1   # Supplementary Information
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3   A treponemal genome from an historic plague victim supports a recent emergence of yaws
4   and its presence in 15th century Europe
5

6   Karen Giffin[1]*, Aditya Kumar Lankapalli[1]*, Susanna Sabin[1], Maria A. Spyrou[1], Cosimo Posth[1],
7   Justina Kozakaitė[2], Ronny Friedrich[3], Žydrūnė Miliauskienė[2], Rimantas Jankauskas[2], Alexander
8   Herbig[1], and Kirsten I. Bos[1]
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10   [1]Max Planck Institute for the Science of Human History, Jena, Germany
11   [2] Vilnius University, Vilnius, Lithuania
12   [3] Curt-Engelhorn-Zentrum Archäometrie, Mannheim, Germany

DNA extraction and qPCR Screening

Sampling, DNA extraction, qPCR reaction preparation, library preparation and indexing reaction preparation were performed in the ancient DNA clean lab facilities of the Max Planck Institute for the Science of Human History. All amplifications were carried out in the modern DNA lab facilities of this institution.

One tooth from each of 26 individuals was sectioned, and the pulp chamber was drilled using a Dremel tool at low setting affixed with a grout-cutting bit. DNA was extracted from ca. 50 mg of powder using a protocol designed to increase the recovery of short fragments, which are typical of ancient DNA[1]. The extraction protocol was modified as per Spyrou et al., 2019. Two extraction blanks were analyzed along with each set of 10 samples, the first as a reagent blank, and the second as a process blank. An inhibition test[2] was performed to rule out the presence of PCR-inhibiting components by spiking 2 µL of sample extract into Illumina library qPCR reactions, with known amounts of standard template molecules, to record deviations from the anticipated amplification behaviour. No inhibition was detected via this method. Reaction chemistry for the *pla* qPCR assay for detection of *Y. pestis* PCP1 plasmid DNA was performed without deviation[3]. Results of the *pla* assay can be found in Figures S1 and S2, and Table S1.

Library preparation and shotgun sequencing

i) Library preparation and sequencing
Double-stranded Illumina libraries were prepared for all 26 individuals and their associated negative controls using 10 µL of DNA extract following established protocols[4]. Additionally, for the 6 putative positive plague samples, double-stranded libraries were prepared using 40 µL of DNA extract to increase template. Prior to adaptor ligation, each enriched library was treated with uracil-DNA-glycosylase (UDG) and Endonuclease VIII (New England Biolabs) to repair damaged DNA bases[5,6]. Two library blanks were analyzed along with each set of samples, the first a reagent blank, and the second a process blank. Quantification of libraries, indexing, and quantifications of the indexed libraries were performed as per Spyrou et al., 2019. The libraries of the UDG and nonUDG samples and blanks were amplified to individual concentrations of approximately $1.3*10^{13}$ DNA copies/uL using Herculase II Fusion DNA Polymerase (Agilent), purified as per Spyrou et al. (2019). Samples and blanks were pooled separately to total concentrations of approximately 10nM for Illumina shotgun sequencing. Concentrations were measured on a TapeStation 4200 (Agilent). Aliquots of the amplified UDG and nonUDG libraries for the 6 putative plague positives and their associated blanks were further amplified for capture to a concentration of 200-400 ng/µL as per Spyrou et al. (2019). Concentrations of capture libraries were measured on a Nanodrop 8000 (Thermo Scientific). In-solution *Y. pestis* capture was

54 performed as previously described[7]. Samples were captured in individual wells, but blanks
55 were pooled and captured together separately from the samples.

56

57 Shotgun libraries and capture products were sequenced to a depth of approximately 10
58 million reads on an Illumina HiSeq4000 using a 50 bp paired-end kit. Screening and captured
59 blanks were sequenced to a depth of approximately 2 million on an Illumina NextSeq500 on
60 either a 75 bp paired-end mid-output kit or on a 75bp single-read kit, except for the *T.
61 pallidum* capture blanks for the first tooth from AGU007, which were sequenced to a depth
62 of 2 million reads on an Illumina HiSeq4000 using a 75bp single-read kit.

63

64 *ii) Illumina read processing*
65 Sequenced shotgun libraries and blanks were demultiplexed and mapped to the human
66 genome (hg19) in EAGER v1.92 to evaluate aDNA preservation[8]. In EAGER, reads had the
67 adapters clipped, and paired reads were merged using AdapterRemoval v2.2[9]. Reads were
68 filtered for a length of ≥ 30 bp and a minimum base quality of 20. Mapping was performed
69 by BWA version 0.7.12 with a seed length (-*l*) of 32 and a mapping stringency setting of 0.01
70 (-*n*). We removed read alignments with a mapping quality (-*q*) below 20 using SAMtools
71 (http://samtools.sourceforge.net/). Duplicates were removed with MarkDuplicates
72 (http://broadinstitute.github.io/picard/) and mapDamage v2.0 was used to examine DNA
73 damage[10]. The 26 samples had 373 – 1,090,755 DNA fragments mapping to hg19 after
74 quality filtering and duplicate removal, and % endogenous DNA ranging from 0.01 to 32.61
75 (Table S2). Negative controls showed between 90 and 24,782 fragments mapping to hg19
76 after quality filtering (Table S2).

77

78 Shotgun libraries for samples and blanks, both UDG and nonUDG, were mapped in EAGER
79 against the CO92 reference genome for *Y. pestis* (NC_003143.1) as a verification of the qPCR
80 screening results[11]. EAGER was applied as described above, however, the BWA mapping
81 parameters were -*l* = 16, -*n* = 0.01 and read alignments with a mapping quality below 37
82 were removed. Results are presented in Table S3.

83

84 *iii) qPCR re-evaluation*
85 On account of the unexpectedly high number of mapping *Y. pestis* fragments in individual
86 AGU007, re-examination of the qPCR assay results revealed that AGU007 had a signal that
87 had been initially dismissed. AGU007's amplification curve indicated a quantity below the
88 lowest standard of 0.2 copies/μL (Figure S1, Table S1), and the melting peak signal was also
89 very low as compared to the amplification standards and the other samples (Figure S2).

90
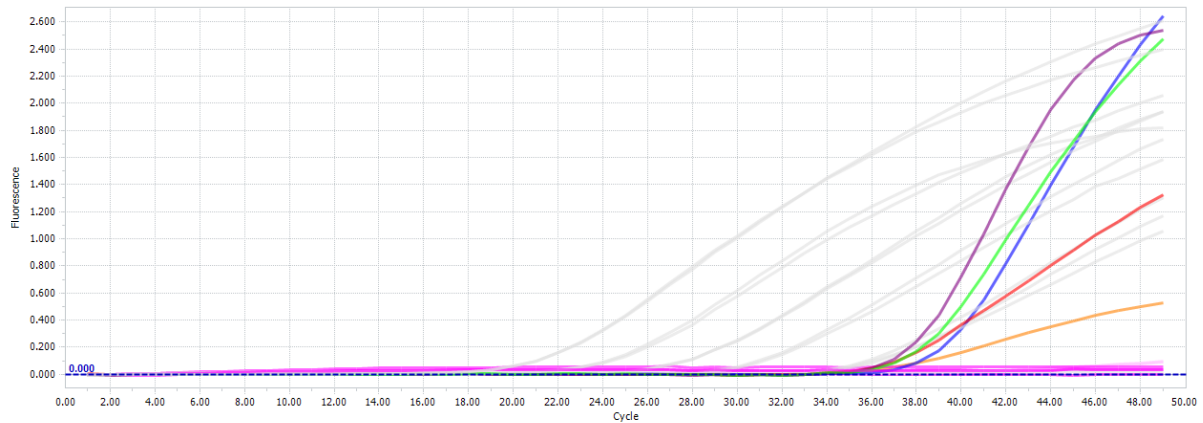
91
92
Figure S1 – Amplification curves of pla Assay. Curves in grey are *pla* standards. Purple, green, blue, red and orange curves are AGU010, AGU009, AGU013, AGU003 and AGU007, respectively. Extraction and qPCR blanks are shown in pink.
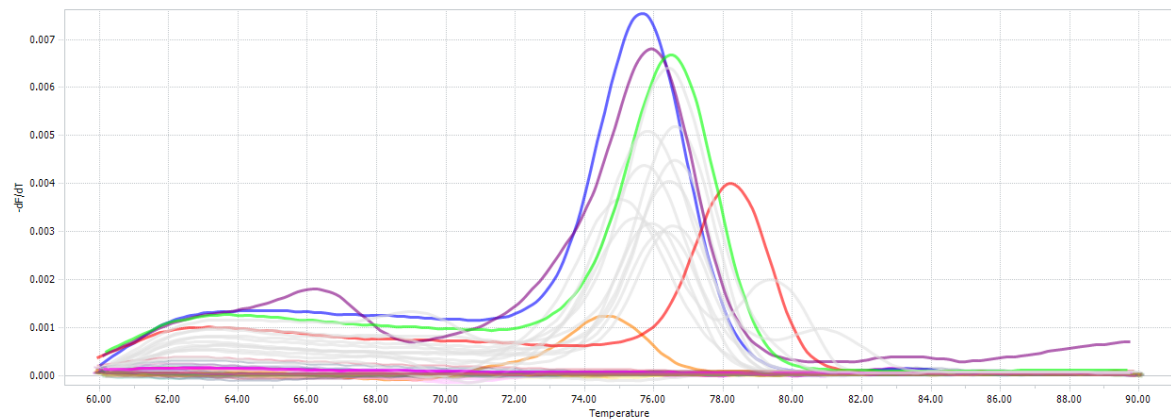
98
99
Figure S2 – Melting peaks of pla Assay. Curves in grey are *pla* standards. Purple, green, blue, red and orange curves are AGU010, AGU009, AGU013, AGU003 and AGU007, respectively. Extraction and qPCR blanks are shown in pink.

A UDG library from 40 µL of extract was prepared for AGU007 and was shotgun sequenced on an Illumina HiSeq 4000 with a 75bp single end kit. Mapping to hg19 and *Y. pestis* was carried out as previously described, though with more stringent parameters (-*l* 32, -*n* 0.1, -*q* 37) for the *Y. pestis* mapping (Table S3).

Pathogen Screening in HOPS

Libraries were screened for the presence of ancient pathogens using the MEGAN Alignment Tool (MALT) as part of the Heuristic Operations for Pathogen Screening (HOPS) pipeline[12,13]. The MALT database used for screening was constructed from a custom RefSeq Genome set in November 2017 that contained bacteria, viruses, and eukaryotes (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/). *Y. pestis* signals were observed in the nonUDG library of AGU007, and in both the UDG-treated and untreated libraries for

AGU010, AGU020 and AGU025. Contrary to the qPCR assay, no *Y. pestis* signals were observed in either the UDG-treated or untreated libraries for AGU003, AGU009, and AGU013, indicating these were likely false positives.

Aside from *Y. pestis*, numerous reads were assigned to other pathogenic taxa, including oral microorganisms (Figure S3). Verification of these signals would require targeted enrichment for species and strains of the relevant taxa, and was not pursued here.

In addition to *Y. pestis*, the UDG library for AGU007 also demonstrated a signal for *Treponema pallidum* that had not been observed in the initial screening of the nonUDG library. Closer examination of the MALT data of the nonUDG library disclosed the presence of 7 fragments matching to *T. pallidum* and 11 fragments matching to the *Treponema* genus (Figure S3). This was not reported as positive identification by HOPS since the level of damage in the reads precluded proper evaluation of taxon assignment.

Organism	AGU007.A		AGU010.A		AGU020.A		AGU025.A	
	nonUDG	UDG	nonUDG	UDG	nonUDG	UDG	nonUDG	UDG
Yersinia pseudotuberculosis complex	90	83	29	30	36	38	30	26
Yersinia pestis	19	17	9	10	8	8	9	7
Tannerella forsythia							1055	1602
Treponema pallidum pallidum	7	12						
Treponema	11	21						18
Streptococcus anginosus							4	
Streptcoccus agalactiae							2	
Streptococcus							15	
Schistosoma mansoni							2	
Salmonella enterica enterica								9
Porphyromonas gingivalis							118	159
Parvimonas micra							160	226
Neisseria						18		11
Mycobacterium tuberculosis complex					5			
Mycobacterium leprae					4			
Mycobacterium intracellulare	26							
Escherichia coli			260	228				
Enterobius vermicularis		115		13				
Clostridium botulinum BKT015925							5	
Borreliaceae								2

Figure S3 – Pathogen screening data produced in HOPS from the putative *Y. pestis* positive screening libraries. Numbers in each cell correspond to assigned reads. Cell fill colour indicates degree of taxon assignment evaluation: gold pass the set edit distance filter, green pass this filter and also have terminal C to T damage, and blue shows that reads with terminal C to T damage pass the edit distance filter. Grey indicates no positive taxon assignment evaluation in HOPS.

Both the untreated and UDG-treated libraries were mapped in EAGER against the Nichols syphilis reference genome (NC_021490.2) using the same programs and parameters as previously described for mapping to CO92[14]. The untreated library was found to contain 27 mapping fragments after duplicate removal and quality filtering and the UDG-treated library was determined to have 36 mapping fragments following duplicate removal and quality

143   filtering (Table S11). The other 25 AGU samples had mapping fragment numbers ranging
144   from 0-8 after duplicate removal and quality filtering. Screening blanks were also mapped in
145   EAGER using screening library parameters and programs, and were found to have 0-3
146   fragments mapping to Nichols (NC_021490.2) after duplicate removal and quality filtering,
147   consistent with the other samples found to be negative via screening.
148
149   For targeted enrichment of *Treponema pallidum*, DNA probes were designed on the basis of
150   *Treponema pallidum* subsp. *pallidum* strains Nichols (NC_000919.1), SS14 (NC_021508.1),
151   Sea 81-4 (NZ_CP003679.1), Mexico A (NC_018722.1), *T. pallidum* subsp. *endemicum* strain
152   Bosnia A (NZ_CP007548.1), and *T. pallidum* subsp. *pertenue* strain Fribourg-Blanc
153   (NC_021179.1). The probes were designed with a 1bp tiling and a length of 52 bp with an
154   additional 8bp linker sequence (CACTGCGG) as described in Fu et al. (2013)[15]. Duplicated
155   probes and probes with low sequence complexity were removed. This resulted in 1,125,985
156   unique probe sequences. This probe set was spread on two Agilent one-million feature
157   SureSelect DNA Capture Arrays. The capacity of the two arrays was filled by randomly
158   duplicating probes from the probe set. The arrays were turned into an in-solution DNA
159   capture library as described elsewhere[15].
160
161   The treated and untreated libraries for AGU007 and its associated blanks were then in-
162   solution captured for whole genomic *Treponema pallidum* DNA using the capture library
163   described above. The *T. pallidum*-captured untreated and UDG-treated libraries were
164   captured in individual wells and the associated blanks were pooled and captured separately
165   from the samples.
166
167   **Mapping of captured products for *Y. pestis* and *T. pallidum* (first tooth)**
168
169   Reads from *Y. pestis*-captured samples were mapped in EAGER against the CO92 reference
170   genome (NC_003143.1)[11] with stringent BWA mapping parameters (-*l* 32, -*n* 0.1, -*q* 37).
171   Samples had 119 – 156,123 fragments mapping to CO92 after quality filtering and duplicate
172   removal, with mean genomic coverages ranging from 0 to 1.98-fold (Table S4 and S5).
173   Untreated libraries showed expected damage patterns (Figure S4). Samples AGU003,
174   AGU009 and AGU013 had mean genomic coverages of 0 – 0.01-fold following capture,
175   supporting the negative MALT/HOPS results. Samples AGU010, AGU020 and AGU025 had
176   low mean genomic coverages ranging from 0.28 to 0.77-fold. Captured blanks had between
177   5 and 90 mapping fragments, with duplication (cluster) factors ranging from 2.3 to 2645.7,
178   indicating that complexity of the library had been adequately explored. These numbers are
179   consistent with previously reported *Y. pestis* capture blank data in Bos et al. 2016.
180
181   The *T. pallidum*-captured untreated and UDG-treated libraries for AGU007 were sequenced
182   on a HiSeq4000 to a depth of approximately 40 million reads with a 75 bp paired-end kit.
183   The samples were mapped in EAGER to the Nichols genome (NC_021490.2) with the same

184 parameters as the *Y. pestis* mapping. The libraries had 31,882 and 126,552 mapping
185 fragments, respectively, following duplicate removal and quality filtering (Table S12). The
186 mean coverages of the untreated and UDG-treated libraries were 1.78X and 6.67X,
187 respectively, with the untreated library displaying the expected aDNA damage pattern
188 (Figure S5). The UDG-treated library covered 96.1% of the genome at 1X and 76.1% at 5X.
189 Captured blanks, sequenced on a HiSeq4000 with a 75bp single read kit, had 4 or fewer
190 reads mapping to the Nichols genome after quality filtering.
191
192
193 **Processing and analysis of Second Set of Teeth from AGU**
194
195 Further data analysis awaited the arrival of the second set of teeth: one each from
196 individuals AGU007, AGU010, AGU020 and AGU025. Following arrival, sampling, DNA
197 extraction, library preparation, indexing, amplification, and *Y. pestis* and *T. pallidum* capture
198 protocols were followed as previously described. No qPCR screening was performed for
199 these samples. Remaining aliquots of unindexed AGU010 and AGU020 UDG-treated libraries
200 from the first tooth were indexed and captured as part of the same batch following the
201 same processes. Untreated and UDG-treated screening libraries were sequenced on
202 separate runs on a HiSeq4000 using single-end 75 bp kits. *Y. pestis*-captured libraries were
203 paired end sequenced to a depth of 20 million reads on a NextSeq500 using a 75bp mid
204 output kit and *T. pallidum*-captured libraries were sequenced to a depth of approximately
205 40 million on a HiSeq4000 using a 75 bp paired-end kit. All negative controls were
206 sequenced to a depth of approximately 2 million on separate NextSeq500 runs using 75 bp
207 mid-output paired end kits.
208
209 Mapping of the untreated and UDG-treated libraries of the tooth samples to hg19 was
210 performed following the same procedures described previously. Shotgun sequencing
211 yielded endogenous DNA contents of 0.07-59%, which corresponded to 6,381 – 6,986,467
212 mapping fragments after duplicate removal and quality filtering (Table S6). Both AGU007
213 and AGU010 had better human DNA preservation in the second tooth, but the opposite was
214 observed for AGU020 and 025. Negative controls showed between 683 and 6,886 fragments
215 mapping to hg19 after quality filtering. Mapping of the untreated and UDG-treated shotgun
216 libraries to the *Y. pestis* CO92 genome was also performed, yielding 22 – 6,394 mapping
217 fragments following duplicate removal and quality filtering (Table S7). UDG-treated shotgun
218 libraries were mapped using more stringent parameters than untreated shotgun libraries (-*l*
219 32, -*n* 0.1 and -*q* 37, and -*l* 16, -*n* 0.01 and -*q* 37, respectively). Negative controls had 11 or
220 fewer fragments mapping after quality filtering.
221
222 Mapping of shotgun libraries from the second set of teeth against the Nichols genome
223 (NC_021490.2) yielded 1 – 181 fragments following duplicate removal and quality filtering
224 (Table S13). Mapping parameters used were the same as for the shotgun library mapping to

225 *Y. pestis*. Blanks showed no indication of the presence of *T. pallidum* DNA after quality
226 filtering.

228 Shotgun libraries for these second teeth were all screened using MALT/HOPS. As expected
229 both *T. pallidum* and *Y. pestis* were detected in the UDG-treated and untreated libraries for
230 AGU007. *Y. pestis* was detected in AGU010, AGU020 and AGU025, though for AGU020 it
231 was detected in only the UDG-treated library, suggesting that *Y. pestis* DNA levels were
232 minimal.

234 In-solution *Y. pestis* capture was performed on all libraries and their associated blanks using
235 previously described methods[7]. Libraries for AGU007 and its blanks were also captured for
236 *T. pallidum* as described above. The captures for both *Y. pestis* and *T. pallidum* were initially
237 sequenced together on an Illumina HiSeq4000. The *Y. pestis* captures were subsequently re-
238 sequenced separately on an Illumina NextSeq500.

240 Mapping of the *Y. pestis*-captured samples and blanks in EAGER against CO92 yielded 3978 –
241 2,344,695 mapping fragments and mean coverages of 0.04X to 38.01X after duplicate
242 removal and quality filtering (Table S8). Captured nonUDG libraries showed the expected
243 ancient DNA damage pattern (Figure S4). AGU020 had the lowest coverage, 0.04X and 0.09X
244 for its untreated and UDG-treated libraries, respectively. By contrast, the UDG treated
245 AGU007 had a mean coverage of 38.01-fold with 93.7% of the genome covered at 5-fold.
246 AGU010 had a mean coverage of 20.76-fold with 92.0% of the genome covered at 5-fold.
247 AGU025.B had a mean coverage of 14.36-fold with 92.1% of the genome covered at 5-fold.
248 For maximum coverage, all available sequencing data for the first tooth of AGU020 from the
249 UDG library aliquots of both captures were merged. This resulted in a coverage of 1.68-fold.
250 *Y. pestis*-captured blanks had 3-24 fragments mapping to *Y. pestis* after duplicate removal
251 and quality filtering, with cluster factors between 1 and 1092.

253 Verification of the presence of *Y. pestis* plasmids pMT1 and pCD1 was also performed.
254 Mapping in EAGER using previous stringent parameters yielded coverages for the pMT1
255 from 4.02 to 63.75-fold, with 6284 to 79,105 fragments mapping after quality filtering. As
256 expected, the lowest values were observed for AGU020 and the highest for AGU007 Table
257 S9). No more than 4 uniquely mapping fragments were observed in the captured negative
258 controls. Mapping to pCD1 displayed similar results with the lowest values found in
259 AGU020, with 6983 unique mapping fragments and 6.08-fold coverage, and the highest in
260 AGU007, with 68,941 uniquely mapping fragments covering the pCD1 at 80.90-fold (Table
261 S10). No uniquely mapping fragments were found in the negative controls.

263 The *T. pallidum*-captured libraries of AGU007 and its associated blanks were mapped in
264 EAGER against Nichols (NC_021490.2) using the same parameters and programs as the *Y.*
265 *pestis* captures. AGU007 had 146,398 – 330,505 fragments mapping after duplicate removal

266 and quality filtering, mean coverages of 9.3 to 21.3-fold and 92.1% to 97.6% of the genome
267 covered at 5-fold (Table S14). The nonUDG library showed the expected ancient DNA
268 damage pattern (Figure S5). One mapping fragment was detected in three of the captured
269 blanks after quality filtering. Each mapping fragment was analyzed using BLASTn and found
270 to map to regions conserved in multiple bacterial taxa. None of the top 10 matches were to
271 *T. pallidum.*

272
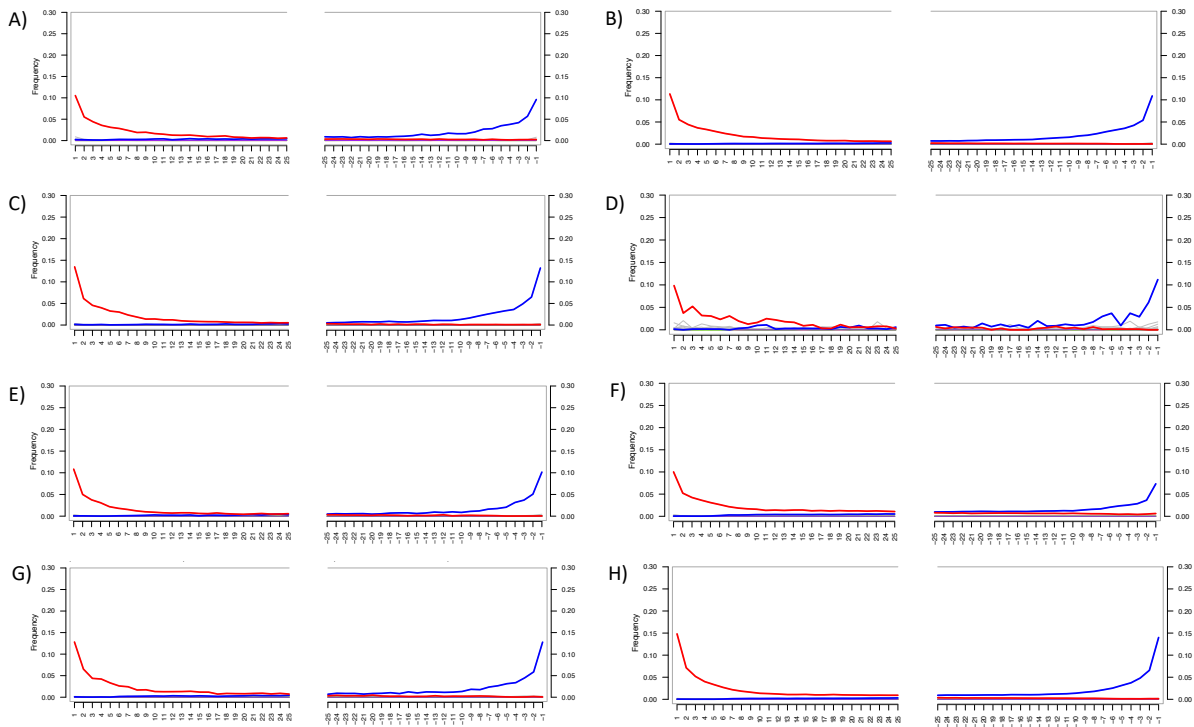
273
274 Figure S4 – *Y. pestis* DNA damage plots, generated by Mapdamage 2.0 in the EAGER pipeline, of captured
275 libraries of A) AGU010 tooth 1, B) AGU010 tooth 2, C) AGU020 tooth 1, D) AGU020 tooth 2, E) AGU025 tooth 1,
276 F) AGU025 tooth 2, G) AGU007 tooth 1 and H) AGU007 tooth 2.

277
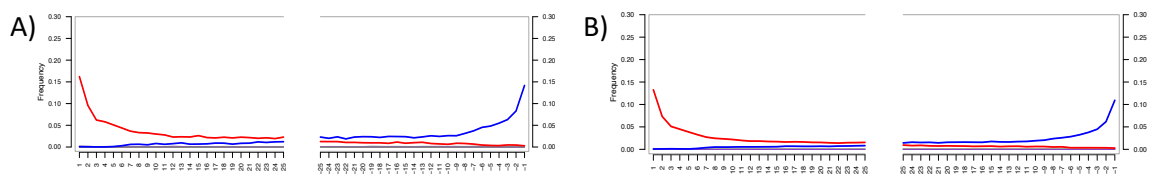
278
279 Figure S5 – *T. pallidum* DNA damage plots for captured libraries of A) AGU007 tooth 1 and B) AGU007 tooth 2.

280
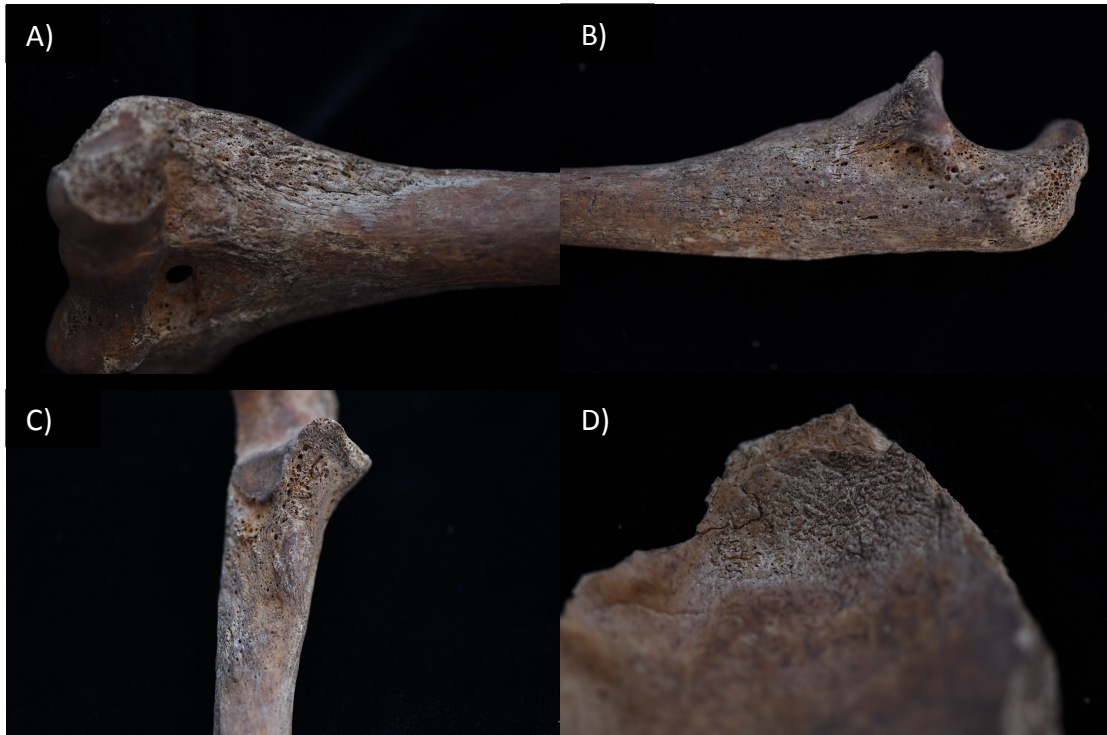
281

282
283   Figure S6 - Paleopathological Features of the skeleton of AGU007, A) right humerus, B) lateral view of right
284   ulna, C) anterior view of the olecranon process of the right ulna, D) parietal bone (photographs courtesy of
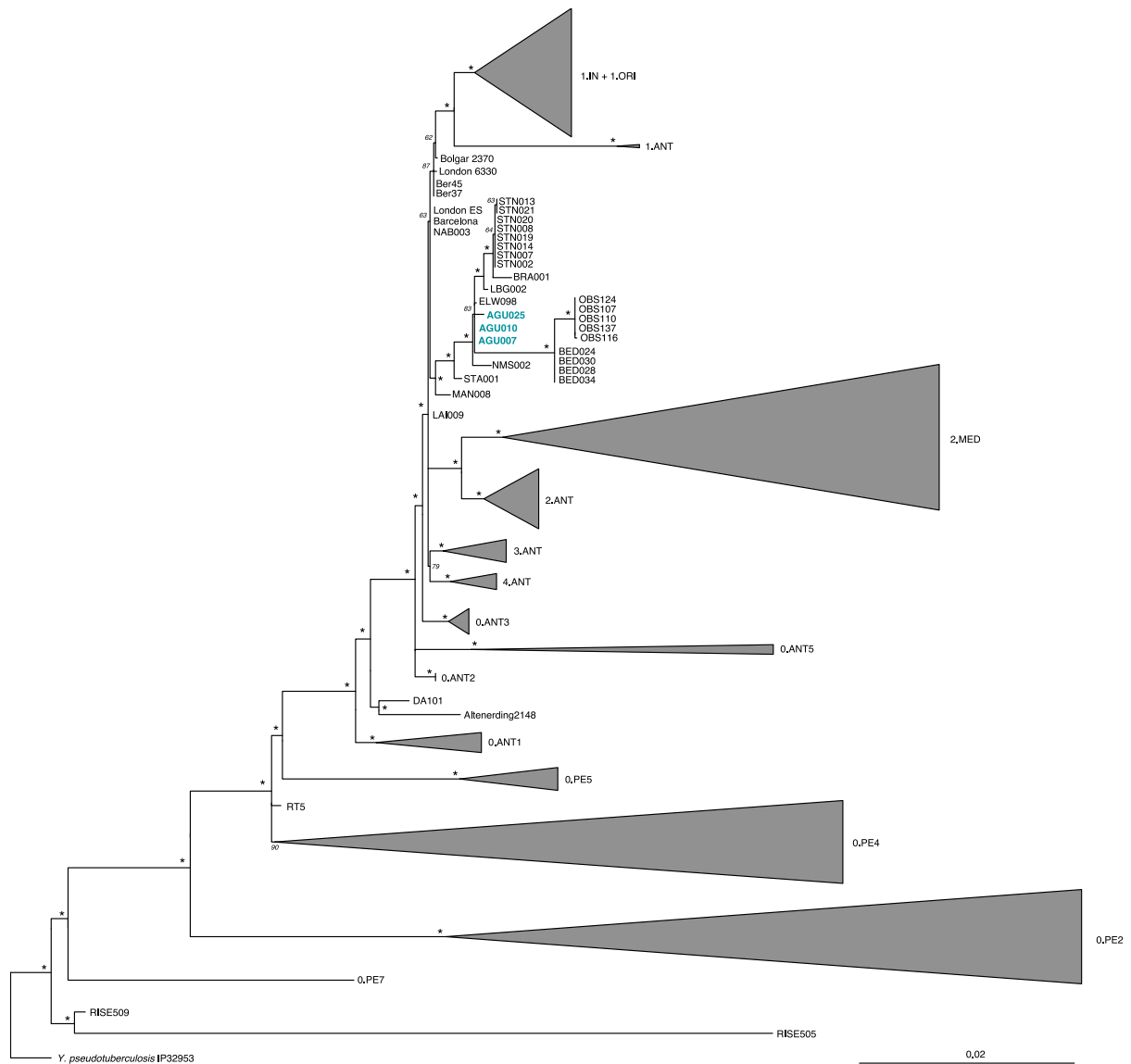285   Justina Kozakaitė).
286

287   **Phylogenetic Analysis of *Yersinia pestis***

288   Phylogenetic assessment was made following methods described in the main manuscript
289   with a genome dataset defined in Table S15. *Y. pseudotuberculosis* was used as the
290   outgroup. A full phylogeny is presented in figure S7, and a zoomed in phylogeny of the
291   post-Black Death lineages only is shown in Figure 4.

292

Figure S7: Maximum likelihood tree of post-Black Death genomes of *Y. pestis*. Constructed from 275 genomes with the Generalised Time Reversible (GTR) model, 1000 bootstrap replicates, and a 98% partial deletion filter (considering 5801 SNPs). Bootstrap values of 95 or greater are indicated with an asterisk (*). Scale denotes substitutions per site.

Phylogenetic Analysis of *Treponema pallidum pertenue*

Sequencing reads for 70 samples from Arora *et al.*, 2016[16], 8 samples from Marks *et al.* 2018[17], and 8 samples from Knauf *et al.*, 2017[18] were analysed along with simulated reads generated from complete *T. pallidum* genomes downloaded from NCBI of 1st April,2019 (Table S19). Each simulated read is 100bp long with 1bp tiling to the successive read. Adapters of sequenced reads were trimmed and poor-quality reads were filtered. Overlapping reads were merged and processed in EAGER version 1.92.58[19]. For AGU007,

raw reads from the first and second rounds of capture were filtered for sequencing quality independently. The resulting *fastq* reads were combined to form the raw data for the AGU007 merged dataset. These reads were mapped against reference genome *Treponema pallidum* subsp. *pallidum* Nichols (NC_021490.2) with mapping quality of 37, seed length of 32 with 0.1 fraction of missing alignment against the reference. Duplicated reads with the same 5' end, but of lower quality were excluded using MarkDuplicates (http://broadinstitute.github.io/picard/). Variants were called using GATK[20]. Using a threshold of five-fold mean coverage, 33 genomes covering 90 percent of the reference Nichols genome covered were of sufficient quality to be carried forward in our analysis, and their vcf files were combined and compared for downstream analysis. Variant positions were identified from a set of 2 *pallidum* genomes, 2 *endemicum* genomes and 25 *pertenue* genomes (Table S20) using MultiVCFAnalyser (https://github.com/alexherbig/MultiVCFAnalyzer). SNP calling was based on a minimum coverage of five reads with a minimal mapping quality of 30 and at least 90 percent of the reads supporting the major allele; failure to meet this threshold resulted in assignment of an ambiguous base 'N' for both variant or invariant alleles with respect to the Nichols reference genome. Full genome alignment for these 29 genomes with respect to the Nichols reference and the SNP alignment from concatenated variant positions were generated. A phylogenetic tree was built in RAxML[21], using the maximum likelihood approach with a GTR+GAMMA substitution model and eight gamma categories of evolutionary rates for 1000 bootstrap replicates. ClonalFrameML[22] was used to detect possible sites of recombination from a full genome alignment. The maximum likelihood (ML) tree is provided as a clonal genealogy. We identified homoplastic SNPs between the yaws clade and the Bejel/SS14 clades by filtering for positions were the SS14 and/or the Bejel clade show the derived allele and at least one yaws strain shows the ancestral allele, while at least one other yaws strain shares the derived allele. We identified 13 positions that fulfill these criteria. These positions, in addition to those in recombinant positions identified by ClonalFrame, were excluded from further analysis (https://github.com/AdityaLankapalli/Recombination_tools). Positions with missing data ('N') were also removed. For these two datasets, ML trees were constructed as described above and trees were visualized (Figure 6).

SNP effects

The *Y. pestis* SNPs were called using UnifiedGenotyper in the Genome Analysis Toolkit (GATK) in EAGER, using the emit all sites option[23]. The resulting vcf file was processed with MultiVCFAnalyzer v0.85 (https://github.com/alexherbig/MultiVCFAnalyzer), to produce a SNP table[24]. Two hundred and seventy-five (275) genomes, consisting of both ancient and modern varieties, were used in the analysis, [25-36] (Table S15). MultiVCFAnalyzer parameters

included a minimum SNP coverage of 3-fold, genotyping quality of 30, and homozygosity calling at 90% support. An N was inserted in positions where no base call could be made based on the above parameters. Excluded regions are described elsewhere[37]. A total of 6,949 variant positions were identified in this set (Table S16). The unique variants associated with AGU025 were analyzed using SnpEff[38] (Tables S17 and S18). The 4 SNPs in AGU025 affected four different proteins. Two of these changes, at positions 4,047,235 and 4,171,875, affecting the *yjcD* and *aceB* genes respectively, were synonymous. The SNP at position 3,789,518, present in only AGU025, affected the *mrcB* gene, which is a penicillin binding protein. The SNP at position 4,232,523 affects the *fre* gene - NADPH (FMN reductase) and is present in both AGU025 and AGU020. One unique SNP in AGU025 at position 1577025 was disregarded following visual inspection: It was restricted to the terminal ends of reads at the edge of an uncovered region of the genome, and hence is more likely to result from mismapping than actual biological variation.

The above procedures were followed for the treponemal dataset with 31 genomes (Table S19), with exclusion of SNPs filtered for sites identified as recombinant (see above). The unique variants observed in AGU007 were analyzed using SnpEff version 4.3t. The variant type, its annotation, and effect were estimated using SnpSift and snpEff respectively. The two unique variants 39267 and 523975 cause non-synonymous changes in the amino acid residues of genes TPANIC_RS00150 (molecular chaperone GroEL), TPANIC_RS02370 (methyl-accepting chemotaxis protein) respectively.

Dating of the Yaws Clade

As input for TempEst, we used a maximum likelihood tree generated using RAxML[39] and an AGU007 tip date of 1464 CE (oldest sigma 2 values as determined from radiocarbon dating). The relationship between root-to-tip distance and date yielded an R^2 of approximately 0.053 (Figure S7). The MEGA maximum likelihood clock test was configured using MEGAproto with a general time reversible (GTR) substitution model with 4 gamma categories

The BEAST model used for molecular dating was configured with BEAUti[40]. Tip sampling for AGU007 and correction for static positions in the genome was enabled through manual editing of the configuration file. The best-fitting substitution model for the dataset as calculated by ModelGenerator[41] was the transversion model (TVM), which assumes variable base frequencies, variable transversion rates, and equal transition rates. This was implemented in BEAUti by selecting the GTR model and fixing the AG operator, with a relaxed clock and constant coalescent tree model.
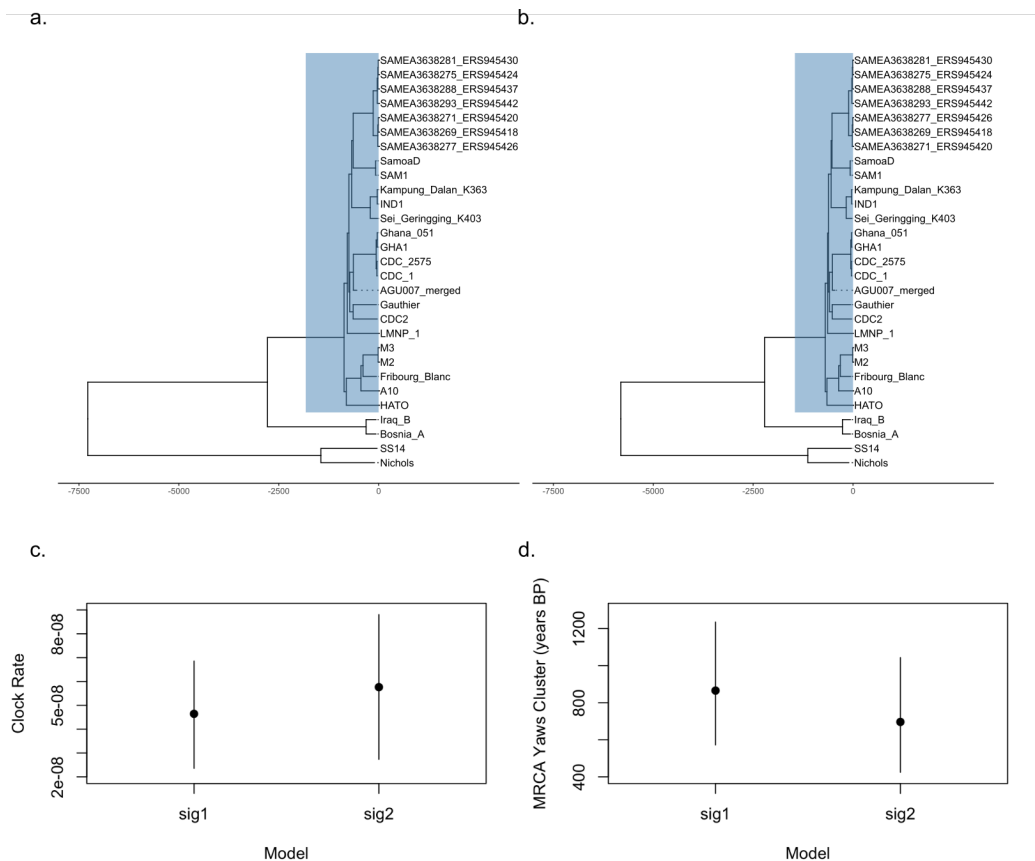
Figure S8: Molecular dating of yaws cluster with BEAST. a) MCC tree for sig1 model with yaws cluster. b) MCC tree for sig2 model. In both panel (a) and panel (b) the yaws cluster (*Treponema pallidum pertenue*) is highlighted in blue and the temporal scale at the bottom of each tree is in years before present. c) Comparison of clock rate estimates between models sig1 and sig2. The black dots indicate the estimated mean clock rate across the tree for each model, and the black lines represent the 95% HPD interval for each model. The y-axis is in substitutions per site per year. d) Comparison of the MRCA date for the yaws cluster according to each model. The black dots indicate the mean MRCA date, and the black lines represent the 95% HPD interval of the date for each model.

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