not without issue. The date obtained from an individual can be influenced by differences in the rate of bone formation in the cortical and trabecular bone tissue, which can produce lower or higher radiocarbon values. The age of the individual can also affect the radiocarbon content of the sample. Older individuals display a greater gap between radiocarbon content of their bones and the values consistent with their time of death (18, 19). As the individual died in his 30s or 40s, this dating issue could conceivably push the 2-sigma date outside the Black Death time-scale. However, the identification of the specific *Y. pestis* strain in the skeleton indicates that this is

not the case, and that the 2-sigma date is in fact correct. By using the radiocarbon dates in conjunction with the evidence for the presence of *Y. pestis,* we can perhaps re-evaluate the different hypotheses for the spread of the Black Death in eastern Norway, strengthening the interpretation for a 1350 outbreak.

Sample preparation and aDNA extractions

In addition to previously screened samples (20), we screened six teeth stemming from individual SLC1006 from Saint-Laurent-de-la-Cabrerisse, 41 teeth from a total of 20 individuals from the site of Abbadia San Salvatore, 18 teeth from the site of Oslo (Norway) and three more teeth from three previously published individuals from the site of Bergen op Zoom. Regarding the samples from Abbadia San Salvatore, the skeletons unearthed in the Abbey are stored in a permanent repository at the Soprintendenza Archeologica della Toscana, Laboratorio di Archeo-antropologia, Florence, Italy, and are accessible with permission. The material from Oslo was made available for sampling courtesy of the Museum of Cultural History (University of Oslo) after application to the museum and the Norwegian National Committee for Research Ethics on Human Remains. All necessary permits were obtained for these and further studies, which complied with all relevant regulations.

Lab work was performed at the Paleogenetics Laboratories at the University of Mainz, (Germany) and at the Ancient DNA Laboratory at the University of Oslo (Norway). Both laboratories are solely dedicated to the analysis of ancient samples and are subjected to strict anti-contamination protocols including full overnight UV irradiation. Target enrichment was performed at the post-PCR capture laboratory of CEES, University of Oslo, Oslo.

The teeth were decontaminated, sandblasted and milled to fine powder, as previously described (20). aDNA was extracted using either a formerly published phenol-chloroform protocol (20) or modified versions of silica-based protocols based on Brotherton et al. (21) or

Dabney et al. (22). We used 0.2-0.5g of tooth powder for phenol chloroform extractions and 0.1-0.26 g for the silica based extraction methods. Two samples from Bergen op Zoom (Ber37, Ber45), six samples from Saint-Laurent-de-la-Cabrerisse and 39 samples from Abbadia San Salvatore were extracted by phenol chloroform extraction (Protocol A). Three teeth from Saint-Laurent-de-la-Cabrerisse, two teeth from Abbadia San Salvatore and 18 teeth from Oslo were extracted via silica extraction based on Brotherton et al. (21) (Protocol B), and one tooth from Abbadia San Salvatore based on Dabney et al. (22) (Protocol C). All extractions included negative milling and extraction controls.

Protocol A: extraction as described in Hänsch et al. (20).

Protocol B (modified after Brotherton et al. (21)): 0.1-0.26 g of tooth powder was incubated under rotation, overnight, in 4.31 ml of lysis buffer (0.5 M EDTA, pH 0.8; 0.5% N.-Laurylsarcosine; 0.25 mg/mL Proteinase K). A silica suspension was prepared as detailed in Brotherton et al. (21).

The lysates were pelleted, and supernatants were transferred into a 50ml falcon tube and mixed with 125 µl silica suspension and 16 ml binding buffer (13.5 ml Qiagen QG Buffer and 2.86 ml of solution made up of 1x Triton X100, 20 mM NaCl and 0.2 M acetic acid). The samples were then incubated under rotation for 2 hours at room temperature. Subsequently, the samples were centrifuged for 2 minutes at 13,000 rpm and most of the supernatant was discarded. The silica pellet was transferred into 2 ml safe-lock tubes in the remaining supernatant and pelleted again, prior to discarding the remaining supernatant. The pellets were then washed three times with 1 ml ethanol 80% and dried at 37°C for approx. 30 minutes. In order to avoid cross contamination, bleached and UV-ed cotton mull or aluminum foil was used to cover the open tubes during the drying steps. Dried pellets were eluted in 150 µl of pre-warmed (50°C) Qiagen EB buffer, incubated for 10 minutes on a thermomixer at 37°C, and finally centrifuged for 1 minute at 10,000 rpm. Eluates were kept at -20°C until

further use.

Protocol C (modified after Dabney et al. (22)): 120 mg of tooth powder was incubated under rotation, overnight, in a lysis buffer made up of 0.25 mg/mL Proteinase K and 0.45 M EDTA at 38°C. The lysates were then pelleted and 13 ml binding buffer (6x QG Buffer Qiagen and 4x Isopropanol) was added to the lysis supernatants. The samples were processed on a Qiagen Qiavac Vacuum Manifold using Qiagen MinElute Spin Columns and Zymo-Spin-V 15 ml reservoirs, and washed twice with Qiagen PE Buffer. After a dry spin the samples were eluted in two steps in 50 µl pre-warmed Qiagen EB buffer (50°C).

Screening (PCR/Shotgun sequencing)

All extracts, which had not previously been screened for *Y. pestis* in Hänsch et al. (20), were screened for human and *Y. pestis* DNA using previously published primers: pla YP11D/YP10R as published in Raoult et al. (23), caf1 caf1U2/L2 as published in Hänsch et al. (20) and human mitochondrial HVR1 primers L16209 (24) and H16348 (25). PCR conditions were as described in Hänsch et al. (20). Positive samples were shotgun sequenced on an Illumina HiSeq2500 system (125bp PE) at the Norwegian Sequencing Centre (NSC) at the University of Oslo.

We routinely screened milling blanks and extraction controls using the described bacterial and human mitochondrial primers, and did not detect any signs of contamination. Eluates were kept at -20°C until further use.

Library preparation

Library preparation was done following a modified Meyer and Kircher (26) protocol. We used 10-50 µl of extract to build double stranded, single indexed library products. The following modifications were made to the original protocol: 1) all purification steps were performed using the Qiagen MinElute PCR purification kit with 5x Qiagen PB buffer and one

wash with Qiagen PE Buffer; 2) Following the adapter fill-in step, the samples were incubated at 80°C for 20 min for *Bst* denaturation and were not purified before the indexing PCR setup; 3) 1,25 µM of Adaptermix was used during the adapter ligation step; 4) Amplitaq Gold Polymerase was used for the indexing PCR setup. 40 µl of denatured adapter fill-in reactions were split in three reactions and added to 20 µl of indexing PCR mastermix (1.2x AmpliTaq Gold Buffer, 3 mM MgCl2, 0.05 U/µl AmpliTaq Polymerase, 0.4 mg/mL BSA, 200 µM dNTPs (Qiagen), primer IS4/indexing primer 200 µM, H_20). Via indexing PCR individual 7bp indices were attached to the libraries over 12 cycles.

PCR conditions were the following: initial denaturation at 95° for 6 min followed by 12 cyclers of denaturation step 95°C 40 sec, annealing step 60°C 40 sec, elongation step 72°C 40 sec and a final elongation step at 72°C for 10 min.

Amplified products were purified using commercial kits (Stratec PCRapace or Qiagen MinElute PCR purification kits followed by a AMPureXP beads purification) and subsequently quantified on a Bioanalyzer 2100 expert dsDNA High Sensitivity Chip and using a Qubit HS kit. When necessary, re-amplifications were performed with IS5 and IS6 primers following the original protocol by Meyer and Kircher (26).

Target enrichment

Positive samples, screened via standard PCR and/or shotgun metagenomics, were enriched for *Y. pestis* DNA. Over the course of this study, we used two different custom baits kits from different manufacturers for in-solution target enrichment: MYBaits from MYcroarray and SureSelectXT from Agilent. In both cases, we used RNA probes at 3-5x tiling density.

Bait design A - MYBaits (MYcroarray): We used *Y. pestis* CO92 as a reference genome. Most of the highly repetitive regions and ribosomal DNA regions were excluded from the design. In total 215,512 RNA (100bp) baits were designed based on the chromosomal

assembly of *Y. pestis* CO92 (NCBI accession number NC_003143), 4388 based on plasmid pMT1 (NC_003134), 3197 based on plasmid pCD1 (NC_003131) and 363 based on plasmid pPCP (NC_003132) with 5x tiling density over 20 bp intervals. Some of the baits were moved up- or downstream to reach a smooth overall coverage, especially before and after excluding regions and specific SNP positions.

Bait design B - SureSelectXT (Agilent): For the design of 120 bp RNA probes we used 7 *Y. pestis* strains: strain CO92 (GCF_000009065.1), strain Antiqua (GCF_000013825.1), strain A1122 (GCF_000222975.1), strain KIM10+ (GCF_000006645.1), strain Microtus9001 (GCF_000007885.1), strain Nepal516 (GCF_000013805.1) and strain PestoidesF (GCF_000016445.1). The main design (193,712 baits) was based on *Y. pestis* strain CO92. 3844 baits based on all other listed strains were designed for regions with low identity to or absent from the CO92 reference assembly. Overall, 197,560 baits were designed with 187,297 baits based on the chromosome, 3569 based on plasmid pMT1, 286 based on plasmid pPCP and 2560 based on plasmid pCD1. The designed baits were 120bp long at a 3x tiling density for the plasmids and 5x tiling density for the chromosomal regions.

Libraries selected for target enrichment were first concentrated using SpeedVac to 3.4-7 µl, depending on the protocol used. Ber45, OSL1A and all SLC1006 samples were enriched with MYBaits (1.3.8) according to manufacturer's instructions. DNA and baits were hybridized for 24 hours at 55°C for Ber45 and SLC1006. For library OC1, the hybridization time and temperature were 40 hours and 55°C, and for all other OSL1A libraries hybridization time and temperature were 30 hours and 60°C. Three of SLC1006 libraries (SLC1006b_C4/9, SLC1006c_C5/10 and SLC1006h_C8/11) were re-captured, i.e. after initial enrichment a second round of target enrichment followed (hybridization at 65°C for 24 hours). Bss31d libraries were enriched using an updated version of the MYBaits kit (3.01) half of aliquots were used for MYbaits Baits, and MYBaits Block 1-3 and 2) the hybridization ran at 60°C

for 30 hours for the Bss31d_B.C5 capture reaction and 65°C for 30 hours for the Bss31d_B.C6 and Bss31d_B.C7 capture reactions, which stem from the same library. The Ber37b library was target enriched with the SureSelectXT kit (Agilent; protocol version B4) using probes from baits design B. Since Agilent only provides short blocking oligos that do not cover complete adapter sequences we used Block#1 and Block#3 from MYBaits® kit instead.

After target enrichment, samples were amplified in 2-3 reactions over 10-16 cycles using Herculase II Fusion Polymerase (with annealing temperature set at 60°C), purified with AmpureXP beads and then quantified on a Bioanalyzer 2100 expert chip and Qubit® ds High Sensitivity Assay. Captured products SHC1-8 were measured using Nanodrop 1000. Where necessary, samples were diluted down to 10nM for qPCR and subsequent sequencing.

High throughput sequencing (125bp PE) was performed on an Illumina HiSeq2500 system at the NSC at the University of Oslo. Capture products from Ber45 were pooled on one lane, SLC1006 products were split over two lanes (single capture and double capture products were sequenced separately), Bss31d and Ber37c products were each sequenced and pooled with other samples on different lanes and flow cells.

Heterozygous profile

For each sample, the heterozygous profile was calculated by filtering each vcf file based on the PL tag. This tag represents the normalized phred-scaled likelihood of the possible genotype. The PL field contains three numbers, corresponding to the three possible genotypes:0/0 (homozygous reference), 0/1 (heterozygous), and 1/1 (homozygous altered). The PL of the most likely genotype (assigned in the GT field) is 0 in the Phred scale. The result of this analysis is summarized in Fig. S9 (Table S4). Some modern samples have a high ratio (> 0.3), which could reflect possible mixed infection. These samples were isolated from animal hosts that could be more exposed to plague transmission through fleas. Regarding the

aDNA, only 3 samples, the one from Barcelona and the two samples from Bergen-op-Zoom, have a higher ratio (< 0.35).

Figures

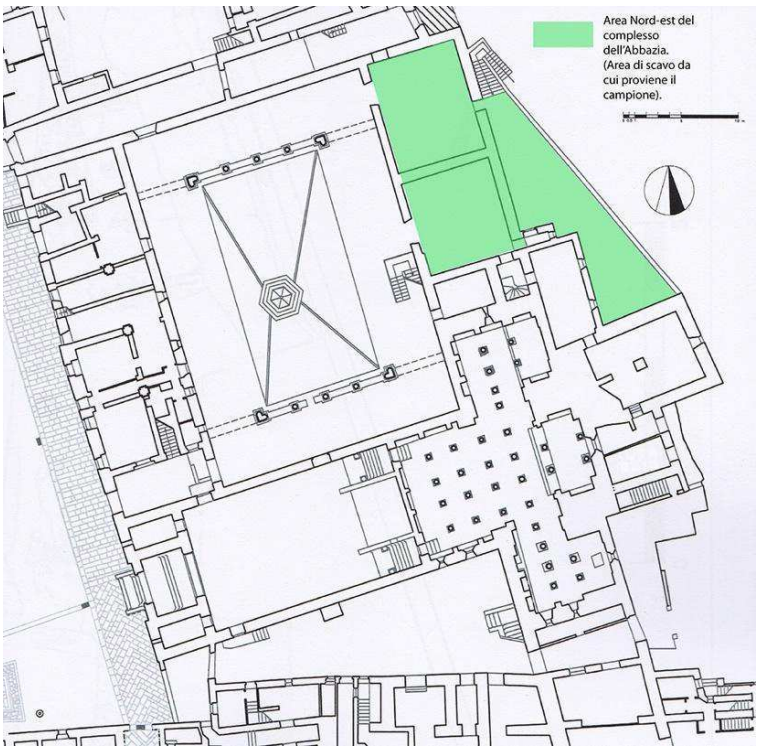

Figure S1. Map of the monastery in San Salvatore. The green area represents the burial area

Figure S2. Two examples of burials excavated at the abbey of San Salvatore, with longitudinal ditches intersected by later depositions.

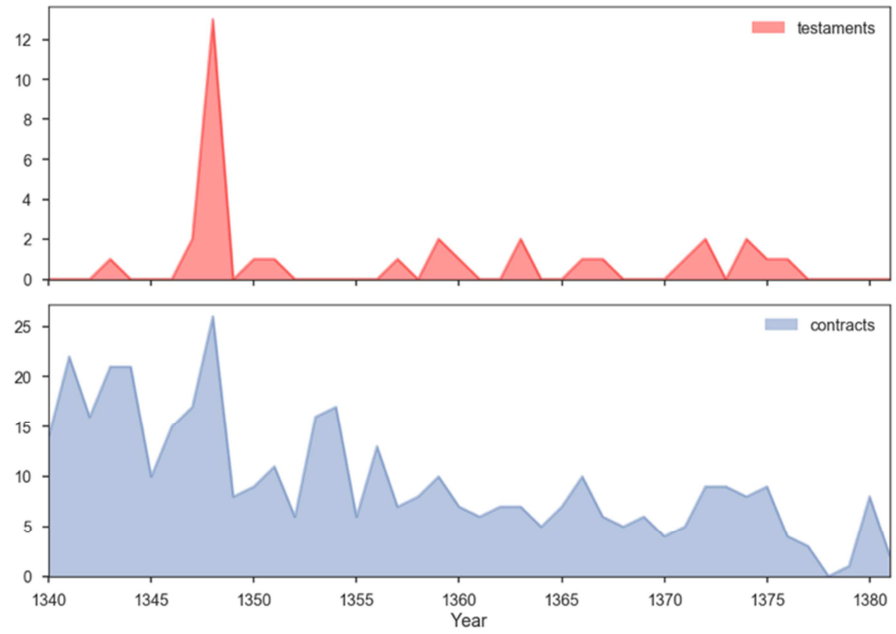

Figure S3. Number of contracts and testaments from 1340 to 1381 from Monte Amiata.

Figure S4. Map of the excavation site (left) where the burial of the skeleton OSL1/SZ14604 (right) was found.

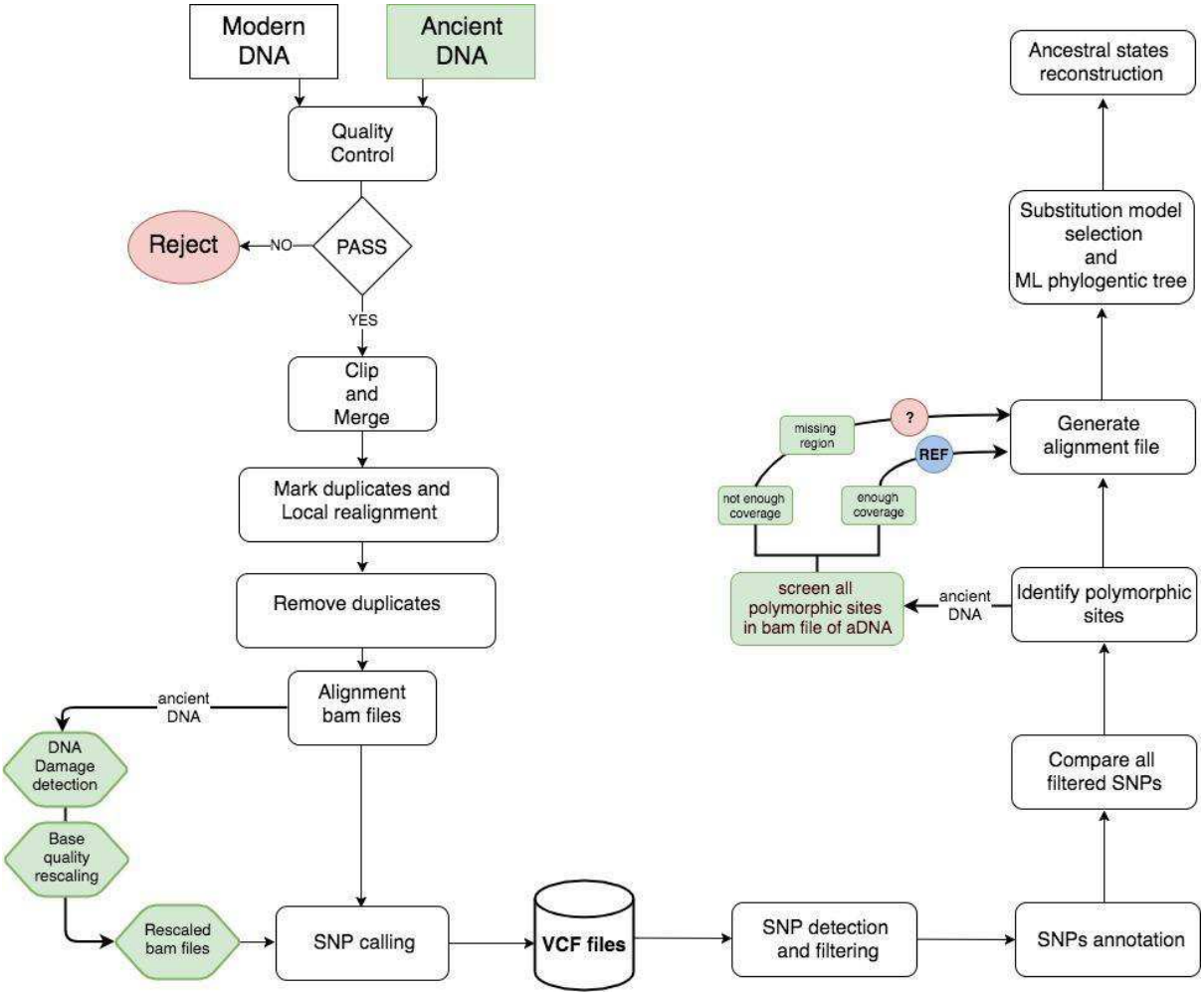

Figure S5. Bioinformatics pipeline.

Schematic workflow showing the different steps from data collection to phylogenetic tree construction applied in this study. Modern and aDNA data were processed through the same pipeline with the exception that DNA damage was estimated on bam files. When necessary, quality scores were rescaled before SNPs calling. "REF" refers to the reference allele and "?" refer to missing region. The final generated alignment file is in fasta format and contains the four nucleotides A, T, C and G in addition to '?' to reflect missing data.

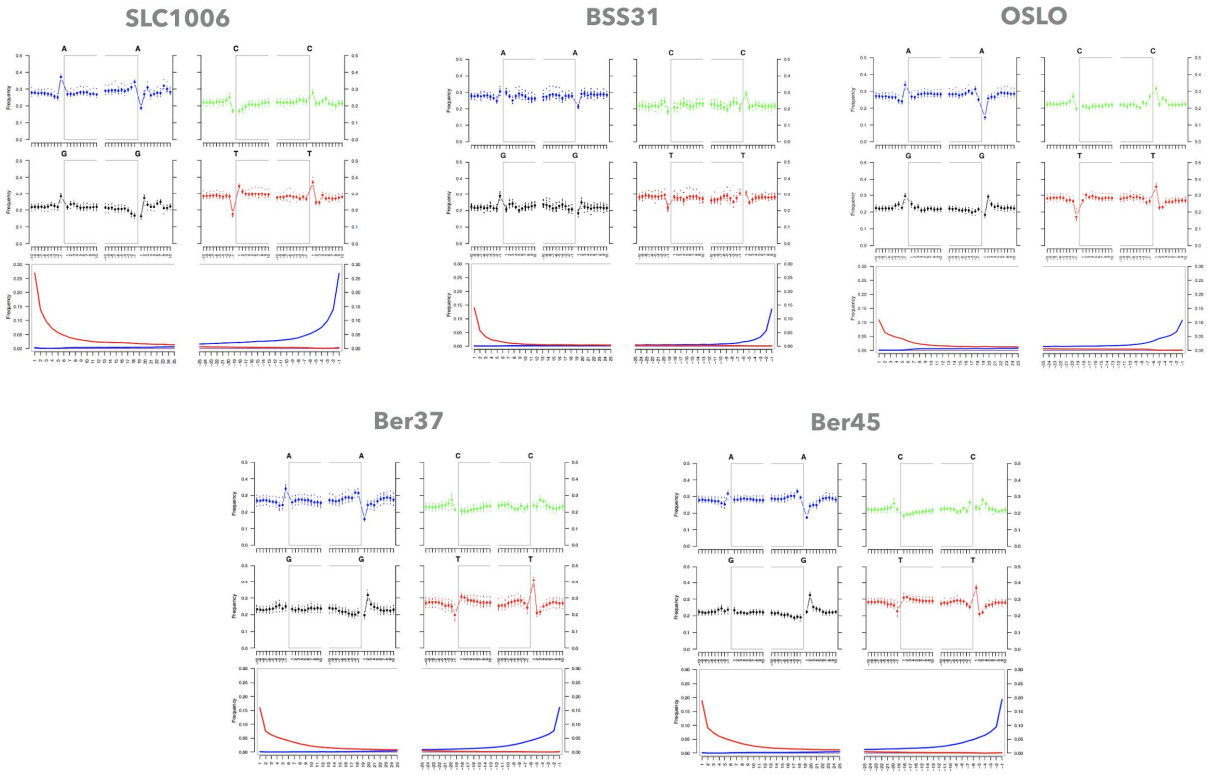

Figure S6. DNA damage profiles.

DNA damage patterns for newly described genomes were generated using MapDamage 2.0 (27). The typical DNA damage patterns from C to T and G to A are reported in red and blue, respectively. The frequencies of all possible mismatches are reported according to the Y. pestis CO92 chromosome.

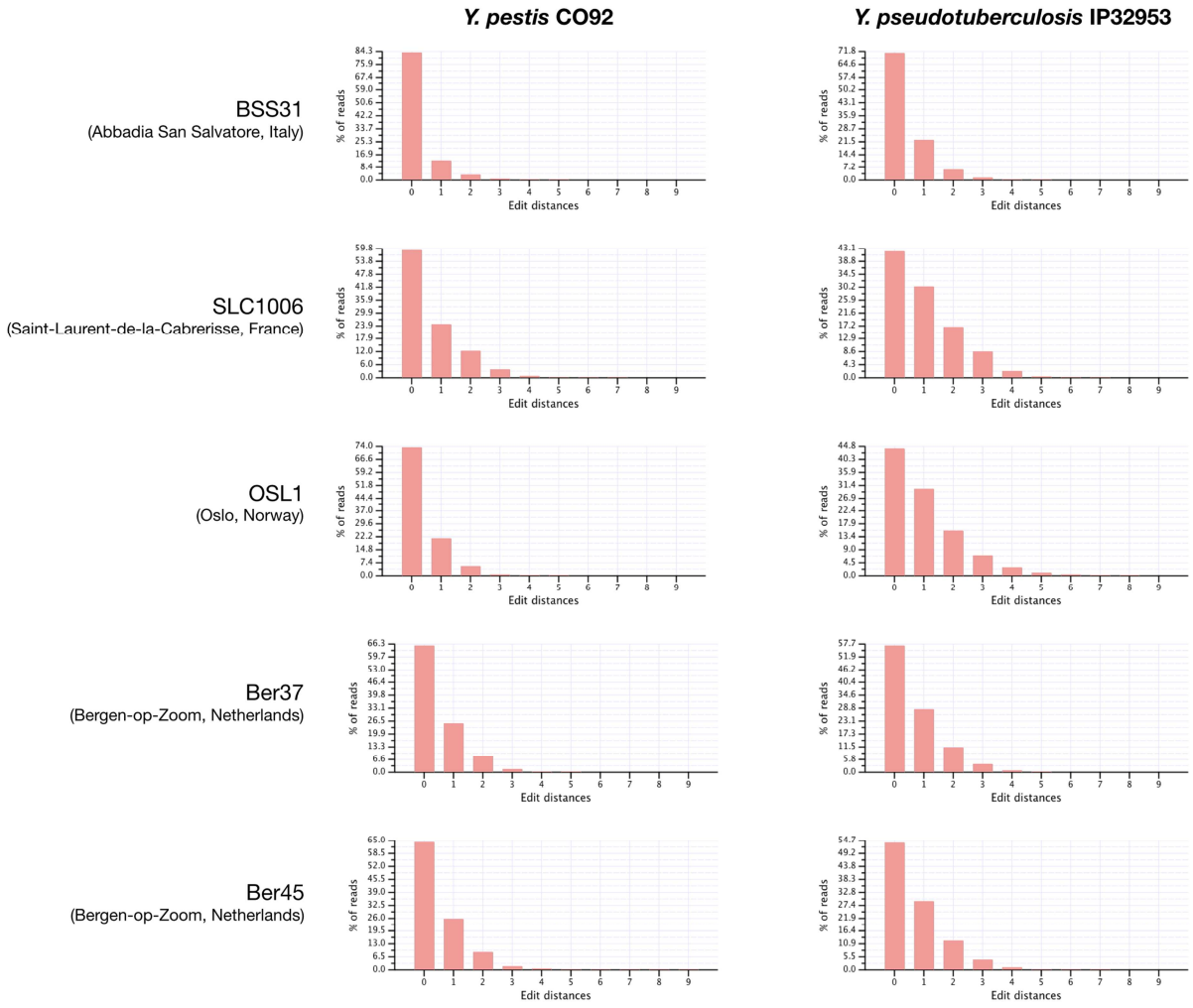

Figure S7. Edit distance. The histograms of each sample dataset show the edit distance and the percentage of reads after alignment to *Y. pestis*, strain CO92 and *Y. pseudotuberculosis*, strain IP32953. For all novel samples reported in this study, the percentage of reads with an edit distance of 0 is higher when aligned to *Y. pestis*.

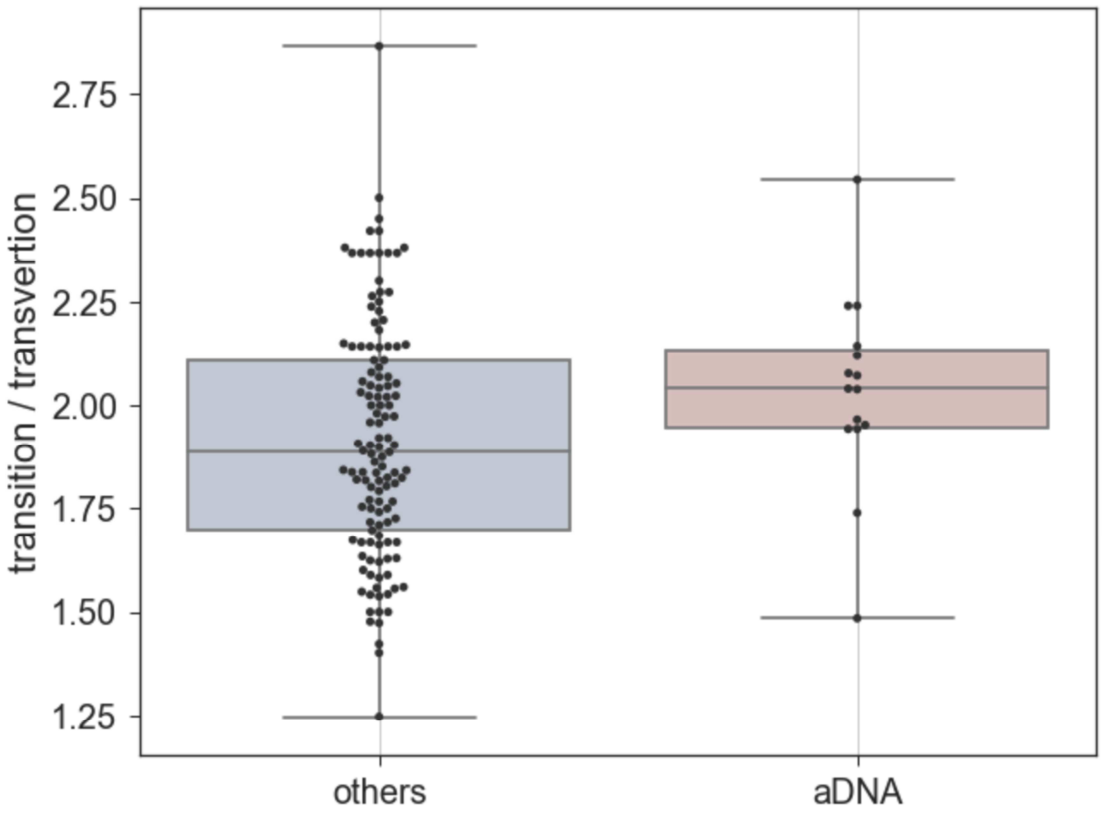

Figure S8. Transition to transversion ratio boxplot. Transition to transversion ratio was calculated for each sample included in this study and indicated by a black dot in the figure. For aDNA samples, the ratios fall into the range of most modern samples.

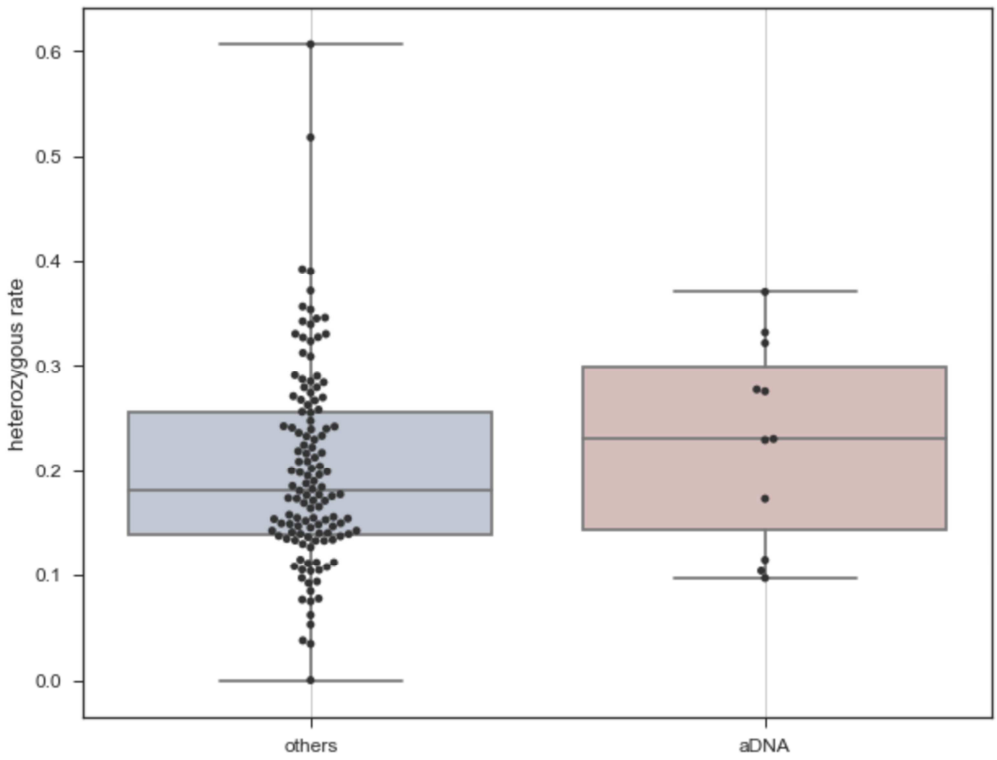

Figure S9. Heterozygous profile boxplot. The heterozygous rate was computed by filtering vcf files using the PL field, which represents the normalized phred-scaled likelihood of the possible genotype. The PL field contains three numbers, corresponding to the three possible genotypes:0/0 (homozygous reference), 0/1 (heterozygous), and 1/1 (homozygous altered). The PL of the most likely genotype (assigned in the GT field) is 0 in the Phred scale. Each sample is represented by a black dot in the figure.

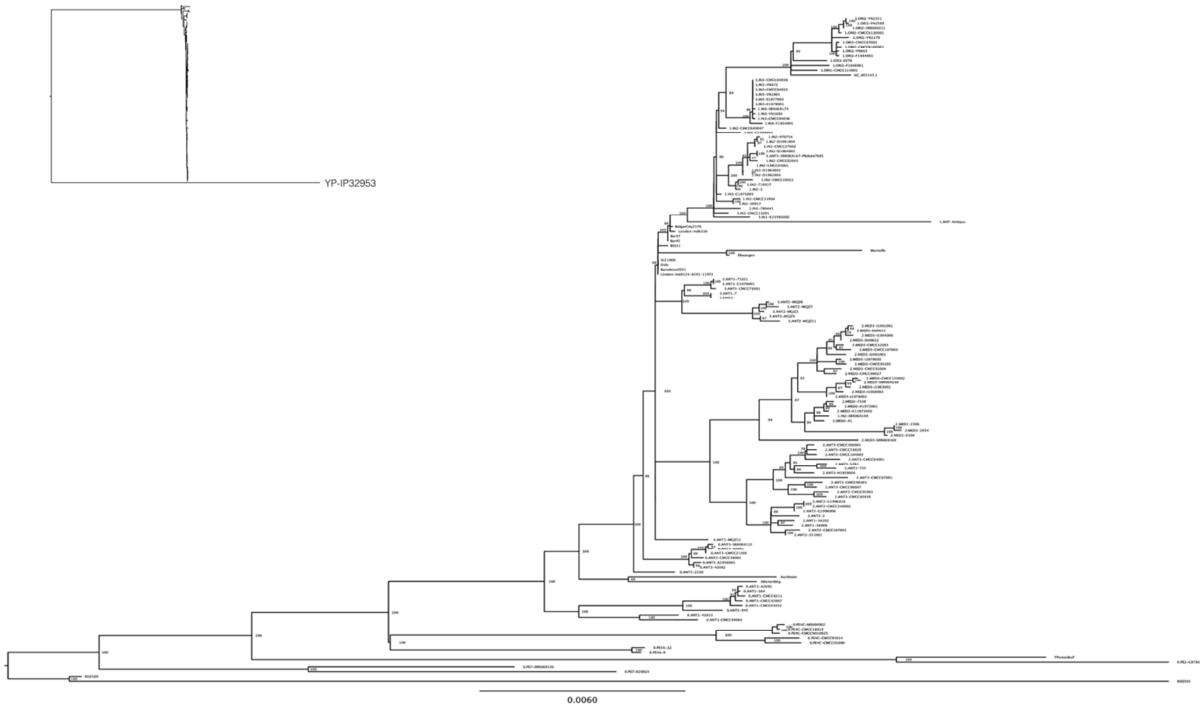

Figure S10. *Y. pestis* phylogeny. The phylogenetic tree was built using a set of 2826 polymorphic sites. The values at each node indicate the bootstrap values at 1000 replicates. The ML tree, was visualized and edited using FigTree (version 1.2.1, http://tree.bio.ed.ac.uk/software/figtree/). The panel on the top left side shows the phylogenetic tree with *Y. pseudotuberculosis*, strain IP-32953, as outgroup.

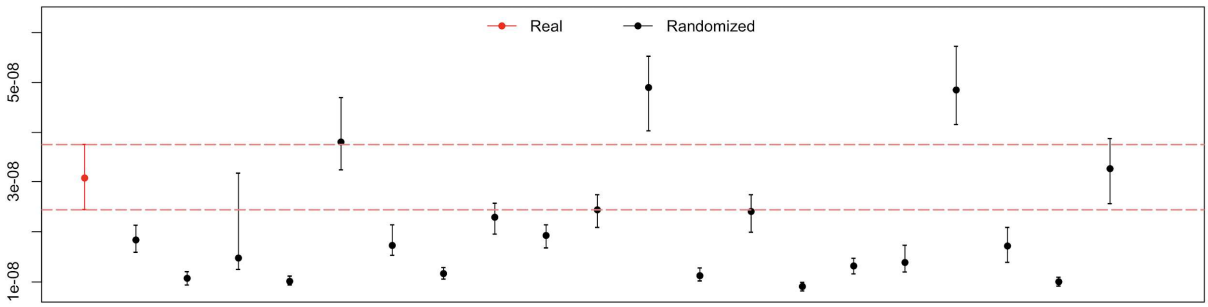

Figure S11. Data randomization test performed on mean substitution rate. Data randomized datasets 3, 5, 8, 10, 13 and 20 are overlapping with the original dataset denoted as "real" in the figure. The DRT test failed to find a temporal signal in the dataset.

Tables

Table S1. List of all samples included in this study and used to build the phylogenetic tree.

Table S2. Contracts and testaments redacted at the Badia di Monte Amiata between 1340 and 1381.

Table S3. Number of mapped reads and fraction of the Y. pestis CO92 genome covered for all analyzed ancient DNA datasets.

Table S4. Heterozygous ratio

Table S5. Lists of polymorphic sites used to build the phylogenetic tree.

Table S6. List of homoplastic sites, including their position and resulting products.

Table S7. C14 dating results for individual OSL1/SZ14604.

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