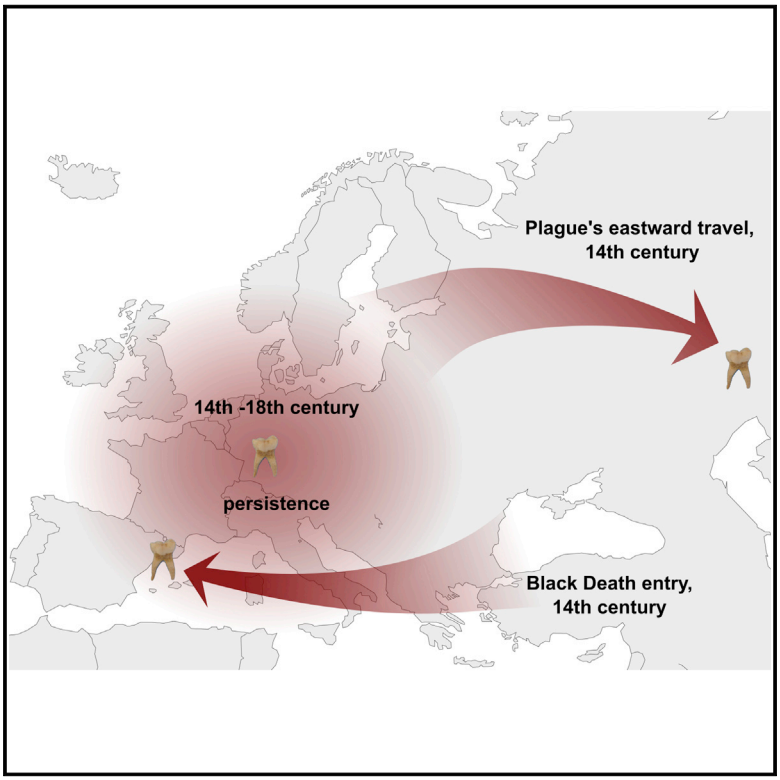
Short Article

Cell Host & Microbe

Historical *Y. pestis* Genomes Reveal the European Black Death as the Source of Ancient and Modern Plague Pandemics

Graphical Abstract



Highlights

- Three historical *Yersinia pestis* genomes from the second plague pandemic in Europe
- Low genetic diversity of the pathogen during the Black Death
- Indication for link between the Black Death and 19th century plague pandemic lineages
- Connection between post-Black Death outbreaks in Europe supports a local plague focus

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In Brief

Spyrou et al. have sequenced historical *Yersinia pestis* genomes from victims of the Black Death and subsequent outbreaks in Europe. Their data suggest a connection between the Black Death and the modern-day plague pandemic as well as the persistence of plague in Europe between the 14th and 18th centuries.

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Historical *Y. pestis* Genomes Reveal the European Black Death as the Source of Ancient and Modern Plague Pandemics

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SUMMARY

Ancient DNA analysis has revealed an involvement of the bacterial pathogen *Yersinia pestis* in several historical pandemics, including the second plague pandemic (Europe, mid-14th century Black Death until the mid-18th century AD). Here we present reconstructed *Y. pestis* genomes from plague victims of the Black Death and two subsequent historical outbreaks spanning Europe and its vicinity, namely Barcelona, Spain (1300–1420 cal AD), Bolgar City, Russia (1362–1400 AD), and Ellwangen, Germany (1485–1627 cal AD). Our results provide support for (1) a single entry of *Y. pestis* in Europe during the Black Death, (2) a wave of plague that traveled toward Asia to later become the source population for contemporary worldwide epidemics, and (3) the presence of an historical European plague focus involved in post-Black Death outbreaks that is now likely extinct.

INTRODUCTION

Yersinia pestis evolved from the closely related zoonotic enterobacterium *Y. pseudotuberculosis* (Achtman et al., 1999) to become one of the most virulent pathogens known to humans. Its recent identification in ancient human material from Altai, Siberia suggests it caused human infections as early as 5,000 years ago, though its ability for flea-borne transmission leading to bubonic disease might have been absent in these older, divergent lineages (Rasmussen et al., 2015). To our knowledge, bubonic plague, and presumably also the pneumonic and septicemic forms, have been the likely culprit of three major pandemics, namely the Plague of Justinian (Eastern Roman Empire, 6th and 8th centuries AD), the second-wave plague pandemic (Europe, mid-14th century Black Death until the mid-18th century AD), and the third plague pandemic that started during the late 19th century in China. Differences in mortality rate and epidemiology of the three pandemics initiated controversy over whether they shared a common etiologic agent (Cohn, 2008). In recent years, however, ancient DNA (aDNA) has confirmed a *Y. pestis* involvement in both historical pandemics (Bos et al., 2011; Haensch et al., 2010; Wagner et al., 2014).

The Black Death claimed up to 50% of the European population between 1347 and 1353 (Benedictow, 2004). The disease is thought to have arisen from plague foci in East Asia and to have spread into Europe via trade routes (Morelli et al., 2010). Its origin, however, is still contentious due to a lack of convincing archeological or documentary evidence from the early 14th century in East Asia (Sussman, 2011). Ancient *Y. pestis* genomes obtained from medieval victims have indicated the presence of a radiation event immediately preceding the Black Death that gave rise to most of the strain diversity circulating in the world today (Bos et al., 2011; Cui et al., 2013). Based on the relationship of ancient European and modern genomes, it was recently suggested that a wave of plague might have traveled from Europe toward Asia after the Black Death, eventually settling in China and later giving rise to the third pandemic (Wagner et al., 2014). Genomes from its purported route are, however, missing in the discussions, and are needed to add legitimacy to the model.

After the Black Death, plague continued to strike Europe for another four centuries through subsequent outbreaks that ceased at the end of the 18th century (Benedictow, 2004). The reasons for its sudden disappearance in Europe are unknown. Sylvatic plague foci have a nearly worldwide presence today, but are absent in Europe (Gage and Kosoy, 2005; Tikhomirov, 1999). The question of whether the recurrent European plague outbreaks of the 14th to 18th centuries were the result of multiple reintroductions of plague into Europe, or rather were attributed to now-extinct European plague foci, is still being explored. Previous studies that draw upon aDNA and climatic data favor the former hypothesis. Through a SNP-based PCR approach,

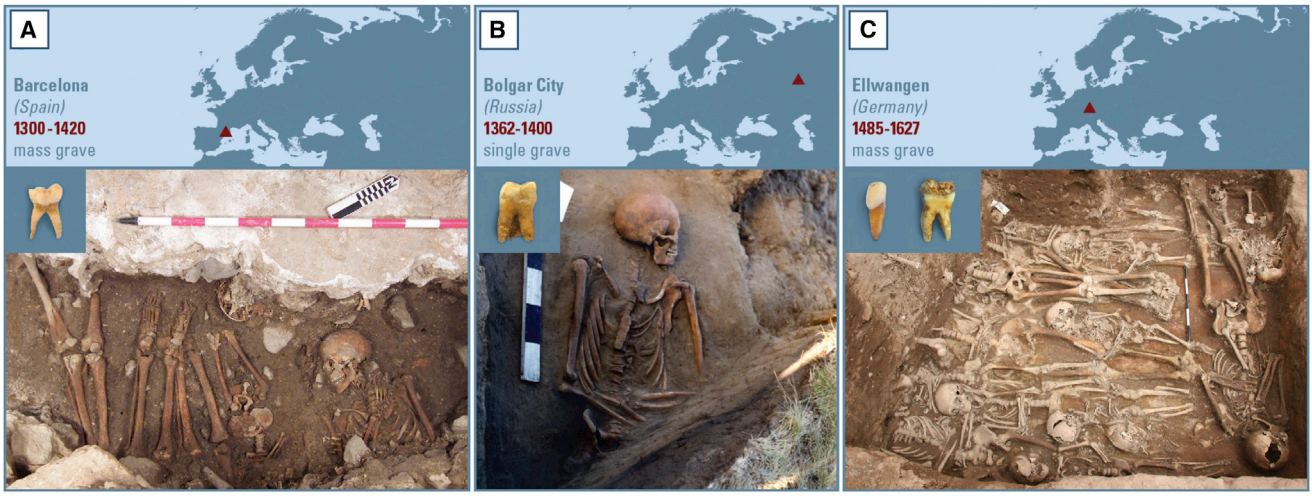

Figure 1. Samples and Their Respective Locations

(A) Tooth sample that was positive for *Y. pestis* (3031) and mass grave picture from the plague burial in Barcelona.

(B) *Y. pestis*-positive tooth sample and picture of infected individual (2370) from the Ust'-Ierusalimsky tomb of Bolgar City.

(C) Picture of mass grave in Ellwangen, and two tooth samples from individual 549_O, found positive for the plague bacterium.

purportedly distinct plague lineages were identified in different areas of Europe during the 14th century and were thought to have entered via different pulses (Haensch et al., 2010). In addition, plague outbreaks documented in some of the main Mediterranean ports were found to coincide with extreme climate fluctuations in Central Asia, suggesting that recurrent maritime imports of plague from Asia might have been responsible for post-Black Death plague outbreaks (Schmid et al., 2015). By contrast, others have suggested a long-term persistence of plague in Europe (Seifert et al., 2016). Using a PCR SNP-typing approach of putative plague material from Southern and Northeastern Germany, identical *Y. pestis* SNP profiles were identified in strains circulating within Europe between the Black Death and 17th century AD (Seifert et al., 2016), implying a single source population for the European plagues of that time period. A further genome-wide analysis of *Y. pestis* strains from the Great Plague of Marseille (1720–1722) has identified a previously uncharacterized lineage of *Y. pestis* that descends from a strain present during the Black Death (Bos et al., 2016). While the lineage is considered to represent an historical plague focus potentially responsible for post-Black Death European outbreaks (Bos et al., 2016), the use of material from a highly operational Mediterranean center that linked Western Europe with the East (Signoli et al., 1998) makes identification of the disease source elusive.

Here, we aim to address three outstanding questions regarding *Y. pestis* history. First, we investigate the possibility of disease entry via multiple pulses during the Black Death by comparing the genotype of a strain from the pandemic's early phase to those circulating in other areas later in the pandemic. Material from Barcelona, Spain, one of the Mediterranean cities through which plague entered southern continental Europe, is compared to Black Death genomes from London. Second, we evaluate the likelihood of the proposed eastward migration of strains from Europe to Asia after the Black Death through the analysis of human remains from a 14th century plague burial in the Volga region of Russia. Third, we take a further step toward understanding the relationship of post-Black Death outbreaks in Europe and evaluate the likelihood of a local reservoir. For this, we investigate a 16th century plague outbreak in Southwestern Germany and compare it to both a London outbreak that occurred soon after the Black Death and to the Great Plague of Marseille, France in 1722. Following the success of previous genomic investigations of ancient bacterial disease (Bos et al., 2011, 2014, 2016; Schuenemann et al., 2011, 2013; Wagner et al., 2014), we employ similar methods of DNA capture and high-throughput sequencing to retrieve the genomes of three historical *Y. pestis* strains. Our results suggest (1) limited *Y. pestis* diversity during the early phase of the Black Death, and likely a single entry into Europe; (2) a wave of plague that traveled eastward after the Black Death and later gave rise to the 19th century pandemic; and (3) an involvement of the same plague lineage in two post-Black Death European epidemics that are 200 years apart.

RESULTS

Archaeological Sites and Dating

Samples were collected from a mass grave in Barcelona, Spain, a single grave in Bolgar City in Russia, and a mass grave in Ellwangen, Germany (Figure 1 and Supplemental Experimental Procedures). Aside from the Bolgar City site that was dated to the second half of the 14th century using coin artifacts known to have been minted after 1362 (Supplemental Experimental Procedures and Figure S1), archaeological dates were not available. To estimate or confirm the historical period during which each of the outbreaks occurred, radiocarbon dates from bone fragments and tooth roots were obtained. The dates yielded were 1300–1420 cal AD for Barcelona, 1298–1388 cal AD for Bolgar City, and 1486–1627 cal AD for Ellwangen (Figure 1 and Table S1).

Screening for *Y. pestis*

A total of 223 DNA extracts from teeth of 178 individuals were evaluated for the presence of *Y. pestis* DNA through a

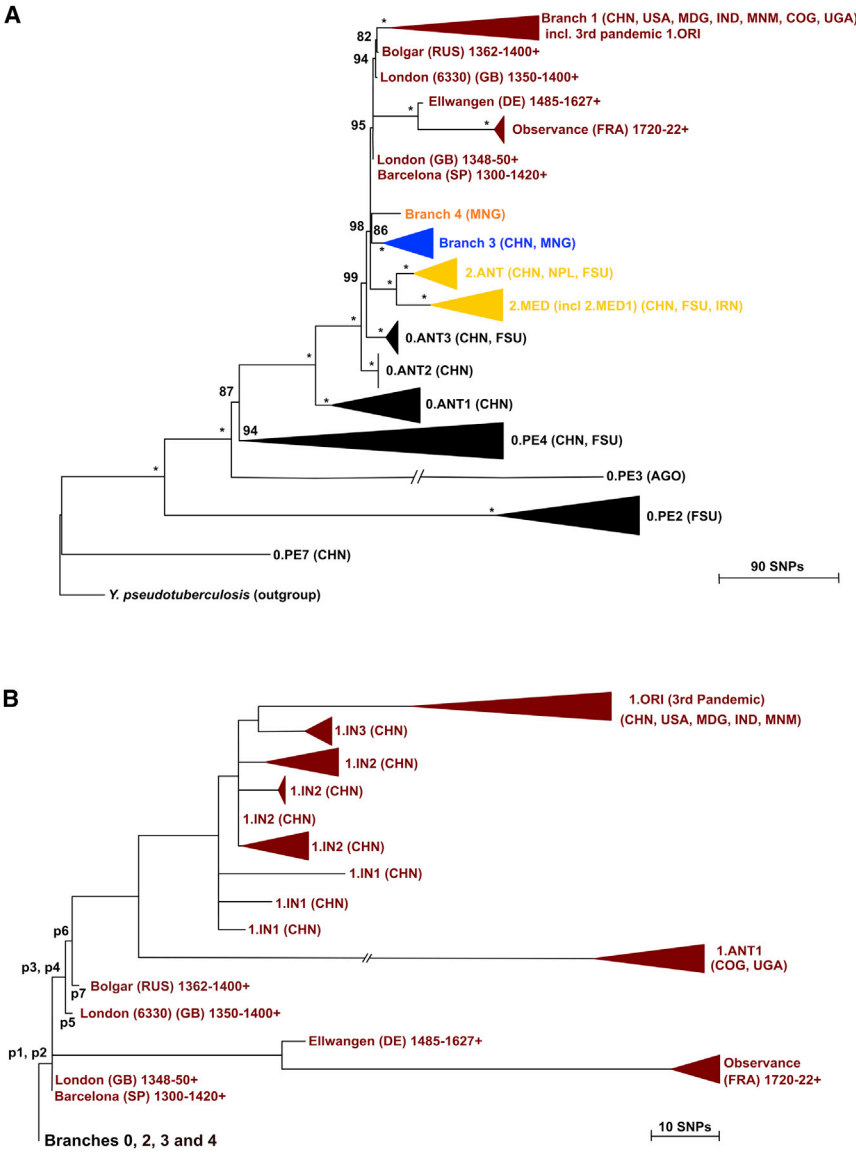

Figure 2. *Yersinia pestis* Phylogeny

(A) Maximum Parsimony phylogenetic tree of 141 modern and 10 historical *Y. pestis* strains. 3,351 SNP positions were considered for the phylogeny. The reconstructed tree shows the topology of the new isolates from Barcelona, Bolgar City, and Ellwangen relative to previously sequenced modern and ancient *Y. pestis* strains. Asterisks (*) indicate bootstrap values of 100. Collapsed branches are represented by triangles, to enhance tree clarity. Strains belonging to Branch 1 are represented in red, Branch 2 in yellow, Branch 3 in blue, Branch 4 in orange, and Branch 0 in black. Ancient Branch 1 strains are indicated by their archaeological or radiocarbon date and by a (+). Because of the great number of derived SNP positions of the 0.PE3 lineage, its branch was reduced to adjust scaling of the tree. Geographic region abbreviations correspond to: CHN (China), USA (United States of America), MDG (Madagascar), IND (India), IRN (Iran), MNM (Myanmar), RUS (Russia), GB (Great Britain), DE (Germany), FRA (France), SP (Spain), MNG (Mongolia), NPL (Nepal), FSU (Former Soviet Union), AGO (Angola), CGO (Congo), and UGA (Uganda).

(B) A magnified version of Branch 1 is shown to enhance its resolution. The branch of lineage 1.ANT was manually reduced to adjust tree scaling. A detailed description of p1-p7 SNPs is given in Table 1 (see also Table S2, Table S3, Table S4 and Figure S2).

species-specific quantitative PCR (qPCR) assay targeting the plasminogen activator (*pla*) gene located on the PCP1 plasmid (Schuenemann et al., 2011) (Supplemental Experimental Procedures). Results indicated 53 potentially positive DNA extracts stemming from 32 individuals. All extraction and PCR blanks were free of amplification products. Amplification products were not sequenced, as samples from potentially positive individuals were directly turned into double-stranded next-generation sequencing libraries and were used for whole-genome array capture. After capture, three individuals had sufficient *Y. pestis* DNA for genome-level analysis. These were tooth specimens 3031 from Barcelona, 2370 from Bolgar City, and 549_O from Ellwangen (Figure 1, Table S1 and Supplemental Experimental Procedures).

Y. pestis Genome-Capture Results

Whole-genome array capture was performed using the chromosome of *Y. pseudotuberculosis* (Chain et al., 2004) and the *Y. pestis* plasmids pMT1 and pCD1 as template for probe design (Supplemental Experimental Procedures). Array captures produced average genomic coverage of 10.3-fold for Barcelona 3031, 19.3-fold for Bolgar City 2370, and 4.9-fold for Ellwangen 549_O (Table S1 and Table S2). Owing to its low coverage, data presented for sample 549_O are from a pool of two independent libraries produced from two teeth of the same individual (Table S1 and Table S2).

Phylogenetic Analysis of Historical *Y. pestis* Genomes

Our ancient genomes were then added to a *Y. pestis* phylogeny constructed from previously published genomes including 130 modern genomes (Cui et al., 2013), 7 historical genomes (Bos et al., 2011, 2016), and 11 newly available modern *Y. pestis* strains from the Former Soviet Union (Zhgenti et al., 2015) (Table S3). Our maximum parsimony tree revealed that the modern Former Soviet Union genomes group with what was previously thought to be diversity restricted in China, specifically lineage 0.ANT3 (Cui et al., 2013). They also add further diversity to the 2.MED1 lineage and, importantly, to the 0.PE2 lineage, which is the second deepest branch in the *Y. pestis* phylogeny (Figure 2A, Figure S2, and Table S3). This reveals a more extensive *Y. pestis* diversity outside of China than was previously thought.

Table 1. SNP Description of Diagnostic Branch 1 Positions in the Newly Sequenced Ancient *Y. pestis* Genomes

SNP Name	Position on Chromosome CO92	CO92 (Reference)	Barcelona	Bolgar City	Ellwangen	Gene
p1	189,227	C	C	C	C	pabA
p2	1,871,476	G	G	G	G	NC[a]
p3[b]	699,494	A	G	A	G	alt (rpoD)
p4	2,262,577	T	G	T	G	YPO1990
p5	4,301,295	G	G	G	G	recQ
p6	3,806,677	C	T	C	T	b0125 (hpt)
p7	3,643,387	G	G	T	G	YPO3271

[a]Non-coding (NC).

[b]The p3 SNP corresponds to the previously described s12 position present in a Black Death plague victim from the Netherlands (Haensch et al., 2010). It is also present in a derived state isolate from London (6330), from which a complete genome is available (Bos et al., 2011).

All three reconstructed historical genomes group in Branch 1, and all possess diagnostic SNP positions here referred to as "p1" and "p2" (Table 1), which were previously identified in historical *Y. pestis* genomes from the Black Death (Bos et al., 2011) (Figure 2B, Table 1). The positioning of the strains reported here in the phylogeny confirms their authenticity as ancient. To date, all *Y. pestis* genomes isolated from the historic 2nd plague pandemic group in Branch 1.

We find no detectable differences between our Black Death strain from Barcelona and three previously genotyped strains from London 1348–1350 (Bos et al., 2011). The Bolgar City strain, however, contains additional differences in four positions compared to Black Death isolates: two of these are shared with London individual 6330 (positions p3 and p4, Figure 2B and Table 1), one is shared with all modern Branch 1 strains (p6), and one is unique to this individual (p7, Figure 2B). Additionally, the Ellwangen strain groups in a sub-branch of Branch 1, together with five strains previously typed from the Great Plague of Marseille (L'Observance), 1720–1722 (Figure 2B) (Bos et al., 2016). Our analysis reveals 20 positions shared with the strains from L'Observance and three unique SNPs (Table S4). That the Ellwangen strain is ancestral to the Observance strains comes as no surprise given the older age of the samples (Figure 2B). This "Ellwangen-Observance" lineage originates from Black Death strains currently represented by the isolates from London and Barcelona. Like the strain from Marseille, that from Ellwangen does not share additional derived positions with other ancient or modern strains (Figure 2B), as no modern descendants have as yet been identified in this sub-branch.

DISCUSSION

Our genomes from Barcelona, Bolgar City, and Ellwangen group on the same phylogenetic branch (Branch 1), adding further legitimacy to the notion that the Black Death and subsequent plague outbreaks in Europe, as well as the worldwide third pandemic, were caused by the same *Y. pestis* lineage (Figure 2, Figure S2, and Figure 3). Further analysis of ancient and modern strains of this branch could reveal important clues to explain why this particular lineage was involved in both the second and third pandemic.

Our analysis reveals that the strain from Barcelona is identical to a previously sequenced Black Death *Y. pestis* strain from London (1348–1350). Barcelona was one of the main entry points for the Black Death into Europe, with historical reports suggesting the disease first entered there in the spring of 1348 (Gottfried, 1983). In London, the earliest reports of the illness are from autumn 1348 (Benedictow, 2004). This indicates a contemporary presence of the same strain in both southern and northern Europe, supporting the notion of a single wave entry, with low genetic diversity in the pathogen. Historical sources indicate that plague first came into view in 1347, with outbreaks in the southern islands of Crete, Sicily, and Sardinia, followed by entry into mainland Europe via the heavily trafficked ports of Genoa and Marseille. Samples from these locations and those further afield from its purported source population in East Asia may provide us with relevant details regarding the microevolution of a highly virulent pathogen at the beginning of a mass pandemic.

The key finding of our study stems from the analysis of an historical *Y. pestis* strain from the Volga region in Russia (Figure 3). This genome has added legitimacy to an important link between the second and third plague pandemics hypothesized elsewhere (Wagner et al., 2014). Under this model, *Y. pestis* spread from Europe to Asia after the Black Death and gave rise to both the 1.IN lineages of the Yunnan Province of China (1.IN3) as well as the 1.ORI strains associated with worldwide spread during the third plague pandemic (Figure 2B, Figure 3, and Table S3) (Wagner et al., 2014). That our sample from Bolgar City shares one additional Branch 1 derived position with a strain circulating in London during the second half of the 14th century provides solid evidence of plague's eastward travel subsequent to the Black Death (Table 1, Figure 2B, and Figure 3). Of note, the 1.ANT lineage today restricted to Sub-Saharan Africa possesses only an additional ten derived Branch 1 positions compared to our Bolgar lineage (Table S4). A compelling possibility is that this plague lineage was introduced via European presence in the continent: its shared ancestry with the Bolgar lineage could imply that it derives from an historical focus that existed along the eastern path that *Y. pestis* traveled after the Black Death. We therefore consider it possible that strains ancestral to these African lineages may have caused disease in Europe during the second wave and may one day be identified in ancient European skeletons.

As the geographical origins of the "p1" and "p2" SNPs are unknown (Figure 2B), the possibility of Branch 1 lineages arising from pre-existing diversity in Asia and independently dispersing into Europe must be considered (Haensch et al., 2010). This model is supported by climatic evidence, where regular

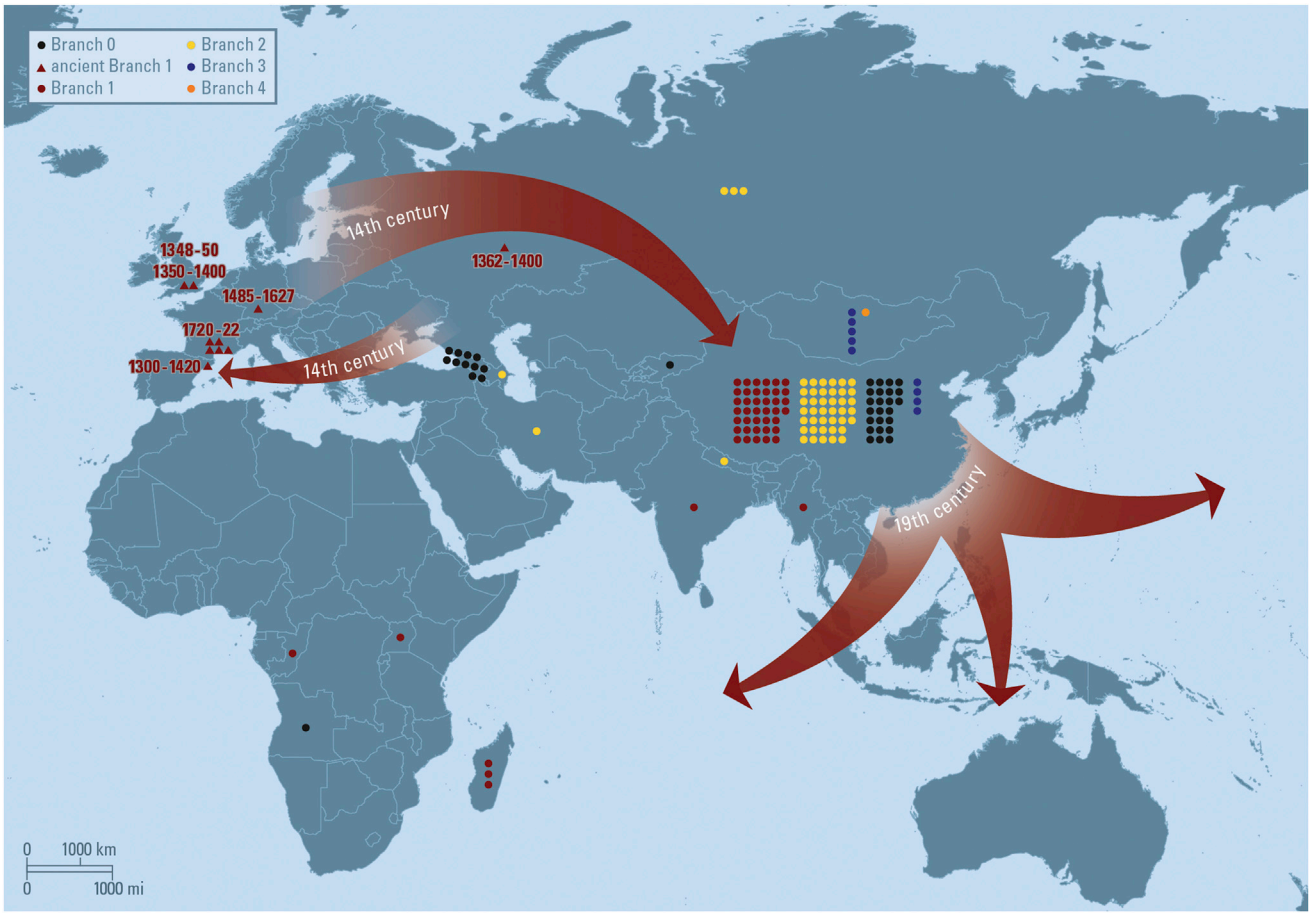

Figure 3. Plague Introduction and Dispersal

Map describing our favored dissemination pattern of *Y. pestis* during the second and third plague pandemics. All strains included in our dataset are depicted as points on the map. Branch 1 strains are in red and include both second pandemic (triangles) and modern (circles) isolates. Branch 2 strains are in yellow, Branch 3 strains are in blue, a single Branch 4 strain is in orange, and Branch 0 strains are in black. Positioning of modern strain distribution on the map corresponds to geographic location, but for the purpose of our study an accurate coordinate system was not necessary. Red arrows indicate Branch 1 cycling through Europe during the 14th century, eastward travel out of Europe after the Black Death, and global dissemination from China during the third plague pandemic (see also Table S3).

westward pulses of plague from an Asian focus throughout the second pandemic are thought possible (Schmid et al., 2015). We find this model for the second pandemic difficult to reconcile with our current data. Although it has been previously shown that *Y. pestis* has an extremely variable substitution rate (Cui et al., 2013), our Russian strain has only two additional derived substitutions (p6, p7, Figure 2) compared to London *Y. pestis* genome 6330 (Bos et al., 2011), dated to 1350–1400. This close genetic similarity suggests that our Russian strain represents a new outbreak subsequent to that which occurred in London after the Black Death. The alternative "Asian origin" model would require a minimum of four separate lineages exiting together from the same focus to account for the level of diversity observed in Europe during the Black Death and its aftermath, i.e., (1) London/Barcelona, (2) London 6330, (3) Bolgar City, and (4) Sub-Saharan Africa. We regard the likelihood of such similar strains leaving Asia in a short time frame to be low, but acknowledge it would be possible if (1) their ancestral focus was in a location particularly conducive to westward travel, or (2) there exists a biological reason for their greater ease in rapid long-distance travel. While the above scenarios could equally explain the sole involvement of Branch 1 in contemporary plague outbreaks outside of China, we regard a single exit followed by an eastward travel as a more parsimonious explanation for the current data. Under this scenario, historical strains carrying the previously described "p3" SNP (Figure 2B) subsequently traveled east to later become established in China, whereas those giving rise to the Ellwangen-Observance lineage did not (Figure 3). Once in the Former Soviet Union, plague likely became established in rodent populations in an area accessible to western Russia and evolved locally, as evidenced by the single unique derived position in our strain from Bolgar City (Figure 2B and Figure 3). Given that all modern Branch 1 lineages descend from a close hypothetical relative of our Russian strain, these European forms may well have given rise to the third plague pandemic in China and beyond.

Consensus has not yet been reached regarding the role played by the Russian region in the introduction of plague into Europe during the Black Death (Alexander, 1980; Benedictow, 2004; McNeill, 1998; Norris, 1977; Schmid et al., 2015). Drawing

upon historical and climatic data, scholars have adopted a "proximal origin" theory, which states that the Black Death erupted from plague foci in the Caucasus and neighboring areas (Alexander, 1980; Benedictow, 2004; Norris, 1977; Sussman, 2011; Varlik, 2015). Molecular investigations of the plague bacillus, however, have pointed to China as both the birthplace of *Y. pestis* itself and the origin of the Black Death (Cui et al., 2013; Morelli et al., 2010). This is difficult to reconcile with the strong East Asian sampling bias of the available data, coupled with the fact that the second most basal *Y. pestis* lineage sampled thus far stems from a rodent focus in the Former Soviet Union (Cui et al., 2013) (Figure 3). In our current investigation, we attempted to partially overcome this limitation by integrating recently sequenced strains from the Caucasus region (Zhgenti et al., 2015) in our *Y. pestis* phylogeny. To our surprise, these strains grouped with some lineages previously thought to be mostly or entirely restricted to China (Figure 2A). We therefore highlight the need to expand the sampling region of both modern and ancient *Y. pestis* to establish a more comprehensive understanding of its evolutionary history and modern ecology.

Our plague strain from the German city of Ellwangen is ancestral to those associated with the Great Plague of Marseille (L'Observance), an epidemic that occurred in France some 200 years later (Figure 2B). This branch descends directly from the strain circulating in both London and Barcelona during the Black Death and does not possess the additional Branch 1 positions present in the London 6330 and Bolgar lineages described above. That the Ellwangen genome shares 20 positions with the Marseille strain and has three unique positions (Table S4) suggests the two share a common genetic history and diverged from the same source population in advance of the 16th century Ellwangen outbreak. A previous study has pointed to natural plague foci in Asia as likely sources of the multiple plague outbreaks in Europe following the Black Death (Schmid et al., 2015). An alternative model, however, proposes a local European source for plague, given the high number of documented sporadic epidemics in isolated rural areas throughout the second wave. Alpine rodent species are considered one possible reservoir (Carmichael, 2014). Both models are explored in recent aDNA analyses of post-Black Death European plague material (Bos et al., 2016; Seifert et al., 2016), though at a resolution too low to strongly favor one hypothesis over the other. Based on modern epidemiological data, no known plague foci exist within Europe; however, several foci are suspected to exist in areas along the former Silk Road, the most prolific of which are immediately to the east of the Caspian Sea (Gage and Kosoy, 2005). The geographical location of the city of Ellwangen, and the seemingly restricted outbreak here, however, makes the introduction of disease via trade routes outside of Europe unlikely. We rather view our data as more supportive of a European reservoir for the disease. As only a small rodent focus with limited exposure to a susceptible host species is thought to be theoretically sufficient to initiate a large-scale human plague epidemic (Keeling and Gilligan, 2000), plague's presence in this proposed European reservoir need not have been large. The Ellwangen-Observance lineage contains no known extant descendants; hence, this focus may no longer exist (Figure 2B), and its extinction may have coincided with the sudden disappearance of plague in Europe. The popular theory of an 18th century domestic rodent replacement of *Rattus rattus* by *Rattus norvegicus* (Appleby, 1980) could still carry some traction. The black rat is a well-known harbinger of plague in several locations where *Y. pestis* infections persist today (Duplantier et al., 2005; Vogler et al., 2011), and though brown Norway rats have a similar susceptibility to plague infection (Anderson et al., 2009), their different ecological niche and comparatively reduced contact with humans in a domestic setting may have slowed the transmission of disease entering from a neighboring sylvatic population.

Our phylogeny is compatible with popular demographic scenarios wherein the Black Death cycled through the Mediterranean (Barcelona), spread to Northern Europe (London), subsequently traveled east into Russia (Bolgar), and eventually made its way into China, its presumed origin and ultimate source of the modern plague pandemic (Figure 3). The most parsimonious interpretation of our data holds that, in the course of its travels, a minimum of one plague lineage was left behind along its route that persisted long enough to later diversify and give rise to at least two subsequent epidemics—one in 16th century Germany and one in 18th century France (Bos et al., 2016). The above proposal, however, is unlikely to explain the full spectrum of *Y. pestis* diversity and plague epidemics during the notorious so-called "second wave" plague pandemic; a unidirectional dispersal of *Y. pestis* is unlikely, as multiple factors are sure to have contributed to its spread in humans and other host species. The epidemics in Germany and France, for example, stemmed from only one of possibly several historical plague foci within Europe or its vicinity. Concurrent plague foci harboring strains related to our Bolgar lineage, to the lineage identified in late 14th century London, or potentially others not yet identified may have been responsible for additional second wave plague outbreaks. Currently there is a lack of ancient *Y. pestis* data from the proposed entry and end points of the Black Death in Europe (Gottfried, 1983). Genetic analyses of putative plague material from these regions would be essential in unraveling additional key features related to the paths traveled by the Black Death and the legacy it left behind.

EXPERIMENTAL PROCEDURES

Array Design and Captures

A one-million-feature Agilent microarray was designed with an in-house probe design software using the chromosome of *Yersinia pseudotuberculosis* (NCBI: NC_006155) (Chain et al., 2004), as well as the *Y. pestis* (CO92) plasmids pMT1 (NCBI: NC_003134) and pCD1 (NCBI: NC_003131). DNA extracts from *pla*-positive samples (Supplemental Experimental Procedures) were turned into double-stranded DNA libraries as described before (Meyer and Kircher, 2010). Serial hybridization-based array capture was performed using previously established methods (Hodges et al., 2009) (Supplemental Experimental Procedures).

High-Throughput Sequencing and Read Processing

Following high-throughput sequencing on Illumina platforms, all pre-processing mapping and genotyping steps were performed using the automated pipeline EAGER (Peltzer et al., 2016). For SNP filtering, the MultiVCFAnalyzer custom java program was applied to all *vcf* files to comparatively filter all detected SNPs (Supplemental Experimental Procedures).

Phylogenetic Reconstruction

A SNP table was used as input for phylogenetic reconstruction. Phylogenetic trees were generated using the Maximum Parsimony (MP) and Maximum Likelihood (ML) methods available in MEGA6.06 (Tamura et al., 2013),

discarding alignment columns with more than 5% missing data. The three newly reconstructed *Y. pestis* strains from Barcelona, Bolgar City, and Ellwangen were analyzed alongside seven previously sequenced historical strains from the second plague pandemic (Bos et al., 2011, 2016) and 141 published modern *Y. pestis* strains (Cui et al., 2013; Zhgenti et al., 2015). A *Y. pseudotuberculosis* strain (IP32953) (Chain et al., 2004) was used as outgroup for rooting the tree, and all its derived SNPs were removed to scale branch lengths (Supplemental Experimental Procedures).

ACCESSION NUMBERS

Raw sequencing reads produced for this study have been deposited at the European Nucleotide Archive (ENA) under accession number ENA: PRJEB13664.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.05.012.

AUTHOR CONTRIBUTIONS

J.K., K.I.B, A.H., and M.A.S. conceived the study; M.A.S., R.I.T., M.F., and K.I.B. performed laboratory work; M.A.S., A.H., K.I.B., and J.K. analyzed data; J.B.d.H., S.A., D.C., J.W., I.R.G., A.G.S., and D.K.N. provided archaeological material and archaeological context information; J.D., S.K., D.C., J.W., and I.R.G. performed anthropological and paleopathological examination; M.A.S., K.I.B., A.H., and J.K. wrote the manuscript with contribution from all co-authors.

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Supplemental Information

Historical *Y. pestis* Genomes Reveal the European Black Death as the Source of Ancient and Modern Plague Pandemics

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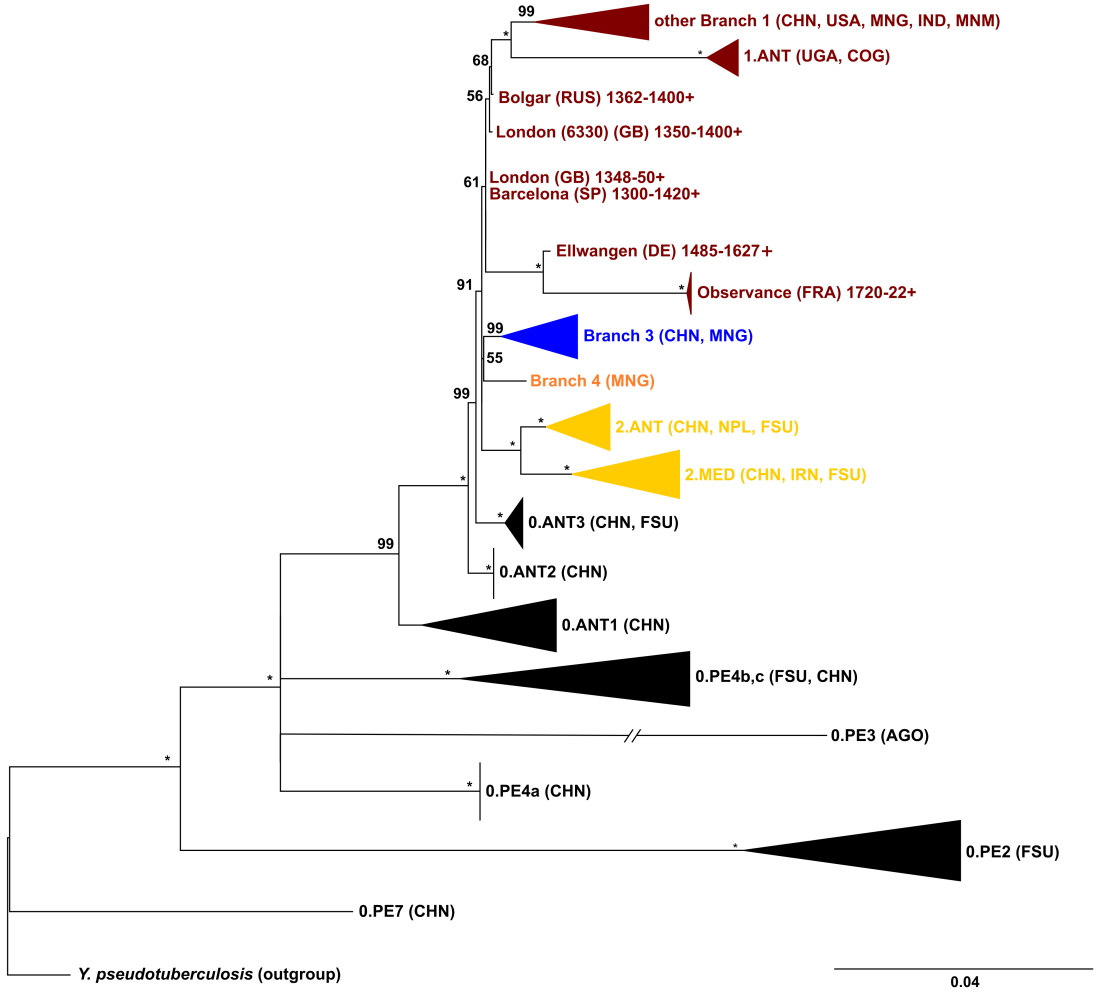
Supplemental Figures and Tables

Figure S1

Supplemental Figure 1, Related to Figure 1. Archaeological artifacts associated with the Bolgar City site.
(A-L) Coin artifacts associated with the burial of individual 2370 from Bolgar City, Ust'Jerusalem tomb, were minted and released during the times of Murad Khan and Abdullah Khan ibn Uzbeg Khan and date the burial to the second half of the 14th century, in the period after 1362 AD.

Figure S2



Supplemental Figure 2, Related to Figure 2 and Figure 3. Maximum Likelihood phylogeny. Maximum Likelihood phylogenetic tree of 141 modern and 10 historical *Y. pestis* strains. A *Y. pseudotuberculosis* strain was used as outgroup (IP32953). 3351 SNP positions were considered for the phylogeny. The reconstructed tree shows the topology of the newly sequenced isolates from Barcelona, Bolgar City and Ellwangen relative to previously sequenced modern and ancient *Y. pestis* strains. Asterisks (*) indicate bootstrap values of 100. Collapsed branches are represented by triangles, to enhance tree clarity. Strains belonging to Branch 1 are represented in red, Branch 2 is represented in yellow, Branch 3 is represented in blue, Branch 4 is represented in orange and Branch 0 is represented in black. Ancient Branch 1 strains are indicated by their archaeological or radiocarbon date and by a (+). Because of the great number of derived SNP positions of the 0.PE3 lineage, its branch was reduced to adjust scaling of the tree. Geographic region abbreviations correspond to: CHN (China), USA (United States of America), MDG (Madagascar), IND (India), IRN (Iran), MNM (Myanmar), RUS (Russia), GB (Great Britain), DE (Germany), FRA (France), SP (Spain), MNG (Mongolia), NPL (Nepal), FSU (Former Soviet Union), AGO (Angola), CGO (Congo) and UGA (Uganda).

Table S1

Supplemental Table 1, Related to Figure 1 and Table S2. Sample description, screening results and sequencing statistics.

Sample ID	Site	Tooth type	Dates cal AD	Archaeological dating	Copies of pla (copies/µl)	CO92 Chromosomal fold-coverage
3031	Barcelona	Molar	1300-1420[a]	-	174	10.3x
2370	Bolgar City	Molar	1298-1388[b]	1362-1400 AD[c]	553	19.3x
549_O	Ellwangen	Molar/ Incisor	1485-1627[b]	-	3/2	4.9x[d]

[a]Published in (Beltrán de Heredia Bercero, 2014)
[b]Radiocarbon dates produced for this study, presented in calibrated year AD (1 sigma)
[c]Archaeological dates point to the second half of the 14th century, and specifically the period after 1362 AD (see also Supplemental Experimental Procedures and Figure S1)
[d]Pooled data from four independent serial capture experiments, using two libraries generated from two tooth samples belonging to the same individual

Table S2

Supplemental Table 2, Related to Figure 2. Read processing and mapping statistics

Sample ID	Site	Number of pre-processed reads before mapping	Number of mapped reads	Duplication factor	Number of mapped reads after map quality filtering	Average coverage on CO92 Chromosome	Percentage of CO92 chromosome covered 3-fold
3031	Barcelona	60,516,261	2,116,962	2.43	870,477	10.3	89.88%
2370	Bolgar	47,595,565	4,183,226	2.49	1,682,954	19.28	91.46%
549_O	Ellwangen	145,645,509	2,120,360	4.79	442,573	4.91	73.56%

Supplemental Table 3, Related to Figure 2 and Figure 3. Geographical regions of *Y. pestis* genomes used for SNP calling and phylogeny

Supplemental Table 4, Related to Figure 2. SNP table including non-unique and unique SNPs of all second pandemic *Y. pestis* strains sequenced to-date

Supplemental Experimental Procedures

Information on archaeological sites and aDNA specimens

Saints Màrtirs Just i Pastor, Barcelona, Spain
The Barcelona mass grave was discovered in 2012 during an excavation campaign performed in the sacristy of the Saints Màrtirs Just i Pastor church, and is likely the first Black Death burial discovered in Spain. The burial was only partially excavated due to security restrictions (i.e. it's proximity to the sacristy's walls) and its western part was destroyed by the foundations of the gothic church. The pit was 1.50 m deep, and estimated to have been 5.5 m long and 4 m wide. Individuals were deposited in 11 successive layers, each layer containing between 4 and 15 skeletons. During the excavation, the skeletons of 120 individuals were recovered, of which 50 non-adults (i.e. less than 20 year-old) and 70 adults, out of an estimated number of 350-400 individuals buried in the pit (Beltrán de Heredia Bercero, 2014). Due to partial excavation of the burial, most of the skeletons recovered were incomplete. Most of the individuals were lying on their back, with their upper limbs in a flexed position (hands on the thorax or abdomen), and their lower limbs extended. None of the individuals were in prone position, and only a few were on their side. Regarding burial methods, no coffins were used. Bodies were directly placed in the grave all together, and the pit was subsequently filled with earth. The excavation, however, revealed textile remains (linen and hemp), suggesting that bodies were wrapped in shrouds. It is noteworthy that a layer of lime was deposited on top of the accumulation of cadavers; this is the only one example of use of such prophylactic material in a medieval plague pit (Kacki, 2014; Schotsmans et al., 2015). For this study, 49 teeth were removed from 18 individuals and used for biomolecular analysis. According to osteological examination of long bone length and dental development, individual 3031 from Barcelona, from which a *Y. pestis* genome was recovered, was most likely a 6-9 year old non-adult, whose sex could not be determined. Although the skeleton was only partly preserved, there was no detectably diagnostic feature of a specific pathological condition. Stress markers such as *cribra orbitalia* and mild developmental dental defects (linear enamel hypoplasia), however, may indicate malnutrition earlier in life.

Ust'-Jerusalem necropolis and Bolgar City mausoleum, Russia
The medieval city of Bolgar was situated on the bank of the Volga River, 30 km downstream from its confluence with the Kama River and some 130 km from modern Kazan (Tatarstan, Russian Federation). Bolgar City was an early settlement of the civilization of Volga-Bolgars, which existed between the 7th and 15th centuries AD and was intermittently capital of Volga Bulgaria between the 10th and 15th centuries (Sitdikov, 2014). Bolgar City was also the first capital of the Golden Horde in the 13th century. The UNESCO World Heritage Committee declared the ancient Bolgar hill fort as a World Heritage Site in 2014. The Ust'-Jerusalem necropolis was excavated between 1996 and 2003, covers an 800 sq. m. area and includes 318 single burials (Vasiliev, 2004). Palaeodemographic analysis revealed a high infant mortality rate (over 57% of the group), which may have been attributed to unfavorable social and environmental conditions, early childbirth in women, and a significant lack of food resources (Boruckaya, 2003). For the current investigation, material was chosen from the anthropological collections of the Ust'-Ierusalimsky tombs (Figure 1b) and the Bolgar city mausoleum (Vasiliev, 2004). A total of 95 teeth were extracted from 93 individuals and used for ancient DNA analysis. A complete skeleton was recovered from the plague victim (2370) of the Ust'Jerusalem tomb in Bolgar City. Anthropological analysis revealed a 35-40 year old male, whose burial was not consistent with medieval Muslim funerary practices (Figure 1b). Coin artifacts that were discovered during excavation of the burial dated the site to the second half of the 14th century. Grave artifacts associated specifically with individual 2370 consisted of 12 silver coins (Figure S1), the earliest of which date to 1362 AD. Such coin types are considered to have been minted and released during the times of Murad Khan and Abdullah Khan ibn Uzbeg Khan of the Golden Horde (Bosworth, 1996), who ruled between 1362 and 1370 AD (Figure S1).

"Marktplatz" Ellwangen, Germany
Excavations of the Ellwangen "Marktplatz" that were initiated as a tribute to the 1250th anniversary of the city, revealed a burial ground proposed to have been used for about a millennium, with human remains unearthed spanning form the 8th to the 18th centuries AD (Arnold, 2014). The cemetery contained 3 mass graves and 14 multiple burials amongst single burials. To-date, a total of 800 individuals has been identified and unearthed. The mass graves included a total of 102 individuals, and presented a case of unstructured burial practices clearly reflecting an event of mass mortality (Wahl, 2014). It is possible that part of the multiple burials were also attributed to the same catastrophic event.

No signs of warfare were detected in the remains, and skeletal indications of infectious disease were unspecific and not uniform across the individuals. Microscopical analyses detected the presence of intestinal parasites among skeletal material, possibly indicating unhygienic living conditions of the population. Within the mass graves, 80% of individuals were determined to be non-adults (<20 years) with an average age of 9.4 years (Wahl, 2014). In addition, the multiple burials contained a total of 73, mostly incomplete, skeletons that were distributed across 14 graves with 2 to 10 individuals in each grave. In this case the average age was much higher, estimated to 17.4 years. For the present study, 79 teeth were removed from 67 individuals for ancient DNA analysis. Individual 549_O from Ellwangen, from which a *Y. pestis* genome was reconstructed, was identified as a 12-14 year old non-adult, whose sex could not be confidently determined. In this case, the skeletal material recovered was also incomplete, with non-specific bone changes and dental defects, including calculus formation (Figure 1c).

DNA Extraction from archaeological material

Extractions were performed for a total of 223 tooth samples, isolated from potential plague victims. 50 mg of pulverized dental pulp, was removed using a dental drill, as preserved pathogen DNA is more likely to reside in the dried blood vessels of the pulp chamber (Schuenemann et al., 2011). All procedures were carried out in the dedicated ancient DNA laboratory of Paleogenetics in the University of Tübingen. DNA extraction was performed according to a previously described protocol (Dabney et al., 2013), with a rotation of 12-16h at 37^{o}C during an initial lysis step. A negative control was included for every 10 samples, and one positive extraction control for every extraction slot.

Screening for *pla*

Initial screening was performed to evaluate the presence of *Y.pestis* DNA in all samples, by using the species-specific gene plasminogen activator (*pla*). 223 DNA extracts were qPCR screened for the presence of the *pla* gene, located in the pPCP1 plasmid using a previously described approach (Schuenemann et al., 2011). 79 samples were from Ellwangen (67 individuals), 49 from Barcelona (18 individuals), 95 from Bolgar city (93 individuals). Amplification products were not sequenced. Potential *Y. pestis* positive samples were subsequently used for further screening using an established whole-genome array capture method (Hodges et al., 2009).

Array design

A one million feature Agilent microarray (Hodges et al., 2009) was designed using an in-house probe design software, both for screening and whole-genome reconstruction purposes. In order to prevent hybridization capture bias for a certain *Y. pestis* reference sequence, probes were prepared using the chromosome of the bacterium *Yersinia pseudotuberculosis* (Accession number: NC_006155), which is the closest known relative of *Y. pestis*, with up to 97% identity in chromosomal genes (Achtman et al., 1999; Chain et al., 2004). The *Y. pestis* (CO92) plasmids pMT1 (Accession number: NC_003134) and pCD1 (Accession number: NC_003131) were also included in the design. A complete set of 976,658 probes was generated for the array.

Array captures

60 µl of extract was used to produce double stranded DNA libraries, as described before (Meyer and Kircher, 2010), for all positive samples. Blank library controls and extraction blanks were also included in every library preparation slot. As the characteristic cytosine deamination accumulating within DNA molecules over time may challenge downstream analyses (Briggs et al., 2007; Sawyer et al., 2012), an initial UDG and endonuclease VIII treatment (USER enzyme) was used to remove uracil residues and subsequently repair DNA fragments (Briggs et al., 2010). Unique double index DNA barcodes (Kircher et al., 2012) were attached onto libraries through a 10-cycle amplification reaction, using universal IS5/IS6 primers. Post indexing, libraries were amplified using AccuPrime *Pfx* or Herculase II Fusion DNA polymerase to accomplish a 19 µg pool of samples. 1ug of a positive control was added to make up a final 20 µg pool that served as template for array capture. A separate pool was made for extraction and library blank controls, and was tested on a separate array to avoid cross talk between samples and blanks. Hybridization-based array capture was performed using previously established methods (Hodges et al., 2009). Hybridization of template to the probes took place over a two-night incubation step at 65 °C. Following array elution, captured template was re-amplified with universal IS5/IS6 primers using Herculase II Fusion DNA polymerase to achieve 20 µg of product that would serve as template for a subsequent capture step (serial capture). Identical array design and methodology were used for the second round of capture. Following elution, products were again

amplified as described above and subsequently diluted down to 10nM for high throughput sequencing. Sequencing was performed on HiSeq 2500 and NextSeq 500 Illumina platforms.

High throughput read pre-processing and mapping pipeline

High throughput sequencing produced up to 123,690,558 raw paired-end reads per sequencing run per library. All pre-processing, mapping and genotyping steps were performed using the automated pipeline EAGER (Peltzer et al., 2016). Adaptors were clipped from all reads produced by the Illumina platforms and overlapping reads were merged. Subsequent quality filtering and length filtering removed reads shorter that 30 bp. All reads were then mapped with BWA (Li and Durbin, 2010) using *Y. pestis* CO92 as a reference genome (Parkhill et al., 2001), the first complete *Y. pestis* genome to be sequenced (Accession number: AL590842.1). To avoid cross mapping from multiple DNA sources, mapping parameters used included a stringency of 0.1 (-n parameter) and a map quality filter of 37 (Table S1).

SNP calling

SNP calling was performed using the UnifiedGenotyper of the Genome Analysis Toolkit (GATK) (DePristo et al., 2011) on the newly produced ancient mapped data, alongside previously published ancient and modern *Y. pestis* data. A total of 152 samples were considered for this study. A *vcf* file was produced for every sample using the "EMIT_ALL_SITES" option, which generates a call for every genomic site. The MultiVCFAnalyzer custom java program was applied to all *vcf* files to comparatively filter all the detected SNPs, and produce a multiple alignment of variable positions in which a SNP was called when present in at least one of the samples in the dataset. Homozygous SNPs were called when covered at least 3-fold with a minimum genotyping quality of 30. Respectively, a reference base was called when supported by 3 independent fragments with the same quality threshold. In case of a heterozygous position, a SNP or reference base was called when at least 90% of the reads covering the position support it. If none of the options was possible, an "N" was inserted at the corresponding positions. In the current dataset, a total of 3,444 variant positions were called.

Phylogenetic reconstruction

After SNP filtering, a SNP table was used as input for phylogenetic reconstruction encompassing a total of 3,444 SNP positons. Phylogenetic trees were generated, using the Maximum Parsimony (MP) and Maximum Likelihood (ML) methods available in MEGA6.06 (Tamura et al., 2013), discarding all alignment columns with more than 5% missing data, which caused the removal of 93 SNP positions. The Maximum Likelihood phylogeny was inferred assuming a General Time Reversible (GTR) model and a Nearest-Neighbor-Interchange (NNI) tree inference option. The total amount of remaining positions to be considered was 3,351. 1000 pseudo-replicates were carried out to assess tree robustness by the bootstrapping method for both phylogenetic methods. A total of 152 samples were used for generation of the phylogeny. The three new strains reported in this study include a Black Death strain from Barcelona, a strain from Bolgar city dating to the second half of the 14th century AD (1362-1400), and a 16th century AD strain from Ellwangen. Data from previously sequenced ancient and modern *Y. pestis* strains included five strains from the 18th century plague of Marseille (Bos et al., 2016), one sequence from Black Death victims from London 1348-1350 (8291-1197-8124), one strain from London 1350-1400 (6330) and 141 modern *Y. pestis* strains (Cui et al., 2013; Zhgenti et al., 2015). A previously published ancient strain recovered from victims of the Plague of Justinian (Wagner et al., 2014) was omitted from the analysis, as it does not contribute to the interpretation of our results, and the low coverage of this genome could negatively influence the robustness of our phylogeny. A *Y. pseudotuberculosis* strain (IP32953) (Chain et al., 2004) was used as an outgroup for rooting the tree, and all its derived SNPs were removed to scale branch lengths.

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