

# 1 The Stone Age Plague: 1000 years of 2 Persistence in Eurasia

3 Aida Andrades Valtueña<sup>1</sup>, Alissa Mittnik<sup>1,2</sup>, Felix M. Key<sup>1</sup>, Wolfgang Haak<sup>1,3</sup>, Raili Allmäe<sup>4</sup>,  
4 Andrej Belinskij<sup>5</sup>, Mantas Daubaras<sup>6</sup>, Michal Feldman<sup>1,2</sup>, Rimantas Jankauskas<sup>7</sup>, Ivor Janković<sup>8,9</sup>,  
5 Ken Massy<sup>10,11</sup>, Mario Novak<sup>8</sup>, Saskia Pfrenkle<sup>2</sup>, Sabine Reinhold<sup>12</sup>, Mario Šlaus<sup>13</sup>, Maria A.  
6 Spyrou<sup>1,2</sup>, Anna Szecsenyi-Nagy<sup>14</sup>, Mari Tõrv<sup>15</sup>, Svend Hansen<sup>12</sup>, Kirsten I. Bos<sup>1,2</sup>, Philipp W.  
7 Stockhammer<sup>1,10</sup>, Alexander Herbig<sup>1,2\*</sup> and Johannes Krause<sup>1,2\*</sup>

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9 <sup>1</sup>Max Planck Institute for the Science of Human History, Jena, Germany

10 <sup>2</sup>Institute for Archaeological Sciences, Archaeo- and Palaeogenetics, University of Tübingen, Tübingen, Germany

11 <sup>3</sup>School of Biological Sciences, The University of Adelaide, Adelaide SA-5005, South Australia, Australia

12 <sup>4</sup>Archaeological Research Collection, Tallinn University, Tallinn, Estonia

13 <sup>5</sup>“Nasledie” Cultural Heritage Unit, Stavropol, Russia

14 <sup>6</sup>Department of Archaeology, Lithuanian Institute of History, Vilnius

15 <sup>7</sup>Department of Anatomy, Histology and Anthropology, Vilnius University, Vilnius, Lithuania

16 <sup>8</sup>Institute for anthropological research, Zagreb, Croatia

17 <sup>9</sup>Department of Anthropology, University of Wyoming, Laramie, USA

18 <sup>10</sup>Institute for Pre- and Protohistoric Archaeology and Archaeology of the Roman Provinces, Ludwig-Maximilians-University  
19 Munich, Munich, Germany

20 <sup>11</sup>Heidelberg Academy of Sciences, Heidelberg, Germany

21 <sup>12</sup>Eurasia Department, German Archaeological Institute, Berlin, Germany

22 <sup>13</sup>Anthropological Center, Croatian Academy of Sciences and Arts, Zagreb, Croatia

23 <sup>14</sup>Institute of Archaeology, Research Centre for the Humanities, Hungarian Academy of Sciences, Budapest  
24 H-1097, Hungary

25 <sup>15</sup>Independent researcher, Estonia

26

27 \* To whom correspondence should be addressed

## 28 Abstract

29 Molecular signatures of *Yersinia pestis* were recently identified in prehistoric Eurasian  
30 individuals, thus suggesting *Y. pestis* caused some form of disease in humans prior to the first  
31 historically documented pandemic. Here, we present six new *Y. pestis* genomes spanning from  
32 the European Late Neolithic to the Bronze Age (LNBA) dating from 4,800 to 3,700 BP. We show  
33 that all currently investigated LNBA strains form a single genetic clade in the *Y. pestis*  
34 phylogeny that appears to be extinct. Interpreting our data within the context of recent ancient  
35 human genomic evidence, which suggests an increase in human mobility during the LNBA, we  
36 propose a possible scenario for the spread of *Y. pestis* during the LNBA: *Y. pestis* may have  
37 entered Europe from Central Eurasia during an expansion of steppe people, persisted within  
38 Europe until the mid Bronze Age, and moved back towards Central Eurasia in parallel with  
39 subsequent human population movements.

## 40 Introduction

41 Plague pandemics throughout human history caused unprecedented levels of mortality that  
42 contributed to profound socioeconomic and political changes. Conventionally it is assumed that  
43 plague affected human populations in three pandemic waves. The first, the Plague of Justinian,  
44 starting in the 6th century AD, was followed by multiple epidemic outbreaks in Europe and the  
45 Mediterranean basin, and has been associated with the weakening and decay of the Byzantine  
46 empire (Russell, 1968). The second plague pandemic first struck in the 14th century with the  
47 infamous 'Black Death' (1347-1352), which again spread from Asia to Europe seemingly along  
48 both land and maritime routes (Zietz and Dunkelberg, 2004). It is estimated that this initial  
49 onslaught killed 50% of the European population (Benedictow, 2004). It was followed by  
50 outbreaks of varying intensity that lasted until the late eighteenth century (Cohn JR, 2008). The

51 most recent plague pandemic started in the 19th century and began in the Yunnan province of  
52 China. It reached Hong Kong by 1894 and followed global trade routes to achieve a near  
53 worldwide distribution (Stenseth et al., 2008). Since then plague has persisted in rodent  
54 populations in many areas of the world and continues to cause both isolated human cases and  
55 local epidemics (<http://www.who.int/mediacentre/factsheets/fs267/en/>).

56 Plague is caused by a systemic infection with the Gram-negative bacterium *Yersinia*  
57 *pestis*. Advances in ancient DNA (aDNA) research have permitted the successful reconstruction  
58 of a series of *Y. pestis* genomes from victims of both the first and second plague pandemic, thus  
59 confirming a *Y. pestis* involvement and providing new perspectives on how this bacterium  
60 historically spread through Europe (Bos et al., 2016, 2011; Feldman et al., 2016; Spyrou et al.,  
61 2016; Wagner et al., 2014). Most recently, a study by Spyrou et al. (2016) suggested that during  
62 the second pandemic, a European focus was established from where subsequent outbreaks,  
63 such as the Ellwangen outbreak (16<sup>th</sup> century Germany) or the Great Plague of Marseille (1720-  
64 1722 France), were derived (Spyrou et al., 2016). The authors also proposed that a descendant  
65 of the Black Death strain travelled eastwards in the late 14<sup>th</sup> century, became established in  
66 East Asia and subsequently gave rise to the most recent plague pandemic that spread the  
67 pathogen around the globe.

68 Our perception of the evolutionary history of *Y. pestis* was changed substantially by a  
69 recent report of two reconstructed genomes from Bronze Age individuals found in the Altai  
70 region (Southern Siberia, dating to ~4,729 cal BP and ~3,635 cal BP, respectively) and  
71 molecular *Y. pestis* signatures in an additional five individuals from Eurasia (~4,500 to 2,800  
72 BP) suggesting the presence of plague in human populations over a diffuse geographic range  
73 prior to the first historically recorded pandemics. Phylogenetic analysis of the two reconstructed  
74 *Y. pestis* genomes from the Altai region shows that they occupy a phylogenetic position  
75 ancestral to all extant *Y. pestis* strains, though this branch was not adequately resolved  
76 (bootstrap lower than 95% (Rasmussen et al., 2015)). Further open questions remain regarding

77 *Y. pestis*' early association with humans. It is not currently known whether the *Y. pestis* lineages  
78 circulating in Europe during the Late Neolithic and Bronze Age were all descended from the  
79 ~5,000 BP Central Eurasian strain or whether there were multiple strains circulating in Europe  
80 and Asia. Furthermore, how did plague spread over such a vast territory during the period  
81 comprising the Late Neolithic and Bronze Age? Could these bacterial strains have been  
82 associated with certain human groups and their respective subsistence strategies and cultures?

83         The Late Neolithic and Early Bronze Age in Western Eurasia (ca. 4,900-3,700/3,600 BP;  
84 cf. Stockhammer et al., 2015) was a time of major transformative cultural and social changes  
85 that led to cross-European networks of contact and exchange (Vandkilde, 2016). Intriguingly,  
86 recent studies on ancient human genomes suggested a major expansion of people from the  
87 Eurasian Steppe westwards into Central Europe as well as eastwards into Central Eurasia and  
88 Southern Siberia starting around 4,800 BP (Allentoft et al., 2015; Haak et al., 2015). These  
89 steppe people with a predominantly pastoral economy carried a genetic component that is  
90 present in all Europeans today but was absent in early and middle Neolithic farmers in Europe  
91 prior to their arrival. The highest amount of genetic 'steppe ancestry' in ancient Europeans was  
92 found in individuals associated with the Late Neolithic Corded Ware Complex (Figure 1) around  
93 4,500 BP (Haak et al., 2015), who show a genetic makeup close to the steppe people  
94 associated with the 'Yamnaya' complex, suggesting a strong genetic link between those two  
95 groups. Furthermore, it could be shown that the 'middle Neolithic farmer' genetic component  
96 also appears in individuals associated with the Andronovo culture in the Altai region around  
97 4,200 BP (Allentoft et al., 2015). These genetic links between humans ranging from Western  
98 Eurasia to Southern Siberia highlight the dimensions of mobility and connectedness at the time  
99 of the Bronze Age.

100         The reasons for the magnitude of the genetic turnover that occurred in Central Europe  
101 around 4800 BP, where around 75% of the local middle Neolithic farmer genetics was replaced  
102 (Haak et al., 2015), have yet to be explained. As in other episodes of human history, infectious

103 diseases may have played a significant role in triggering or catalyzing those major cultural shifts  
104 and human migrations. Here we present six novel *Y. pestis* genomes from Central Europe and  
105 the North Caucasus steppe spanning from the Late Neolithic to the Bronze Age (LNBA).  
106 Through comparative analyses with other ancient and modern *Y. pestis* lineages (Bos et al.,  
107 2016, 2011; Cui et al., 2013; Feldman et al., 2016; Kislichkina et al., 2015; Spyrou et al., 2016;  
108 Zhgenti et al., 2015), we show that all LNBA strains form a single clade in the *Y. pestis*  
109 phylogeny. This indicates a common origin of all currently identified *Y. pestis* strains circulating  
110 in Eurasia during the Late Neolithic and Bronze Age, and reveals a distribution pattern that  
111 parallels human movements in time and space.

## 112 Results

### 113 Screening

114 A total of 563 tooth and bone samples dating from the Late Neolithic to the Bronze Age from  
115 Russia (122), Hungary and Croatia (139), Lithuania (27), Estonia (45), Latvia (10), and  
116 Germany (Althausen 4, Augsburg 83, Mittelelbe-Saale 133) were screened for *Y. pestis* by  
117 mapping DNA sequencing reads ranging in numbers from 700,000 to 21,000,000 against a  
118 multi-fasta reference consisting of the genomes of 12 different *Yersinia* species (Table 1).

119 To assess if an individual was positive for *Y. pestis*, we calculated a score based on the  
120 number of specific reads mapping to *Y. pestis* in comparison to the number of reads mapping to  
121 other *Yersinia* species (See methods). Following this metric, all individuals with a positive score  
122 were identified as possible candidates. Individuals that had a score higher than 0.005, and had  
123 reads mapping to all the three plasmids present in *Y. pestis* were considered 'strong' positives.  
124 In our dataset we identified five strong candidates, all of them tooth samples, from three  
125 different locations spanning from the Late Neolithic to the Early Bronze Age: one individual from

126 the site Rasshevatskiy (RK1001) in the North Caucasus (Russia), one individual from the  
127 Lithuanian site Gyvakarai (Gyvakarai1), one individual from the Estonian site Kunila (Kunilall)  
128 and two individuals from Augsburg, Germany (Haunstetten, Unterer Talweg 85 Feature 1343  
129 (1343UnTal85), and Haunstetten, Postillionstraße Feature 6 (6Post). Additionally, one individual  
130 from the Croatian site Beli Manastir - Popova Zemlja (GEN72, also a tooth sample), which did  
131 not pass the criteria for a strong candidate, was taken along as a potential candidate since it  
132 had the highest number of reads mapping to the *Y. pestis* chromosome and all the plasmids  
133 (chromosome=993, pCD1=243, pMT1=111, pPCP1=22). For a detailed description of all  
134 samples, individuals and archaeological sites see Table 2 and SI.

135

## 136 Genome reconstruction

137 The five strong positive individuals identified during the screening step (Gyvakarai1, Kunilall,  
138 1343Untal85, 6Post, RK1001) were shotgun sequenced to a depth of 379,155,741 to  
139 1,529,935,532 reads. In addition to the shotgun sequencing, RK1001 and GEN72 were  
140 enriched for *Y. pestis* DNA following an in-solution approach (See Methods). After mapping to  
141 the reference genome (*Y. pestis* CO92, NC\_003143.1), we reconstructed genomes from all six  
142 potential candidates with a mean coverage from 3.7 to 12-fold with 86-94% of the reference  
143 genome covered at least 1-fold (Table 2). The reads were independently mapped to the three  
144 plasmids of *Y. pestis* CO92, and we reconstructed the three plasmids for our ancient samples  
145 with mean coverage of: pCD1 7 to 24-fold, pMT1 3 to 14-fold and pPCP1 18 to 43-fold  
146 (Supplementary Table 1).

147 In order to authenticate the ancient origin of the bacterial genomes, we evaluated the  
148 damage patterns of terminal deamination common to ancient DNA (Briggs et al., 2007). All our  
149 samples present typical damage profiles (Supplementary Figure 1). GEN72, RK1001, Post6 and

150 1343UnTal85 only retain damage in the last two bases as these libraries were prepared using a  
151 'UDG-half' protocol (Rohland et al., 2015, See Methods).

152 The six reconstructed genomes and their plasmids were compared to the two Bronze  
153 Age genomes reported previously (Rasmussen et al., 2015). After visual inspection of aligned  
154 reads, our prehistoric genomes from Europe showed similar coverage of the reference genome  
155 CO92, and all regions were also covered in the Bronze Age Altai *Y. pestis* genomes (Figure  
156 2A). The six reconstructed genomes in this study lack the same region of the pMT1 plasmid,  
157 which contains the *ymt* gene (Figure 2), as already identified in the Altai genomes (Rasmussen  
158 et al., 2015). The *ymt* gene codes for the *Yersinia* murine toxin, which is an important virulence  
159 factor in *Y. pestis* related to transmission via the flea vector (Hinnebusch et al., 2002, 2000).  
160 The expression of *ymt* protects the bacteria from toxic blood digestion by-products in the flea's  
161 gut and thus functions to aid in colonization of the flea midgut (Hinnebusch et al., 2002).

162

## 163 Phylogeny and Dating

164 To assess the phylogenetic positioning of the six European LNBA *Y. pestis* genomes with  
165 respect to the modern and ancient *Y. pestis* genomes, Neighbour Joining (NJ, Supplementary  
166 Figure 2A), Maximum Parsimony (MP, Supplementary Figure 2B) and Maximum Likelihood (ML,  
167 Figure 3, Supplementary Figure 2C) trees were computed. Our samples form a distinct clade in  
168 the *Y. pestis* phylogeny together with the previously reconstructed Southern Siberian Bronze  
169 Age *Y. pestis* genomes (Rasmussen et al., 2015). This topology has a high bootstrap support of  
170 >95% in all three methods. The branching point of the LNBA genomes with the main branch  
171 leading towards the modern *Y. pestis* strains represents the most recent common ancestor  
172 (MRCA) of all the extant and ancient *Y. pestis* genomes currently available.

173 To date the MRCA of *Y. pestis* we performed a ‘tip dating’ analysis using BEAST  
174 (Drummond et al., 2012). The MRCA of all *Y. pestis* was dated to 6,078 years (95% HPD  
175 interval: 5,036-7,494 years) suggesting a Holocene origin for plague, which is in agreement with  
176 previous estimates (5,783 years, 95% HPD interval: 5,021–7,022 years, Rasmussen et al.,  
177 2015). The time to the MRCA of *Y. pestis* and *Y. pseudotuberculosis* strain IP 32953 was  
178 estimated to 28,258 years (95% HPD interval: 13,200-44,631 years). A maximum clade  
179 credibility tree was computed (Supplementary Figure 3) supporting the same topology as the  
180 NJ, MP and ML with high statistical support of the branching points of the LNBA plague clade.

## 181 Genetic makeup

182 The effects of Single Nucleotide Polymorphisms (SNPs) detected in our dataset were  
183 determined using the software *snpEff* (Cingolani et al., 2012) and an in-house program  
184 (*MultiVCFAnalyzer*). A total of 423 SNPs were found in the LNBA branch including strain-  
185 specific and shared SNPs. A total of 114 synonymous and 202 non-synonymous SNPs are  
186 present in the LNBA branch. All the LNBA genomes share five SNPs: four non-synonymous and  
187 one stop mutation (Supplementary Table 2).

188 Additionally, nine stop mutations were detected in the ancient branch, which were not  
189 shared by all the LNBA genomes. Most of these mutations were found in the terminal part of the  
190 LNBA branch with six being specific to the youngest Early Bronze Age *Y. pestis* strain  
191 (RISE505), one being shared between RISE505 and Post6, one being GEN72-specific and one  
192 being Gyvakarai1-specific (Supplementary Table 3). Additionally, RISE505 misses the start  
193 codon of the YPO0956 gene, which is involved in iron transport, and one stop codon in  
194 YPO2909, which is a pseudogene.



195 To identify potential homoplasies, a table of all variable SNPs was examined for any that  
196 contradict the tree topology. Nine homoplasies were detected (Supplementary Table 4).  
197 Furthermore, a tri-allelic site was detected at nucleotide position 4,104,762 (A,T,C).

198 The percentage of the gene covered in the LNBA plague genomes was calculated for a  
199 set of genes that are related to virulence, flea transmission and colonization and dissemination  
200 (Figure 2B). We observed the absence of Ypf $\Phi$  (Derbise et al., 2007), a filamentous prophage,  
201 in all LNBA plague genomes. While Ypf $\Phi$  is found in some *Y. pestis* strains of branch 0, branch  
202 1 and branch 2 as a free phage, it has only been fully integrated and stabilized into the  
203 chromosome of the strains 1.ORI which are responsible for the third pandemic (Derbise and  
204 Carniel, 2014). Additionally, the *yapC* gene was lost in the three younger LNBA strains  
205 (1343UnTal85, Post6 and RISE505). YapC was initially thought to be involved in the adhesion  
206 to mammalian cells, autoagglutination and biofilm formation when expressed in *E. coli* (Felek et  
207 al., 2008). However, the *yapC* knockout in *Y. pestis* does not affect those functions. Felek and  
208 colleagues have thus suggested that this is due to either low expression of *yapC in vitro* or by  
209 compensation through other genes (Felek et al., 2008). The only virulence factor located in the  
210 plasmids missing in all the LNBA *Y. pestis* strains is *ymt* (Figure 2). Other virulence factors,  
211 such as *pla* and *caf1*, were already present in the LNBA *Y. pestis* genomes. The *pla* gene is  
212 involved in the dissemination of the bacteria in the mammalian host by promoting the migration  
213 of the bacteria to the lymphatic nodes (Lathem et al., 2007; Sebbane et al., 2006), while the  
214 *caf1* gene encodes the F1 capsular antigen, which confers phagocytosis resistance to the  
215 bacterium (Du et al., 2002). Both genes are absent in the closest relative *Y. pseudotuberculosis*.

216 Urease D (*ureD*) is an important gene that plays a role in flea transmission. When *ureD*  
217 is expressed in the flea vector it causes a toxic oral reaction to the flea killing around 30-40%  
218 (Chouikha and Hinnebusch, 2014). While *ureD* is functional in *Y. pseudotuberculosis*, it is a  
219 pseudogene in *Y. pestis*. The pseudogenization of this gene is caused by a frameshift mutation  
220 (insertion of a G in a six G-stretch) in *Y. pestis* (Sebbane et al., 2001). The LNBA *Y. pestis*

221 genomes were inspected in the search of this specific frameshift mutation. This insertion is not  
222 present in those genomes indicating that this gene was still functional in *Y. pestis* at that time,  
223 suggesting that it was as toxic to fleas as its ancestor *Y. pseudotuberculosis*.

224 Large-scale insertions and deletions (indels) were evaluated by comparison of mapped  
225 data for the LNBA *Y. pestis* genomes, branch 0 strains (0.PE7, 0.PE2-F, 0.PE3, 0.PE4), KIM,  
226 and CO92 using *Y. pseudotuberculosis* IP 32953 (NC\_006155.1) as a reference. Regions larger  
227 than 1 kb were explored as possible indels. We detected two regions present in the LNBA *Y.*  
228 *pestis* genomes that are absent in all the other strains analyzed: a 1kb region (2,587,386-  
229 2,588,553) that contains a single gene (YPTB0714) encoding an aldehyde dehydrogenase, part  
230 of the R3 *Y. pseudotuberculosis*-specific region identified by Pouillot et al., 2008 and a second  
231 region (1.5kb, 3,295,644-3,297,223) that contains a single gene (YPTB2793) encoding a  
232 uracil/xanthine transporter being part of the region orf1 defined by Pouillot et al., 2008, which  
233 was also characterized as *Y. pseudotuberculosis*-specific. Additionally, two missing regions  
234 were detected: one region of 34kb is missing in the three younger genomes of the LNBA lineage  
235 (Post6, 1343UnTal85 and RISE505) and another 36kb region, which contains flagella genes, is  
236 missing in the youngest sample RISE505, as shown by Rasmussen et al., 2015, which contains  
237 flagella genes. These two missing regions contain multiple membrane proteins, which could be  
238 potential virulence factors or antigens recognized by the immune system of the host.

## 239 Discussion

240 The six prehistoric genomes presented here are the first complete *Y. pestis* genomes spanning  
241 from the Late Neolithic to the Bronze Age in Europe. They form a distinct clade with the  
242 previously reconstructed Southern Siberian Bronze Age *Y. pestis* genomes, confirming that all  
243 LNBA genomes identified so far originate from a common ancestor. The previous reported  
244 genome RISE509 (Rasmussen et al., 2015) together with the reconstructed RK1001 genome

245 reported here occupy the most basal position of all *Y. pestis* genomes sequenced to date. This  
246 suggests that Central Eurasia rather than Eastern Asia should be considered as the region of  
247 potential plague origin.

248 The temporal and spatial distribution of the Late Neolithic and Bronze Age *Y. pestis*  
249 genomes allows us to evaluate the evolution and dissemination of plague in prehistory. We  
250 propose two contrasting scenarios to explain the phylogenetic pattern observed in the LNBA *Y.*  
251 *pestis* branch:

252 1. **Plague was introduced multiple times to Europe** from a common reservoir between  
253 5,000 to 3,000 BP. Here, the bacterium would have been spread independently from a  
254 source, most likely located in Central Eurasia, to Europe at least four times during a  
255 period of over 1,000 years (Figure 1A), once to Lithuania and Croatia, once to Estonia,  
256 and two times to Southern Germany. A similar “multiple wave” proposal has been made  
257 for the second pandemic, where climatic fluctuation was considered as driving changes  
258 in rodent populations (Schmid et al., 2015). We do not have such data for the time  
259 periods in question here, and thus cannot speculate on the mechanism.

260 2. **Plague entered Europe once during the Neolithic.** From here it established a  
261 reservoir within or close to Europe from which it circulated, and then moved back to  
262 Central Eurasia and the Altai region/East Asia during the Bronze Age (Figure 1B). This  
263 parallels the scenario of local persistence and eastward movement during the second  
264 pandemic that is gaining support as more genetic data become available (Seifert et al.,  
265 2016; Spyrou et al., 2016).

266 With just a few genomes available it is difficult to disentangle the two hypotheses; however,  
267 interpreting our data in the context of what is known from human genetics and archaeological  
268 data can offer some resolution. Ancient human genomic data point to a change in mobility and a  
269 large scale expansion of people from the Caspian-Pontic Steppe related to individuals  
270 associated with the ‘Yamnaya’ complex, both to the East and the West starting around 4,800

271 BP. These people carried a distinct genetic component that first appears in Central European  
272 individuals from the Corded Ware Complex and then forms/becomes part of the genetic  
273 composition of most subsequent and all modern day European populations (Allentoft et al.,  
274 2015; Haak et al., 2015). It was furthermore shown that there is a close genetic link between the  
275 highly mobile groups of people associated to the Southern Siberian 'Afnasievo Complex', the  
276 'Yamnaya', and the Central and Eastern European Corded Ware Complex (Allentoft et al.,  
277 2015).

278 Our earliest indication of plague in Europe is found in Croatia and the Baltic region and  
279 coincides with the time of the arrival of the genetic steppe component (Allentoft et al., 2015).  
280 The two Late Neolithic *Y. pestis* genomes from the Baltic in this study were reconstructed from  
281 individuals associated with the Corded Ware Complex (Gyvakarai1 and Kunilall). The Baltic and  
282 Croatian *Y. pestis* genomes are genetically derived from a common ancestor of the strain  
283 RK1001, reconstructed from an individual associated to the 'Yamnaya' complex and RISE509  
284 from the 'Afnasievo' complex from the Altai region, suggesting that the pathogen might have  
285 spread with steppe people from Central Eurasia to Eastern and Central Europe during their  
286 large scale expansion. Furthermore, human genomic analyses indicate that the individuals  
287 RISE509, Gyvakarai1, Kunilall and GEN72 carry 'steppe ancestry' (Mathieson et al., 2017;  
288 Mitnik et al., 2017) Evidence for these long distance contacts is also present in the  
289 archaeological record. For example, the Gyvakarai1 burial is characterised by both a specific  
290 set of grave inventory (hammer headed pin) and distinct skeletal morphology, which have no  
291 analogues in earlier local populations (Tebelškis and Jankauskas, 2006).

292 The younger Late Neolithic *Y. pestis* genomes from Southern Germany are genetically  
293 derived from the Baltic strains and are found in individuals associated with the Bell Beaker  
294 Complex. Previous analyses have shown that Bell Beaker individuals from Germany also carry  
295 'steppe ancestry' (Allentoft et al., 2015; Haak et al., 2015). This suggests that *Y. pestis* may  
296 have been spread further southwestwards analogous to the human steppe component. The

297 youngest of the LNBA *Y. pestis* genomes (RISE505), found also in the Altai region, associated  
298 with the Central Eurasian 'Andronovo' complex, descends from the Central European strains,  
299 which suggests a spread back into Southern Siberia. Interestingly, genome-wide human data  
300 shows that human individuals associated to the Sintashta, Srubnaya and Andronovo cultural  
301 complexes in the Eurasian steppes (dating from around 3,700-3,500 BP) carried mixed ancestry  
302 of middle Neolithic European farmers and Bronze Age steppe people, suggesting a backflow of  
303 human genes from Europe to Central Eurasia (Allentoft et al., 2015). From an archeological  
304 perspective there is a close connection of the Abashevo cultural complex and Sintashta, that  
305 might also have included population shifts West to East. In particular, the post-Sintashta  
306 Andonovo cultural complex is an epoch of massive population shifts affecting all the area east of  
307 the Urals to the Western borders of China including populations with European origin  
308 (Koryakova and Epimakhov, 2007; Kuzmina, 2008). The steppe, as a natural corridor  
309 connecting people and their livestock throughout Central and Western Eurasia, might have  
310 facilitated the spread of strains closely related to the European Early Bronze Age *Y. pestis* back  
311 to the Altai region, where RISE505 was found. The patterns in human genetic ancestry and  
312 admixture, in combination with the temporal series within the LNBA *Y. pestis* branch, therefore  
313 support scenario 2, suggesting that *Y. pestis* was introduced to Europe from the steppe around  
314 4,800 BP. Thereafter, the pathogen became established in a local reservoir within or in close  
315 proximity to Europe, from where the European *Y. pestis* strain was disseminated back to the  
316 Altai region in a process connected to the backflow of human genetic ancestry from Western  
317 Eurasia into Southern Siberia. The pathogen diversity, therefore, mirrors the archaeological  
318 evidence, which indicates a strong intensification of Eurasian networks since the beginning of  
319 the Bronze Age (Vandkilde, 2016).

320 Even though *Y. pestis* seems to have been spread following human movements, its  
321 mode of transmission during this early phase of its evolution cannot be easily determined. Most  
322 contemporary cases of *Y. pestis* infection occur via an arthropod vector and stem from a sylvatic

323 rodent population that has resistance to the bacterium. The flea transmission can be  
324 accomplished by one of two mechanisms: the classical blockage-dependent flea transmission  
325 (Hinnebusch et al., 1998) and the recently proposed early-phase transmission (EPT) (Eisen et  
326 al., 2006). In the blockage-dependent model, *Y. pestis* causes an obstruction in the flea  
327 digestive system by producing a biofilm that blocks the pre-gut of the flea within 1-2 weeks after  
328 infection. This blockage prevents a blood meal from reaching the flea's gut, and regurgitation of  
329 the blood by a hungry flea in repeated attempts to feed sheds several live bacteria into the  
330 blood stream of the host (Chouikha and Hinnebusch, 2012; Hinnebusch et al., 1998). It has  
331 been shown that the blockage-dependent transmission requires a functional *ymt* gene and *hms*  
332 locus, and non-functional *rcaA*, *pde2* and *pde3* genes (Sun et al., 2014). *ymt* protects *Y. pestis*  
333 from toxic by-products of blood digestion and allows the bacterium to colonise the mid-gut of the  
334 flea. The *hms* locus is involved in biofilm formation and *rcaA*, *pde2* and *pde3* are the down-  
335 regulators of biofilm formation. However, evidence is emerging that *Y. pestis* can be transmitted  
336 efficiently within the first 1-4 days after entering the flea prior to biofilm formation (Eisen et al.,  
337 2015, 2006), in a process known as the EPT model. Unfortunately this model is currently less  
338 well understood molecularly and physiologically than blockage-dependent transmission, but has  
339 been shown to be biofilm (Vetter et al., 2010) and *ymt* independent (Johnson et al., 2014).

340       Based on the genetic characteristics of the LNBA genomes (i.e. lack of *ymt*, still  
341 functional *pde2* and *rcaA* as shown by previous work (Rasmussen et al., 2015), functional *ureD*  
342 which will kill 30-40% of the flea vectors) it seems most parsimonious that *Y. pestis* was not able  
343 to use a flea vector in a blockage-dependent model. However, since none of these genes seem  
344 to be required for EPT, it remains possible that LNBA *Y. pestis* was transmitted by a flea vector  
345 via this transmission mode. Under this assumption, the transmission would have been  
346 presumably less efficient since a functional Urease D would have reduced the number of fleas  
347 transmitting the bacteria.

348           The presence of genes involved in virulence in the mammalian host such as *pla* and  
349 *caf1*, which are absent in *Y. pseudotuberculosis*, indicates that LNBA *Y. pestis* was already  
350 adapted to mammalian hosts to some extent. *pla* aids in *Y. pestis* infiltration of the mammalian  
351 host (Lathem et al., 2007; Sebbane et al., 2006). The *pla* gene present in the LNBA *Y. pestis*  
352 strains has the ancestral I259 variant, which has been shown to be less efficient than the  
353 derived T259 form (Haiko et al., 2009). *Y. pestis* with the ancestral variant is able to cause  
354 pneumonic disease, however, it is less efficient in colonising other tissues (Zimmler et al., 2015).  
355 This indicates that LNBA *Y. pestis* could potentially cause a pneumonic or a less virulent  
356 bubonic form. In addition to the above noted changes, we detected two regions missing in the  
357 LNBA genomes: a ~34kb region that contains genes encoding membrane proteins missing in  
358 the three youngest *Y. pestis* strains (Post6, 1343UnTal85 and RISE505) and a ~36kb region  
359 containing genes encoding proteins involved in flagellin production and iron transporters missing  
360 in the youngest sample RISE505, as observed elsewhere (Rasmussen et al., 2015). This  
361 genome decay affecting membrane and flagellar proteins potentially involved in interactions with  
362 the host's immune system, can be an indication of adaptation to a new host pathogenic lifestyle  
363 (Ochman and Moran, 2001).

364           Our common understanding is that plague is a disease adapted to rodents, where  
365 commensal species such as *Rattus rattus* and their fleas play a central role as disease vectors  
366 for humans (Perry and Fetherston, 1997). While a rodent-flea mediated transmission model is  
367 compatible with the genomic makeup of the LNBA strains, disease dynamics may well have  
368 differed in the past. The most parsimonious explanation would be that LNBA plague indeed  
369 traveled with rodent species commensal to humans, in keeping with the orthodox model of  
370 plague transmission. The Neolithic is conventionally considered to be a time period where new  
371 diseases were introduced into human groups as they made the transition from a nomadic  
372 lifestyle to one of sedentism, and where the adoption of agriculture and increased population  
373 density acted synergistically to change the disease landscape (Ronald Barrett et al., 1998).



374 Whether commensal rodent populations were large enough to function as reservoir populations  
375 for plague during human migrations at this time is unknown. In central Eurasian Bronze Age  
376 cultures, agriculture, i.e. large scale food storage, is mostly absent (Ryabogina and Ivanov,  
377 2011) However, contact between steppe inhabiting rodents, pastoralists and their herds might  
378 have been frequent when moving within these environments. Alternative models of transmission  
379 involving different host species, perhaps even humans or their domesticates, might carry some  
380 traction, as the ancient disease may have behaved rather differently from the form we know  
381 today.

382 Here, we present the first LNBA *Y. pestis* genomes from Europe. We show that all LNBA  
383 genomes reconstructed so far form a distinct lineage that potentially entered Europe following  
384 the migration of steppe people around 4,800 BP. We find striking parallels between the *Y. pestis*  
385 dispersal pattern and human population movements during this time period. We propose two  
386 scenarios for presence of the bacteria in Europe: a multiple introduction hypothesis from a  
387 Central Eurasian source, or the establishment of a local *Y. pestis* focus within or close to  
388 Europe from where a resident strain ultimately moved back towards Central Asia in the Bronze  
389 Age. On account of the chronology and the tight synergy between the ancient *Y. pestis*  
390 phylogeny and known patterns of human mobility, we find stronger support for the second  
391 scenario.

392 The LNBA period was a time of increased mobility and cultural change. The presence of  
393 *Y. pestis* may have been a promoting factor for the increase in mobility of human populations  
394 (Rasmussen et al., 2015). The manifestation of the disease in Europe could have played a  
395 major role in the processes that led to the genetic turnover observed in the European human  
396 populations, who may have harbored different levels of immunity against this newly introduced  
397 disease. Testing these hypotheses will require more extensive assessment of both human and  
398 *Y. pestis* genomes from the presumed source population before and after migration from the  
399 steppes, as well as in Europe during this period of genetic turnover.



## 400 Authors contribution

401 J.K., A.H. and A.A.V. conceived the study. K.M., R.A., M.D., R.J., M.T., P.W.S., A.B., I.J., M. N.,  
402 S.R., M.S., A.S., S.H. provided the samples and performed archaeological assessment. A.M.,  
403 S.P., M.F., A.A.V. performed laboratory work. A.A.V., A.H., M.A.S., F.M.K. and J.K. analysed  
404 the data. A.A.V, A.H., J.K., P.W.S., K.I.B., W.H. and A.M. wrote the manuscript with  
405 contributions from all co-authors. All authors read and approved the final manuscript.

406

## 407 Competing financial interests

408 The authors declare no competing financial interests.

409

## 410 Data availability

411 Raw sequencing data have been deposited at the European Nucleotide Archive under  
412 accession PRJEBXXXXX

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728

## 729 Figures and table legends

730 **Figure 1: Map of proposed *Yersinia pestis* circulation throughout Eurasia.** A) Entrance of  
731 *Y. pestis* into Europe from Central Eurasia with the expansion of Yamnaya pastoralists around  
732 4,800 years ago. B) Circulation of *Y.pestis* back into the Altai from Europe. Only complete  
733 genomes are shown.

734 **Figure 2:** A) Average coverage plot for the chromosome and plasmids of *Yersinia pestis*, from  
735 the outer ring to the inner ring: *Y. pestis* CO92 (NC\_003143.1, reference), RISE509, RK1001,  
736 GEN72, Gyvakarai1, Kunilall, 6Post, 1343UnTal85 and RISE505. Colours correspond to the  
737 regions where the genomes were recovered from: Altai region (purple), Russia (red), Croatia  
738 (dark yellow), Gyvakarai, Lithuania (blue), Kunila, Estonia (orange), Augsburg, Germany  
739 (green). The average depth of coverage was calculated for 1kb regions for the chromosome and  
740 100bp for the plasmids, each ring represents a maximum of 20X coverage. The figure was  
741 generated with Circos (Krzywinski et al., 2009). B) Percentage covered of virulence factors  
742 located on the *Yersinia pestis* chromosome and plasmids, plotted with in R using the ggplot2  
743 package. [1] *ymt* gene, [2] *pla*, [3] deletion of flagelin genes, [4] filamentous prophage YpfΦ, [5]  
744 *Y. pestis*-specific genes, [\*] region mask in pPCP1 due to high similarity to expression vectors  
745 during enzyme production (Schuenemann et al., 2011).

746 **Figure 3: Maximum Likelihood tree of all *Yersinia pestis* genomes including 1,867 SNPs**  
747 **positions with complete deletion.** Nodes with support equal or higher than 95% are marked  
748 with an asterisk. The colours represent different branches in the *Y. pestis* phylogeny: branch 0  
749 (black), branch 1 (red), branch2 (green), branch 3 (blue), branch 4 (orange) and LNBA *Y. pestis*  
750 branch (purple). *Y. pseudotuberculosis*-specific SNPs were excluded from the tree for  
751 representative matters.

752 **Table 1: Genomes from the NCBI (RefSeq/Nucleotide) database, used in the multi-species**  
753 **reference panel for screening for *Y. pestis* aDNA.**



754 **Table 2: Statistic of the *Y. pestis* genome reconstruction. BP = Before Present.**

## 755 STAR Methods

### 756 Sampling and extraction

757 Sampling of a total of 563 tooth and bone samples (Russia (122), Hungary and Croatia (139),  
758 Lithuania (27), Estonia (45), Latvia (10), and Germany (Althausen 4, Augsburg 83, Mittelbe-  
759 Saale 133)) took place in the clean room facilities of the Institute for Archaeological Sciences at  
760 the University of Tübingen, the Institute of Archaeology RCH HAS in Budapest and of the MPI-  
761 SHH in Jena. After irradiation with UV light to remove surface contamination, teeth were sawed  
762 apart transversally at the border of crown and root, and dentine from inside the crown was  
763 sampled and powdered using a sterile dentistry drill. For the samples processed in Budapest,  
764 whole teeth were powdered. For bone samples, the surface layer from the sampling area was  
765 removed with a dentistry drill prior to obtaining bone powder from the inside of the bone by  
766 drilling. For each specimen we gathered between ~30 and 120 mg of powder to be used for  
767 DNA extraction.

768 Extraction was performed following a protocol optimized for the recovery of small ancient DNA  
769 molecules (Dabney et al., 2013), resulting in 100µl of DNA extract per sample. An aliquot of  
770 20µl of extract was used to generate double-indexed libraries (Kircher et al., 2012; Meyer and  
771 Kircher, 2010). Negative controls were included in the extraction and library preparation and  
772 taken along for all further processing steps.

### 773 Shotgun screening

774 Libraries were PCR-amplified and quantified using an Agilent 2100 Bioanalyzer DNA 1000 chip

775 and pooled at equimolar concentrations prior to paired-end sequencing on a NextSeq500 with  
776 2x101+8+8 and a HiSeq2500 with 2x101+8+8 cycles according to the manufacturer's  
777 instructions to a depth of ~1.5 million reads per library.

## 778 *In-silico* screening

779 The sequencing data for the 170 samples was preprocessed with ClipAndMerge (Peltzer et al.,  
780 2016) to remove adaptors, base quality-trim (20) and merging and filtering for only merged  
781 reads. Reads were mapped using the BWA aln algorithm (Li and Durbin, 2009) to a multi-  
782 species reference panel, containing various representatives of the genus *Yersinia* (Table 2) and  
783 the plasmids of *Yersinia pestis*: pCD1, pMT1 and pPCP1 from *Y. pestis* CO92. The region  
784 comprising 3000-4200bp of the *Y. pestis* specific plasmid pPCP1 was masked in the reference,  
785 since it is highly similar to an expression vector used during the production of enzyme reagents  
786 (Schuenemann et al., 2011).

787

788 Mapped files were then filtered for reads with a mapping quality higher than 20 with  
789 Samtools (Li et al., 2009). PCR duplicates were removed using the MarkDuplicates tool in  
790 Picard (1.140, <http://broadinstitute.github.io/picard/>). The number of reads mapping specifically  
791 to each genome and to the plasmids were retrieved from the bam files using Samtools (Li et al.,  
792 2009) idxstats. An endogenous based score was used to assess the potential of the sample  
793 being 'positive' for *Y. pestis*. It was calculated as follows:

794

$$\frac{(YPS - \max(YS))}{M}$$

795 where YPS is the number of reads specifically mapping to *Y. pestis*; YS is the maximum number  
796 of reads mapping specifically to a *Yersinia* species with the exception of *Y. pestis* and M is the  
797 total number of merged reads in the sample. By using the maximum number of reads mapping  
798 to another species of the genus *Yersinia*, the score takes in account different source of  
799 contamination other than *Y. pseudotuberculosis*. Five samples (RK1001, Gyvakarai1, Kunilall,  
800 6Post and 1343UnTal85) fulfilled the criteria for being considered strong candidates (score  
801 higher than 0.005 and reads mapping to all plasmids). Another samples, GEN72, was also  
802 included in further processing and analysis since it had higher numbers mapping the *Y. pestis*  
803 chromosome and plasmids even though it did not full-fill the score requirements. For a detailed  
804 description of the archaeological sites and individuals see the SI.

## 805 Deep shotgun sequencing

806 The five strong candidate samples detected in screening of the shotgun data were processed  
807 for deep shotgun sequencing as following: For Gyvakarai1 the screening library described  
808 above was pair-end sequenced on two lanes of a HiSeq4000 for 100 cycles, and on a full run of  
809 a NextSeq500 for 75 cycles. The screening library for Kunilall was pair-end sequenced deeper  
810 on 80% of one lane of a HiSeq4000 for 100 cycles. Additionally, 40 µl of DNA extract of Kunilall  
811 was converted in to a library treated with UDG and endonuclease VIII to remove deaminated  
812 bases (Briggs and Heyn, 2012), and pair-end sequenced on one lane of a HiSeq4000 for 75  
813 cycles.

814 For RK1001, Post6 and 1343UnTal85, 60 µl of DNA extract each were converted into DNA  
815 libraries using so-called UDG-half treatment, whereby deaminated bases are partially removed  
816 and retained mostly at the ends of the molecule (Rohland et al., 2015). The library of RK1001  
817 was deep shotgun pair-end sequenced in 8 lanes of a HiSeq4000 for 55 cycles. The libraries of  
818 6Post and 1343UnTal85 were deep shotgun single-end sequenced on 2 and a half lanes of a  
819 HiSeq4000 for 75 cycles. Post6 was additionally pair-end sequenced on a full run of a

820 NextSeq500 for 75 cycles.

## 821 *Y.pestis* in-solution capture

822 *Y. pestis* whole-genome DNA capture probes were designed using as template sequences the  
823 *Y. pestis* CO92 chromosome (NC\_003143.1), *Y. pestis* CO92 plasmid pMT1 (NC\_003134.1), *Y.*  
824 *pestis* CO92 plasmid pCD1 (NC\_003131.1), *Y. pestis* KIM 10 chromosome (NC\_004088.1), *Y.*  
825 *pestis* Pestoides F chromosome (NC\_009381.1) and *Y. pseudotuberculosis* IP 32953  
826 chromosome (NC\_006155.1). We used a 6 bp tiling with a probe length of 52 bp with an  
827 additional 8 bp 3' linker sequence as described in (Fu et al., 2013). Low complexity regions  
828 were masked using dustmasker (Camacho et al., 2009, version 2.2.32+). Redundant probes as  
829 well as probes with more than 20% masked nucleotides were discarded. The procedure  
830 resulted in 816,413 unique probe sequences. A second probe set was created with a coordinate  
831 offset of 3 bp resulting in 827,438 unique probe sequences. In combination the two probe sets  
832 represent an effective tiling density of 3 bp. The two probe sets were ordered on two 1 million  
833 feature Agilent SureSelect DNA Capture Arrays. The full capacity of the arrays was filled up with  
834 randomly selected probes. The two arrays were turned into in-solution DNA capture libraries as  
835 described elsewhere (Fu et al., 2013).

836 For GEN72, 25 µl of DNA extract was converted into DNA libraries using so-called UDG-half  
837 treatment as described above<sup>10</sup>. The UDG-half libraries of RK1001 and GEN72 were enriched  
838 for *Y. pestis* DNA using in-solution DNA capture probes (see above) as described elsewhere  
839 (Fu et al., 2013; Haak et al., 2015; Mathieson et al., 2015). The capture products of RK1001  
840 and GEN72, were sequenced on 1 and 0.6 of the lane, respectively, of the HiSeq4000 for 75  
841 cycles.

## 842 Genome reconstruction and authentication

843 All samples were processed with the EAGER pipeline (Peltzer et al., 2016). Sequencing quality  
844 for each sample was evaluated with FastQC  
845 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and adaptors clipped using the  
846 ClipAndMerge module in EAGER. For paired-end data, the reads were also merged with  
847 ClipAndMerge and only the merged reads were kept for further analysis.

848 Due to variability in the laboratory preparation and sequencing strategies, the sequencing reads  
849 for each sample were treated as follows:

- 850 • Gyvakarai1: two HiSeq lanes and one Next-Seq run paired-end of the non-UDG treated  
851 library were combined and reads mapped to *Y. pestis* CO92 reference with BWA aln (-l  
852 16, -n 0.01, hereby referred to as non-UDG parameters). Reads with mapping quality  
853 scores lower than 37 were filtered out. PCR duplicates were removed with  
854 MarkDuplicates. MapDamage (Jónsson et al., 2013, v2.0) was used to calculate damage  
855 plots. Coverage was calculated with Qualimap (Okonechnikov et al., 2016, v2.2).
- 856 • Kunilall: UDG and the non-UDG libraries were sequenced in 2 HiSeq pair-end lanes and  
857 processed separately until calculation of the coverage. The non-UDG treated libraries  
858 were mapped with non-UDG parameters while the UDG treated library reads were  
859 mapped with more stringent parameters (-l 32, -n 0.1, referred to as UDG parameters).  
860 Reads with mapping qualities less than 37 were filtered out and duplicates were  
861 removed with MarkDuplicates as before. The non-UDG bam file was used to calculate  
862 damage plots as indicated above. After duplicate removal, the UDG- and non-UDG  
863 treated BAM files were merged together and used to calculate the coverage as above.
- 864 • GEN72, Post6 and 1343UnTal85: the UDG-half treated libraries were sequenced in two  
865 HiSeq lanes for Post6 and 1343UnTal85 and 19,777,683 reads were generated in the  
866 HiSeq for GEN72, and two different runs were performed. For the first run, reads without

867 clipping were used to retain miscoding lesions indicative of aDNA. BWA aln was used for  
868 mapping with non-UDG parameters (-l 16 and -n 0.01). Reads with mapping qualities  
869 lower than 37 were filtered and PCR duplicates were removed with MarkDuplicates as  
870 described above. Coverage and damage plots were calculated as above. After clipping  
871 the last two bases with the module ClipAndMerge in eager, potentially affected by  
872 damage, the samples were mapped with UDG parameters.

- 873 • RK1001: UDG-half library was shotgun sequenced pair-end in 8 HiSeq lanes and in-  
874 solution captured and sequenced single end to a depth of 303,148,884 reads sequenced  
875 in the HiSeq. Shotgun and captured data were combined in a fastq file and processed as  
876 described above for GEN72, Post6 and 1343UnTal85.

## 877 SNP calling & phylogenetic analysis

878 Prior to SNP calling in order to avoid false SNP calling due to aDNA damage, the quality scores  
879 of damaged sites in the non-UDG treated samples were downscaled using MapDamage  
880 (Jónsson et al., 2013, v2.0), as performed in previous analysis (Rasmussen et al., 2015). For  
881 the UDG-half data, the files with the two last bases clipped, hence removing the damage signal,  
882 and mapped with UDG parameters were used for SNP calling (see above).

883 SNP calling was performed with GATK UnifiedGenotyper (Van der Auwera et al., 2013) in  
884 EAGER<sup>43</sup> with default parameters and the 'EMIT\_ALL\_SITES' output mode.

885 VCF files of the new ancient samples, along with the two complete genomes from Rasmussen  
886 et al., 2015, the Black Death (Bos et al., 2011), Justinianic Plague (Feldman et al., 2016),  
887 Bolgar (Spyrou et al., 2016) and Observance (Bos et al., 2016) genomes, were combined with a  
888 curated dataset of 130 modern genomes (Cui et al., 2013) in addition to 11 samples from the  
889 Former Soviet Union (Zhgenti et al., 2015) and 19 draft genomes of *Y. pestis* subsp. *microtus*  
890 strains (Kislichkina et al., 2015).

891 The VCF files were processed with an in-house program (*MultiVCFAnalyser*) that  
892 produced a SNP table and an alignment file containing all variable positions in the dataset, in  
893 respect to the reference *Y. pestis* CO92. In order to call a SNP a minimum genotyping quality  
894 (GATK) of 30 was required, with a minimum coverage of 3X, and with a minimal allele frequency  
895 of 90% for a homozygous call. No heterozygous calls were included in the output files.

896 The SNP alignment was curated by removing all alignment columns with missing data  
897 (complete deletion). The curated SNP alignment was then used to compute NJ and MP trees  
898 with MEGA6 (Tamura et al., 2013) and a ML tree using PhyML 3.0 (Guindon et al., 2010) with  
899 the GTR model used in previous *Y. pestis* work (Cui et al., 2013; Rasmussen et al., 2015), with  
900 4 gamma categories and the best of NNI and SPR as tree branch optimization. The specific  
901 variants of *Y. pseudotuberculosis* were removed from the analysis to improve the visual  
902 resolution of the tree.

## 903 Dating analysis

904 The SNP alignment after complete deletion was used for molecular dating using BEAST 1.8.2  
905 (Drummond et al., 2012). The modern sample 0.PE3, also called Angola, was removed from the  
906 dataset due to its long branch.

907 For tip dating, all modern genomes were set to an age of 0. The dates of the ancient  
908 samples presented in this study plus the two complete genomes from Rasmussen et al., 2015  
909 were recalibrated with Calib 7.1 (<http://calib.gub.ac.uk/calib/>) to the IntCal13 calibration curve.  
910 The ancient samples were given the median calibrated probability as their age, and the 2 sigma  
911 interval was used as the boundaries for a uniform prior sampling (Supplementary Table 5). The  
912 dates published for previous historical genomes were transformed to cal BP assuming 1950 as  
913 age 0 and given the mean as the age with the interval as the boundaries of a prior uniform  
914 distribution: Black Death 603 (602-604, Bos et al., 2011); Observance 229 (228-230, Bos et al.,

915 2016), Bolgar 569 (550-588, Spyrou et al., 2016) and Justinian 1453 (1382-1524, Feldman et  
916 al., 2016).

917 The molecular clock was tested and rejected using MEGA6. Therefore, we followed  
918 previous work and used an uncorrelated relaxed clock with lognormal distribution (Cui et al.,  
919 2013; Rasmussen et al., 2015) with the substitution model GTR+G4. Tree model was set up to  
920 coalesce assuming a constant population size and a rooted ML tree was provided as a starting  
921 tree. Two independent 1,000,000,000 MCMC chains were computed sampling every 5,000  
922 steps. The two chains were then combined using LogCombiner from BEAST 1.8.2 (Drummond  
923 et al., 2012) with a 10 percent burn-in (100,000,000 steps per chain). The ESS of the posterior,  
924 prior, treeModel.rootHeight, tMRCA\_allpestis are 4,589, 4,087, 1,054 and 7,571 respectively.  
925 The trees files for the 2 chains were combined with LogCombiner with 100,000,000 of burning  
926 and resampled every 20,000 steps giving a total number of 90,000 trees, that were used to  
927 produce a Maximum Clade Credibility tree using TreeAnnotator from BEAST 1.8.2 (Drummond  
928 et al., 2012).

## 929 SNP effect analysis and virulence factors analysis

930 The SNP table from *MultiVCFAnalyzer* was provided to *SnpEff* (Cingolani et al., 2012) and the  
931 effect of the SNPs within genes present in the dataset was evaluated. Additionally the SNP  
932 table was manually assessed for possible homoplasies.

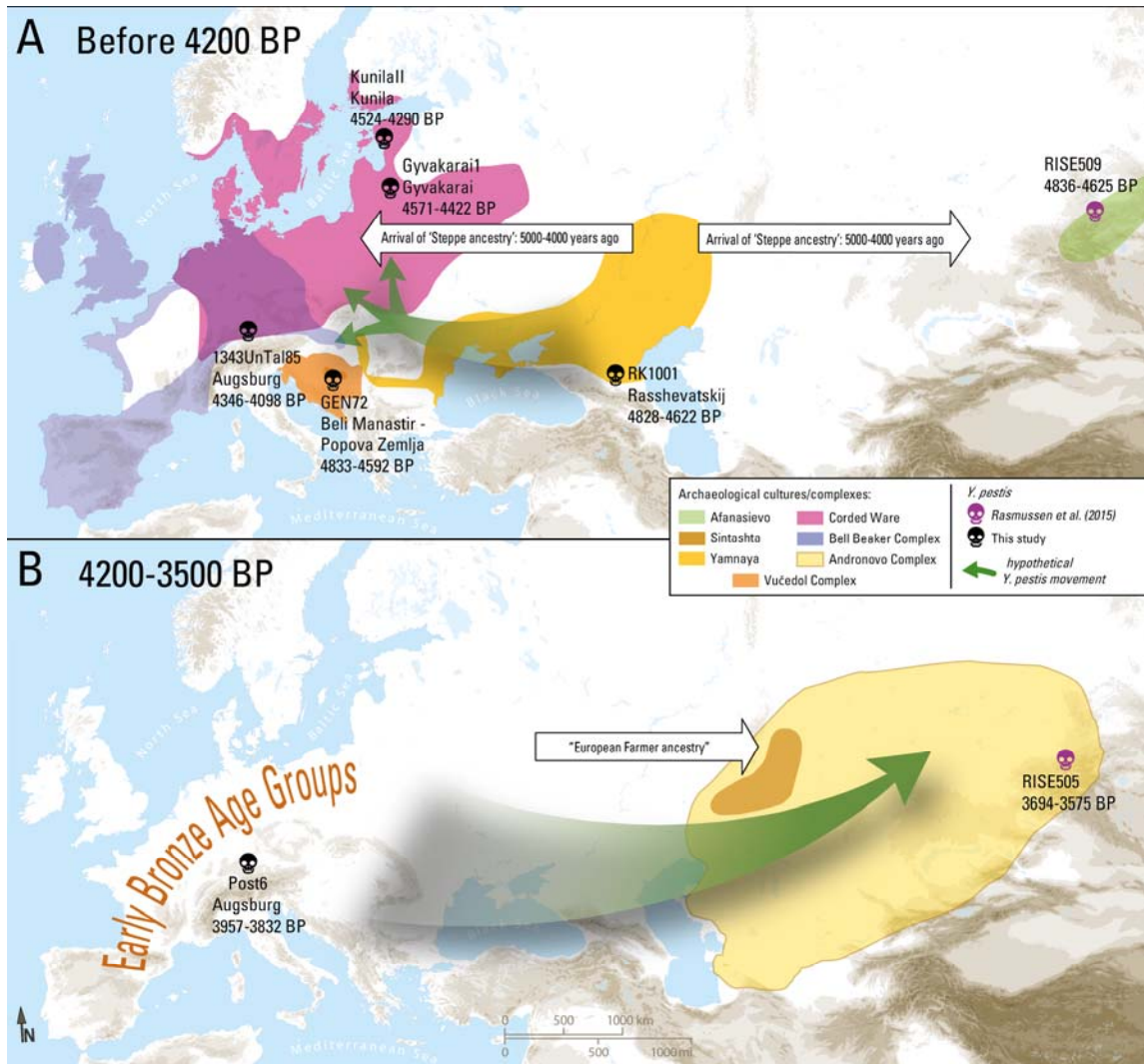
933 For the virulence factors, the samples were mapped as indicated above but without  
934 applying quality filtering and the percentage of coverage was calculated for each region using  
935 bedtools (Quinlan and Hall, 2010) and plotted using the package ggplot2 (Wickham, 2009) in R  
936 (R Development Core Team, 2008). Additionally, *ureD* was manually explored for SNPs using  
937 IGV (Thorvaldsdóttir et al., 2013).



## 938 Indel analysis

939 The samples including the two complete Bronze Age genomes (Rasmussen et al., 2015) were  
940 mapped against *Y. pseudotuberculosis* IP 32953 with bwa with non-UDG parameters (-n 0.01, -l  
941 16), except for RK1001, GEN72, 1343UnTal85 and 6Post that were mapped with bwa with UDG  
942 parameters (-n 0.1, -l 32),. The modern genomes from branch 0 (0.PE7, 0.PE2, 0.PE3 and  
943 0.PE4), *Y. pestis* CO92 and *Y. pestis* KIM10 were *in-silico* cut in 100 bp fragments with 1bp  
944 tiling and mapped to *Y. pseudotuberculosis* reference using bwa with UDG parameters (-n 0.1, -  
945 l 32). The non-covered regions were extracted using the bedtools genomecov function. Missing  
946 regions larger than 1kb were comparatively explored in order to identify indels. Using the  
947 bedtools intersect function, we extracted regions missing in the Neolithic genomes and present  
948 in the modern ones and also the regions missing in the modern ones but still present in the  
949 Neolithic genomes. The results were check by manual inspection in IGV (Thorvaldsdóttir et al.,  
950 2013).

951 Figures and Tables

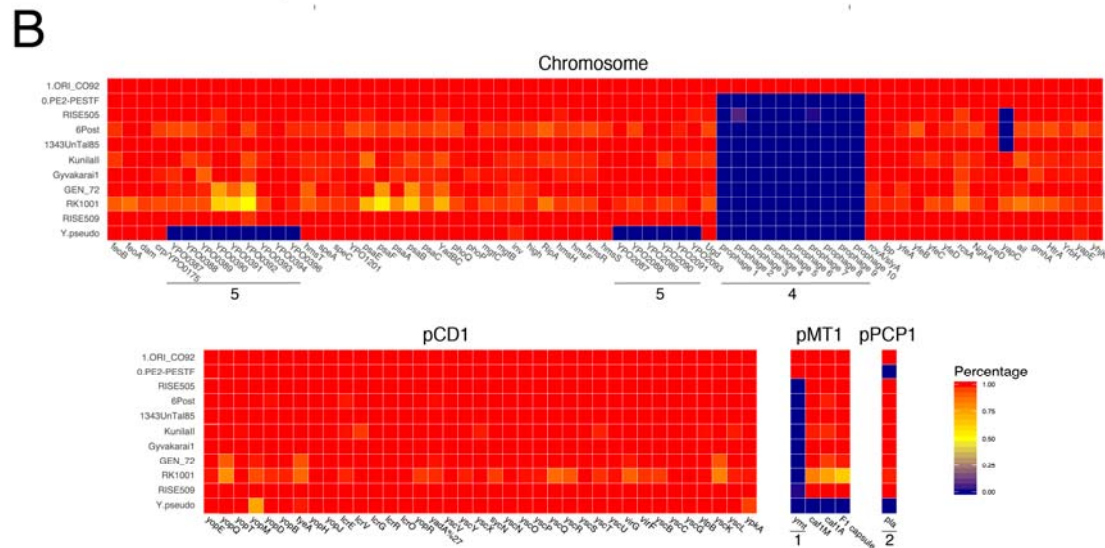
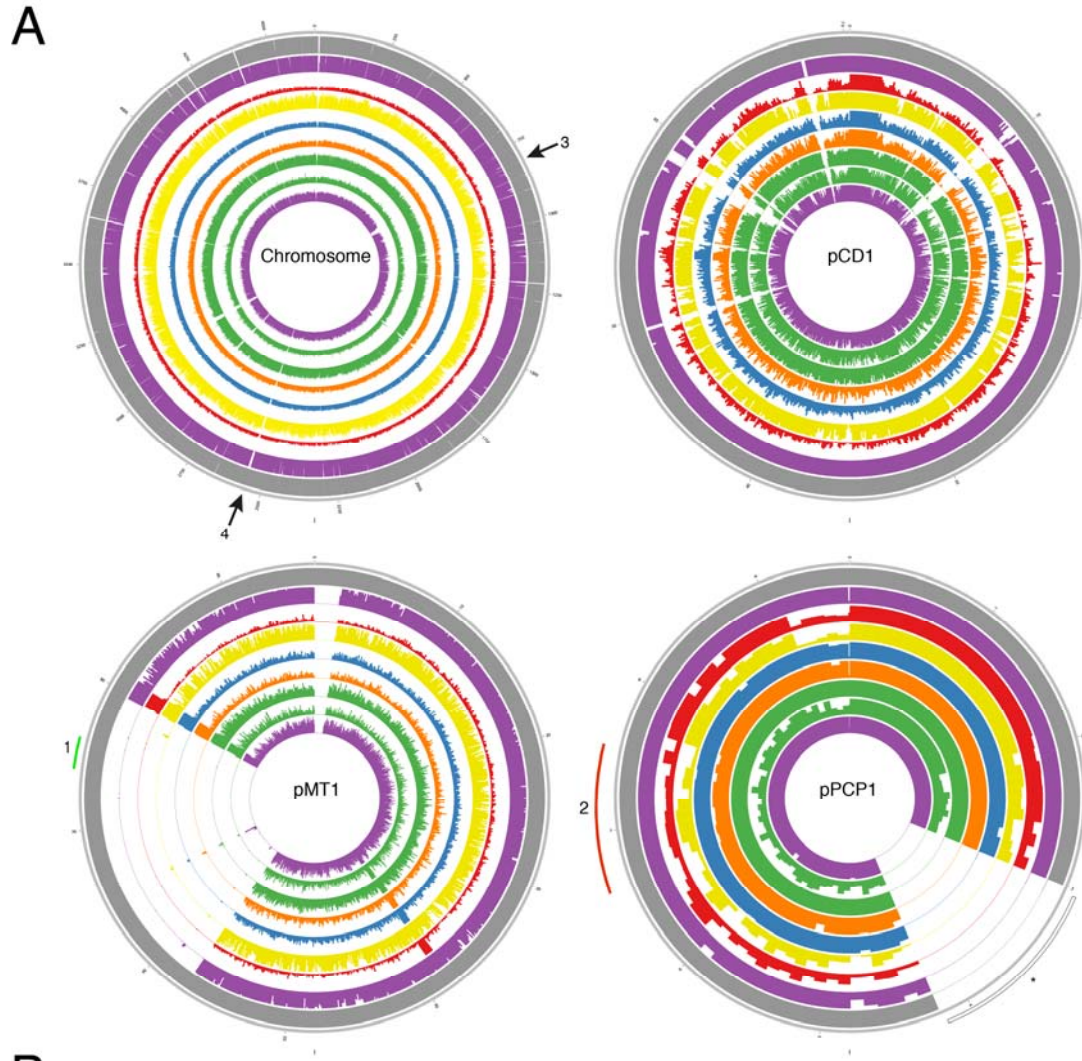


952

953 Figure 1

954

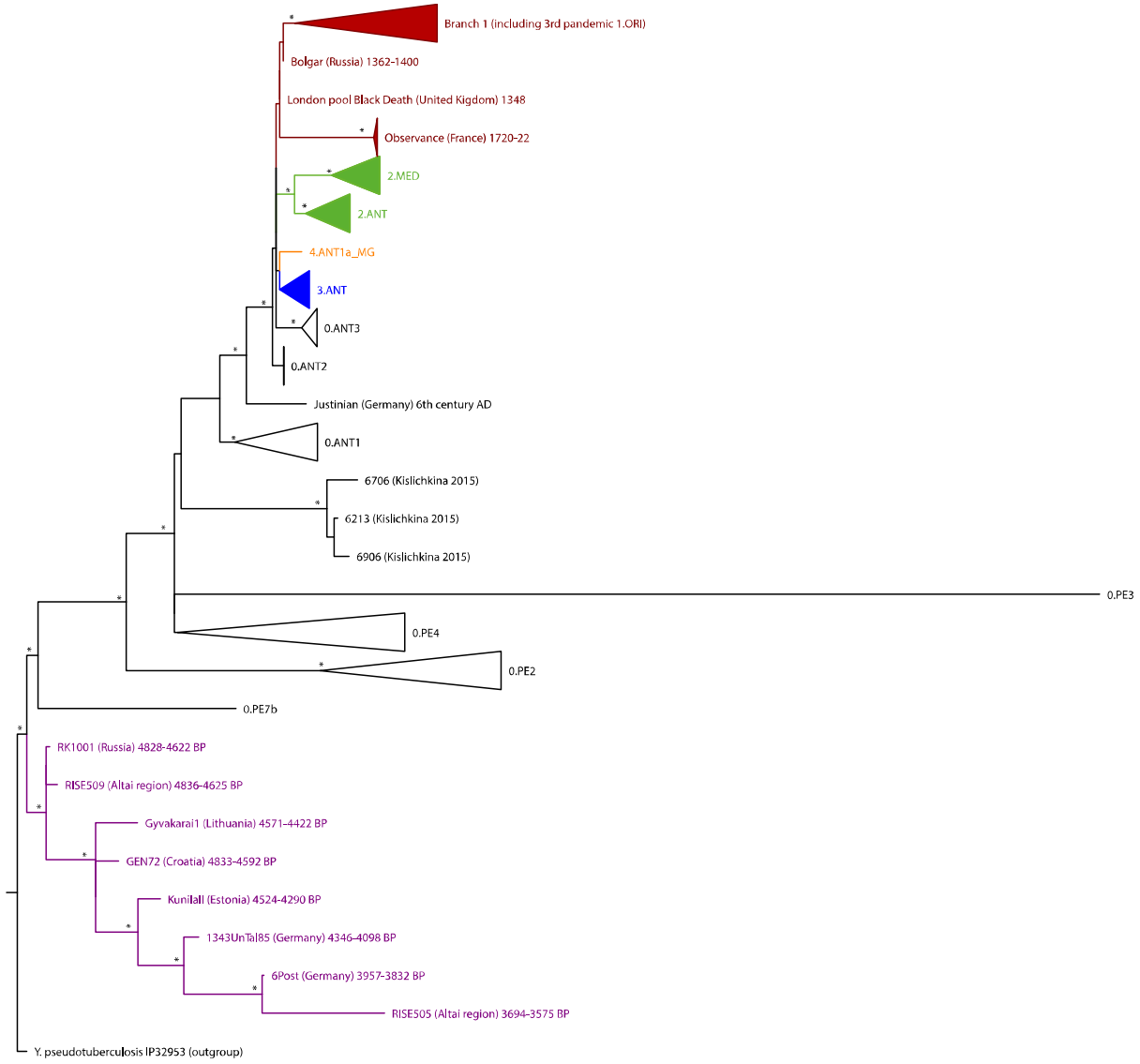
955



957 Figure 2

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962 Figure 3

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966 **Table 1**

Species name	Strain	NCBI Accession number
<i>Y. pestis</i>	CO92	NC_003143.1
<i>Y. pseudotuberculosis</i>	IP 32953	NC_006155.1
<i>Y. enterocolitica</i>	subsp. enterocolitica 8081	NC_008800.1
<i>Y. aldovae</i>	ATCC 35236	NZ_ACCB01000210.1
<i>Y. bercovieri</i>	ATCC 43970	NZ_AALC02000229.1
<i>Y. frederiksenii</i>	ATCC 33641	NZ_AALE02000161.1
<i>Y. intermedia</i>	ATCC 29909	NZ_AALF02000123.1
<i>Y. kristensenii</i>	ATCC 33638	NZ_ACCA01000153.1
<i>Y. mollaretii</i>	ATCC 43969	NZ_AALD02000179.1
<i>Y. rohdei</i>	ATCC 43380	NZ_ACCD01000141.1
<i>Y. ruckeri</i>	ATCC 29473	NZ_ACCC01000174.1

967

968 **Table 2**

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Individual	Tissue sampled	Site	Country	Dating (Median cal BP)	In-solution Capture	Clipped, merged and quality-filtered reads before mapping	Unique reads mapping to <i>Y. pestis</i> reference	Endogenous DNA (%)	Mean Coverage	Coverage (%)				
										>=1X	>=2X	>=3X	>=4X	>=5X
RK1001	Tooth	Rasshevatskiy	Russia	4720	no	1,529,935,532	119,540	0.01	1.0213	58.11	27.24	10.87	3.90	0.85
					yes	303,148,884	383,900	0.85	3.3984	82.83	69.42	55.3	41.95	30.7
					Combined shotgun/capture	1,833,084,416	418,581	0.17	3.6816	86.16	73.67	59.63	45.88	33.3
GEN72	Tooth	Beli Manastir-Popova Zemlja	Croatia	4721	yes	19,777,683	1,321,320	24.36	12.6549	91.65	89.15	86.61	83.84	80.5
Gyvakarai1	Tooth	Gyvakarai	Lithuania	4427	no	1,021,452,137	473,207	0.05	5.2245	94.07	90.96	84.12	73.1	59.7
Kunilall	Tooth	Kunila	Estonia	4203	no	379,155,741	546,243	0.16	5.5418	92.48	86.65	77.58	66.49	54.7
1343UnTal85	Tooth	Augsburg	Germany	3873	no	1,174,989,269	1,165,435	0.14	10.5745	93.69	93.29	92.59	91.29	89.0
6Post	Tooth	Augsburg	Germany	3635	no	419,717,299	598,030	0.17	5.3062	89.71	81.66	71.14	59.82	48.9

