Ancient *Yersinia pestis* and *Salmonella enterica* genomes from Bronze Age Crete

**Highlights**

- We provide genetic evidence of *Y. pestis* and *S. enterica* from Bronze Age Crete
- The *Y. pestis* genome is part of an extinct lineage of non-flea-adapted strains
- Ancient *S. enterica* genomes cluster with contemporary non-host-adapted strains
- The isolates coincide with societal changes ca. 2,000 BCE in Eastern Mediterranean

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**In brief**

Neumann et al. reconstruct ancient *Y. pestis* and *S. enterica* genomes from Crete around 2,000 BCE, a period of significant societal change in the region. Both strains are part of now-extinct lineages, but their existence suggests the importance of considering infectious diseases as a contributing factor to societal transformations.
Ancient *Yersinia pestis* and *Salmonella enterica* genomes from Bronze Age Crete

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SUMMARY

During the late 3rd millennium BCE, the Eastern Mediterranean and Near East witnessed societal changes in many regions, which are usually explained with a combination of social and climatic factors.1–4 However, recent archaeogenetic research forces us to rethink models regarding the role of infectious diseases in past societal trajectories.5 The plague bacterium *Yersinia pestis*, which was involved in some of the most destructive historical pandemics,5–8 circulated across Eurasia at least from the onset of the 3rd millennium BCE,9–13 but the challenging preservation of ancient DNA in warmer climates has restricted the identification of *Y. pestis* from this period to temperate climatic regions. As such, evidence from culturally prominent regions such as the Eastern Mediterranean is currently lacking. Here, we present genetic evidence for the presence of *Y. pestis* and *Salmonella enterica*, the causative agent of typhoid/enteric fever, from this period of transformation in Crete, detected at the cave site Hagios Charalambos. We reconstructed one *Y. pestis* genome that forms part of a now-extinct lineage of *Y. pestis* strains from the Late Neolithic and Bronze Age that were likely not yet adapted for transmission via fleas. Furthermore, we reconstructed two ancient *S. enterica* genomes from the Para C lineage, which cluster with contemporary strains that were likely not yet fully host adapted to humans. The occurrence of these two virulent pathogens at the end of the Early Minoan period in Crete emphasizes the necessity to re-introduce infectious diseases as an additional factor possibly contributing to the transformation of early complex societies in the Aegean and beyond.

RESULTS AND DISCUSSION

The Hagios Charalambos cave, situated on the Lasithi plateau of Crete, Greece (Figure 1), was used as a secondary burial site from the Late Neolithic (ca. 4th millennium Before Common Era [BCE]) to the Middle Minoan II period (18th century BCE)14–16. Secondary depositions of deceased individuals were a common practice during these periods on Crete,17,18 but the Hagios Charalambos cave stands out as an ossuary that contains one of the largest corpora of human remains. In contrast to the low rates of ancient DNA (aDNA) recovery in the Eastern Mediterranean, Hagios Charalambos recently became known for its outstanding preservation of human aDNA,19 probably due to its low and stable temperatures. Additionally, previous osteological analysis has identified a great number of pathologies in individuals deposited in this cave.20,21 However, many diseases cannot be diagnosed by visual inspection of the remains alone because they do not leave any traces or lesions on bones, for example, in acute infections that do not last long enough or those occurring in different tissues of the body.20 As teeth are highly vascular-ized during life-time, they can provide an excellent source for the detection of blood-borne pathogens in archaeogenetic studies.22,23 In order to investigate which pathogens were present during the Bronze Age and their possible impact on early state societies in Crete, we analyzed 68 human teeth from the Hagios Charalambos cave. The selected specimens correspond
to a minimum of 32 individuals (based on anthropological assessment), of which ten were radiocarbon-dated to between 2290 and 1909 calibrated BCE (calBCE, 2 sigma range; Table S1). The teeth were recovered from room 5 and had been found loose or in bone fragments on the floor. After extracting and shotgun sequencing the DNA from all 68 teeth, an estimation of human DNA preservation showed endogenous DNA percentages ranging from 0.1% to 34% (Table S2). A subsequent metagenomic analysis with the screening pipeline HOPS for the investigation of pathogen presence in these teeth and shotgun sequencing the DNA from all 68 teeth, an estimation of human DNA preservation showed endogenous DNA percentages ranging from 0.1% to 34% (Table S2). A subsequent metagenomic analysis with the screening pipeline HOPS for the investigation of pathogen presence in these specimens revealed traces of aDNA from a variety of common oral bacteria, such as the members of the so-called red complex (Tannerella forsythia, Porphyromonas gingivalis, and Treponema denticula) (Table S3), which passed the authentication criteria of (1) a declining edit distance distribution of reads that mapped to the reference genomes, (2) exhibiting a typical aDNA damage pattern in the form of C to T substitutions, and (3) an even distribution of mapped reads along the reference genomes. Intriguingly, we also detected the presence of ancient Y. pestis DNA in individuals HGC009 and HGC068, as well as Salmonella enterica DNA in individuals HGC004 and HGC040. All four individuals fulfilled the aforementioned criteria of the screening pipeline as possible candidates for whole-genome capture, with 14 non-duplicate reads in HGC009 assigned to the Y. pestis node, 30 reads in HGC068 assigned to the Y. pseudotuberculosis complex node, and 157 and 1,722 reads assigned to the Salmonella enterica subsp. enterica node for HGC004 and HGC040, respectively (Figure S1).

After whole-genome S. enterica enrichment of HGC004 and HGC040, reading mapping against the Paratyphi C reference strain RKS4594 yielded a mean genomic coverage of 6.7- and 30.9-fold, respectively (Table 1). In a maximum likelihood (ML) phylogeny with other modern and ancient S. enterica genomes, both strains form a now-extinct clade together with SUA004 from Bronze Age Sardinia (3250–2060 calBCE) and the Late Neolithic OBP001 from Switzerland (3367–3118 calBCE) (Figure 2A). This clade branches off basal to the Para C lineage, which includes the serovars Paratyphi C, Cholerasuis, Typhisuis, and Lomita. Similar to SUA004, both HGC genomes lack the Salmonella pathogenicity island SPI-7 (Figure S2), which is gained subsequently on the Para C lineage and is thus far identified among medieval and modern genomes of the Paratyphi C branch. However, the tcf-operon, which is located on SPI-6 and lost in these medieval and modern Paratyphi C genomes, is still present in HGC004 and HGC040.

For Y. pestis analysis, libraries of HGC009 and HGC068 were enriched for Y. pestis DNA and reads mapped against the CO92 reference genome. For HGC009, this resulted in a mean coverage of 13.1-fold for the chromosome, 18.6-fold for the plasmid pMT1, 27.3-fold for pCD1, and 23.3-fold for pPCP1 (Table 1). Because the mean coverage of HGC068 was only 0.5-fold for the chromosome (pMT1: 0.6-fold, pCD1: 0.9-fold, pPCP1: 1.7-fold), with only 2.9% of the genome covered at least 3-fold, this sample was excluded from further analysis. An ML phylogeny showed HGC009 placement among previously published Y. pestis strains from the Late Neolithic and Bronze Age (LNBA), within a basal branching Y. pestis lineage that has no modern descendants (Figure 2B). More specifically, HGC009 is positioned among the youngest identified LNBA isolates, being most closely related to the genome 6POST (2007–1882 calBCE) from Augsburg in Germany (SNP distance d = 14). In addition, consistent with all other ancient genomes of the LNBA lineage, HGC009 shows an absence of the ymt gene on the pMT1 plasmid (Figure S3) and exhibits the active forms of the genes PDE-3, PDE-2, ureD, rscA, and thiD (Figure S4). The latter are pseudogenized through insertions or nonsense mutations in all modern and historical Y. pestis genomes, but not in genomes of the LNBA lineage and the Middle Neolithic. Inactivation of these genes in addition to the gain of ymt were important evolutionary steps for Y. pestis in its adaptation to the flea vector. As such, the mode of transmission of the bacterium during its early evo-

Figure 1. Location of archaeological sites with evidence of Y. pestis and S. enterica subsp. enterica from the LNBA
(A) Map of Eurasia indicating relevant LNBA sites with genetic evidence of Y. pestis (circles) and S. enterica subsp. enterica (triangles). Hagios Charalambos in pink, previously published sites in black.
(B) Map of Crete showing the location of Hagios Charalambos (pink) and important Bronze Age palatial sites (black).
individuals from whom ancient Y. pestis genomes were isolated carried steppe-related ancestry in their genome and that the phylogeny of the LNBA Y. pestis genomes indicated the same directional spread. To investigate this observed correlation, we additionally enriched the HGC009 and HGC068 genomic libraries, as well as those of HGC004 and HGC040, for circa 1.24 million ancestry-informative SNPs on the human nuclear genome ("1240K SNP capture") of which 583,793 were successfully recovered in HGC009 and 523,608 in HGC040. Lower SNP yields were recovered for HGC068 (5,508 SNPs) and HGC004 (2,968 SNPs), and therefore, these specimens were not included in downstream analyses. Given this low number of SNPs, it cannot be excluded that samples HGC009 and HGC068 were teeth from the same individual, while the unique SNPs of the HGC004 and HGC040 S. enterica genomes indicate that these were two distinct strains, therefore infecting two different individuals. For HGC009 and HGC040, we performed a principal-component analysis (PCA) using modern West Eurasian populations as a scaffold onto which we projected HGC009, HGC040, and other relevant ancient populations. Our results revealed that both HGC009 and HGC040 cluster with other individuals from Hagios Charalambos and Moni Odigitria from Bronze Age Crete. They are all shifted from the cluster of Greek Neolithic genomes in the direction of Chalcolithic-Bronze Age Anatolia (Figure 3A). With qpAdm, we showed that similar to the other Bronze Age individuals from Crete, HGC009 and HGC040 can be adequately modeled with additional ancestry from eastern sources, notably Chalcolithic Anatolia and the Caucasus (Figure 3B) or Chalcolithic Iran (Iran_C) (p values > 0.05). However, only models with Chalcolithic Anatolia fit when the target is the group of all the other individuals from Hagios Charalambos. Models with sources representing the Bronze Age “steppe” ancestry (e.g., Samara-Caucasus Steppe), or LNBA Central Europeans who received gene flow from the former (e.g., C. Europe associated with the Corded Ware phenomenon), consistently failed at all times (p values for fit model to the data <<0.05). In contrast to the evidence from the rest of Europe, this indicates that if the disease reached Bronze Age Crete through contact with mobile peoples from outside Crete and non-related to Anatolia, they were small in number and left no trace in the Early

Table 1. Metadata and summary statistics for pathogen reconstruction (2 sigma calBCE = 95% confidence interval calibrated Before Common Era based on AMS dating). See also Table S1

| Individual | 14C-dating (2 σ calBCE) | Reference genome | Raw reads | Unique mapped reads | Mean coverage chromosome (X) | Coverage ≥ 3 X [%] |
|------------|-------------------------|------------------|-----------|--------------------|-----------------------------|-----------------|
| HGC009     | 2036–1909               | Y. pestis CO92 (chromosome) | 76,670,971 | 1,259,2843         | 13.1                        | 88.5            |
| HGC068     | N/A                     | Y. pestis CO92 (chromosome) | 17,991,665 | 556,391.1          | 0.5                         | 2.9             |
| HGC004     | 2196–2034               | S. enterica Paratyphi RKS4594 | 32,405,434 | 523,608            | 6.7                         | 80.6            |
| HGC040     | 2280–2039               | S. enterica Paratyphi RKS4594 | 44,129,039 | 2,966,002          | 30.9                        | 92.5            |

Figure 2. ML phylogenies of S. enterica and Y. pestis. Genomes from Hagios Charalambos in pink, previously published ancient genomes in blue, and modern genomes in black. Asterisks indicate bootstrap values of >95.

(A) S. enterica subsp. enterica ParaC lineage phylogeny based on 51,358 SNPs. OBQP001 was added manually because of its low coverage, and the dashed line indicates its approximate position according to Key et al. (B) Y. pestis phylogenetic tree based on 5,317 SNPs with 98% partial deletion. The LNBA branch including HGC009 is colored in pink. Goekhem2 was added manually due to the low coverage of its genome, and the dashed line shows its approximate position according to Rascovan et al. See also Figures S1–S4.
Bronze Age archaeogenetic record of the island. Whereas direct human-to-human contact based on the island’s interconnectedness through mobility and trade is one possible way, the involvement of wild and/or domestic animals as hosts also needs to be considered, as plague is a zoonotic disease.

The genetic data suggest that the individuals investigated here were most probably inhabitants of Crete, living at the end of the 3rd millennium BCE (2290–1909 calBCE). This period is of special interest because it witnessed a series of major transformations across the Eastern Mediterranean, most notably the collapse of the Egyptian Old Kingdom and the Near Eastern Akkadian State.1,2 The reasons for these developments have long been under debate, whereby climatic factors and especially severe droughts were emphasized as consequences of the so-called 4.2 ka BP climatic event.2 In the Aegean, the late 3rd millennium (Early Helladic III) witnessed the decline of the complex interconnected societies that had emerged around 2700 BCE on the Greek mainland (Early Helladic II).3,34 This drastic change was commonly explained by various reasons, such as environmental factors (e.g., erosion due to excessive land use), or climate and the migration or even invasion of people.4,34,35 but only rarely were infectious diseases taken into consideration.43 Additionally, the proposed events are not clearly visible across all potentially affected regions. On Crete, there is no apparent crisis at the end of the Early Minoan II period, and the transition between Early Minoan III and Middle Minoan IA periods (ca. 2300–2000/1900 BCE) is not clearly understood. These periods are nevertheless of particular importance because they directly preceded the emergence of the well-known Old Palaces during Middle Minoan IB at the very latest. Although some scholars have described continuity for this period,37,38 others have argued for a phase of societal and population decline in large parts of Crete during Early Minoan III. They report the abandonment of a number of settlements and the subsequent formation of new ones, shortly before the building of the first palaces started in Middle Minoan IB around 1900 BCE.39,40

Both pathogens, *Y. pestis* and *S. enterica* subsp. *enterica*, which were identified in this study at the site of Hagios Charalambos from this time of transition, can cause severe epidemics in human populations. *Y. pestis* was responsible for at least three major pandemics in human history: the Justinianic plague, or first pandemic (540/41–750 CE), the Black Death/second pandemic (ca. 1346 until the 18th century CE), and the third pandemic (1855 until mid-20th century CE).5 Archaeogenetic studies have shown that this pathogen was already present in a wide geographic range between central Europe and Siberia from the Middle Neolithic onward and especially during the Bronze Age.9–13 However, genetic evidence for the presence of *Y. pestis* in the Eastern Mediterranean during this period has been lacking so far. The *S. enterica* genomes from Hagios Charalambos are part of the so-called Para C lineage, which comprises the three serovars Paratyphi C, Typhisuis, and Cholerasis. At present, *S. enterica* Paratyphi C, together with Typhi, Paratyphi A and B, account for ca. 6 million cases of enteric fever in humans annually, with an estimated 54,000 deaths worldwide.41 Ancient genomes from the Paratyphi C serovar were recently isolated from individuals of two medieval mass burials in the north German city of Lübeck, dating to the second half of the 14th century, suggesting an outbreak of paratyphoid fever.42 It was also identified at a 16th century mass burial site in the area.
require blockage of the flea gut through biofilm formation,\textsuperscript{44–47} the less efficient early phase transmission, which does not
might have caused a different form of plague than the bubonic
adapted to effective transmission via the flea vector and thus

dogenes than other non-host restricted
In addition, these ancient genomes are lacking the
suggesting that these strains were rather host generalists.\textsuperscript{25,58}

However, the frequency of pseudogenes in ancient strains
from this lineage, such as ETR001 and XBQM20/XBQM90, as well as SUA004, with which HGC004 and HGC040 form a distinct branch, is in the range of non-host adapted serovars, suggesting that these strains were rather host generalists.\textsuperscript{25,58}

In addition, these ancient genomes are lacking the Salmonella pathogenicity island SPI-7, which comprises a series of virulence genes including the Vi capsular polysaccharide operon.\textsuperscript{59,60} It is associated with evasion of the immune system and typhoid fever and is present in only a few serovars, such as the human restricted serovars Typhi and Paratyphi C.

Therefore, the virulence and mode of transmission of these two pathogen strains from Hagios Charalambos remains uncertain, as does their potential to cause epidemic events. Moreover, their impact on the population and societies of Crete, especially during the transition from the Early Minoan III to Middle Minoan I, is difficult to infer from a restricted dataset derived from a single archaeological site, specifically in the case of Hagios Charalambos where all human remains were recovered from secondary burials. Future screening of more individuals from this region and period for the presence of pathogens will be essential for providing a more detailed picture of infectious disease impact in this region during the end of the 3rd millennium BCE.

Nevertheless, the occurrence of these two pathogens on the island of Crete is of significance. Both \textit{Y. pestis} and \textit{S. enterica} were previously shown to have had a wide distribution in Eurasia during the same period,\textsuperscript{10,11,13,25,58} and given the existence of well-established trading networks in the Bronze Age Eastern Mediterranean,\textsuperscript{61–63} our findings suggest that both pathogens may have also been circulating in neighboring areas from which they were introduced. Further, if both pathogens were present in remote areas such as the Lasithi plateau, they possibly also reached larger settlements in other parts of Crete. There, higher population densities could have facilitated the transmission to a great number of individuals.

While it is unlikely that \textit{Y. pestis} or \textit{S. enterica} were the sole culprits responsible for the societal changes observed in the Mediterranean at the end of the 3rd millennium BCE, we propose that, given the aDNA evidence presented here, infectious diseases should be considered as an additional contributing factor; possibly in an interplay with climate and migration, which has been previously suggested.\textsuperscript{36} Incoming peoples with their livestock could have introduced both diseases to which the local population may have not been previously exposed. Moreover, the massive droughts described in association with the 4.2 ka BP climatic event could have resulted in a shortage of clean drinking water and an immunologically weakened population with higher susceptibility to infectious diseases.\textsuperscript{36,64} As infections by some pathogens, such as \textit{Y. pestis} and \textit{S. enterica}, are not manifested osteologically, these diseases and their impacts have often been unnoticed in the archaeological record in the absence of other evidence (e.g., multiple burials). Therefore, archaeogenetic studies provide an important tool to identify pathogens that affected past populations and, as a result, reveal a more complete picture of their lives and health as well as the pathogens’ evolution.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

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AUTHOR CONTRIBUTIONS

G.U.N., M.A.S., E.S., J.K., and P.W.S. designed the research. G.U.N., M.A.S., and E.S. performed the research. P.P.B. and P.J.P.M. provided samples. G.U.N. and E.S. analyzed the data. G.U.N., P.W.S., M.A.S., and E.S. wrote the paper with contribution by all co-authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** | | |
| Human archaeological remains | This study | HGC001-HGC068; ENA: PRJEB52494 |
| **Chemicals, peptides, and recombinant proteins** | | |
| 0.5 M EDTA, pH 8.0 | Thermo Fisher Scientific (Life Technologies) | Cat#EP0052 |
| Proteinase K | Sigma-Aldrich | Cat#P2308-100MG |
| Guanidine hydrochloride | Sigma-Aldrich | Cat#G3272-500g |
| Ethanol | Merck | Cat#1009832511 |
| 2-Propanol | Merck | Cat#1070222511 |
| 3M Sodium Acetate buffer pH 5.2 | Sigma-Aldrich | Cat#S7899-500ML |
| TE buffer pH 8.0 low EDTA | Panreac AppliChem | Cat#A8569,0500 |
| Tween 20 | Sigma-Aldrich | Cat#P9416-50ML |
| Water HPLC Plus | Sigma-Aldrich | Cat#34877-2.5L-M |
| ATP | New England Biolabs | Cat#P0756S |
| BSA 20 mg/ml | New England Biolabs | Cat#B9000S |
| Bst 2.0 DNA Polymerase | New England Biolabs | Cat#M0537S |
| dNTP Mix 25 mM each | Thermo Scientific | Cat#R1121 |
| T4 DNA Polymerase | New England Biolabs | Cat#M0203L |
| T4 Polynucleotide Kinase | New England Biolabs | Cat#M0201L |
| Buffer Tango 10x | Thermo Scientific | Cat#BY5 |
| USER Enzyme | New England Biolabs | Cat#M5505L |
| 5 M Sodium chloride (NaCl) | Sigma-Aldrich | Cat#S5150-1L |
| Denhardt’s solution | Sigma-Aldrich | Cat#D9905-5ML |
| Tris-HCl, pH 8.0 | Thermo Fisher Scientific (Life Technologies) | Cat#15568025 |
| Pfu Turbo Cx Hotstart DNA Polymerase | Agilent Technologies | Cat#600412 |
| Herculase II Fusion DNA Polymerase | Agilent Technologies | Cat#600679 |
| T4 RNA ligation Buffer | New England Biolabs | Cat#B0216L |
| 10% Criterion™ TBE-Urea Polyacrylamide Gel, 18 well, 30 μl | BioRad | Cat#3450089 |
| 2x TBE-Urea Sample Buffer | BioRad | Cat#1610768 |
| Oligo Length Standards 20/100 Ladder | IDT | Cat#51-05-15-02 |
| UltraPure™ TBE Buffer, 10X | Thermo Fisher Scientific (Life Technologies) | Cat#15581044 |
| 20x SCC Buffer | Thermo Fisher Scientific (Life Technologies) | Cat#AM9770 |
| Dynabeads MyOne Streptavidin C1 | Thermo Fisher Scientific (Life Technologies) | Cat#65002 |
| SYBR® Gold Nucleic Acid Gel Stain (10,000X Concentrate in DMSO) | Thermo Fisher Scientific (Life Technologies) | Cat#S33102 |
| Polyethylene glycol 8000 50% | Jena Bioscience | Cat#CSS-256 |
| FastAP Thermosensitive Alkaline Phosphatase | Thermo Scientific | Cat#EF0652 |
| T4 DNA-Ligase | Thermo Scientific | Cat#EL0013 |

(Continued on next page)
| REAGENT or RESOURCE                        | SOURCE                        | IDENTIFIER          |
|-------------------------------------------|-------------------------------|---------------------|
| Klenow fragment                           | Thermo Scientific             | Cat#EP0052          |
| 20% SDS Solution                          | Serva                          | Cat#39575.01        |
| Silica Magnetic Beads                     | G-Bioscience                   | Cat#GENO786-915     |
| D1000 ScreenTapes                         | Agilent Technologies           | Cat#5067-5582       |
| D1000 Reagents                            | Agilent Technologies           | Cat#5067-5583       |
| Sodiumhydroxide Pellets                   | Fisher Scientific              | Cat#10306200        |
| Sera-Mag Speed CM                         | GE Healthcare Lifescience      | Cat#GE65152105050250|
| Dynabeads MyOne Streptavidin T1           | Thermo Fisher Scientific       | Cat#65601           |
| GeneRuler Ultra Low Range DNA Ladder       | Thermo Fisher Scientific       | Cat#SM1211          |
| 10x GeneAmp PCR Gold Buffer and MgCl2     | Thermo Fisher Scientific       | Cat#4379874         |
| Human Cot-1 DNA                           | Thermo Fisher Scientific       | Cat#15279011        |
| UltraPure™ Salmon Sperm DNA Solution       | Thermo Fisher Scientific       | Cat#15632011        |
| PEG 8000 Powder, Molecular Biology Grade   | Promega                        | Cat#V3011           |

**Critical commercial assays**

| NAME                                      | SOURCE                        | IDENTIFIER          |
|-------------------------------------------|-------------------------------|---------------------|
| High Pure Viral Nucleic Acid Large Volume Kit | Roche                          | Cat#5114403001      |
| DyNAmo Flash SYBR Green qPCR Kit          | Thermo Fisher Scientific      | Cat#F415L           |
| MinElute PCR Purification Kit             | QIAGEN                         | Cat#28006           |
| Quick Ligation Kit                        | New England Biolabs           | Cat#M2200L          |
| Oligo aCGH/Chip-on-Chip Hybridization Kit | Agilent Technologies           | Cat#5188-5220       |
| HiSeq 4000 SBS Kit (50/75 cycles)         | Illumina                       | Cat#FC-410-1001/2   |
| HiSeq® 3000/4000 PE Cluster Kit           | Illumina                       | Cat#PE-410-1001     |
| QIAquick Nucleotide Removal Kit           | Qiaqagen                       | Cat#28304           |
| Oligo aCGH/Chip-on-Chip Hybridization Kit | Agilent Technologies           | Cat#5188-5220       |

**Deposited data**

| NAME                                      | SOURCE                        | IDENTIFIER          |
|-------------------------------------------|-------------------------------|---------------------|
| Raw and analyzed data                     | This study                    | ENA: PRJEB52494     |

**Oligonucleotides**

| NAME                                      | SOURCE                        | IDENTIFIER          |
|-------------------------------------------|-------------------------------|---------------------|
| IS5 (AATGATACGGGCGACCACCGA)               | Sigma-Aldrich                 | N/A                 |
| IS6 (CAAGCAGAACGCGCATACAGA)               | Sigma-Aldrich                 | N/A                 |
| IS7 (ACACTCTTTCCTACACGACGC)               | Sigma-Aldrich                 | N/A                 |
| IS8 (GTGACTGGAGTTCAGACGTGTCG)             | Sigma-Aldrich                 | N/A                 |
| BO4.P7.part1.R (GTGACTGGAGTTCAGACGTGTCG) | Sigma-Aldrich                 | N/A                 |
| BO6.P7.part2.R (CAAGCAGAACGCGCATACGAGAT) | Sigma-Aldrich                 | N/A                 |
| BO8.P5.part1.R (GTGACTGGAGTTCAGACGTGTCG) | Sigma-Aldrich                 | N/A                 |
| BO10.P5.part2.R (AGATCCGGAAACGCGCATACGAGAT) | Sigma-Aldrich                 | N/A                 |

**Software and algorithms**

| NAME                                      | SOURCE                        | IDENTIFIER          |
|-------------------------------------------|-------------------------------|---------------------|
| HOPS v0.2                                 | Hübner et al.                 | 24                   |
| MALT v0.4.0                               | Vågene et al.                | 43                   |
| MaLExtract v1.5                           | Hübner et al.                 | 24                   |

(Continued on next page)
## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Philipp W. Stockhammer (philipp.stockhammer@lmu.de)

### Materials availability

This study did not generate unique reagents

### Data and code availability

- Raw sequencing data have been deposited at the European Nucleotide Archive under the accession number PRJEB52494.
- This study did not generate any unique code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| MEGAN-LR v6.21.16   | Huson et al. | https://uni-tuebingen.de/fakultaeten/mathematisch-naturwissenschaftliche-fakultaet/fachbereiche/informatik/ehrstuehle/algorithms-in-bioinformatics/software/megan6/ |
| nf-core/eager v2.2.1 / v2.3.5 | Yates et al. | https://github.com/nf-core/eager |
| AdapterRemoval v2   | Schubert et al. | https://github.com/MikkelSchubert/adapterremoval |
| Fastx_trimmer v0.0.14 | http://hannonlab.cshl.edu/fastx_toolkit/license.html | http://hannonlab.cshl.edu/fastx_toolkit/license.html |
| Bwa v0.7.17         | Li and Durbin | http://bio-bwa.sourceforge.net/ |
| GATK v3.5/v4.1.7    | DePristo et al. | https://gatk.broadinstitute.org/hc/en-us |
| Samtools v1.3       | http://www.htslib.org/doc/1.1/samtools.html | http://www.htslib.org/doc/1.1/samtools.html |
| bmap v38.86         | https://sourceforge.net/projects/bbmap | https://sourceforge.net/projects/bbmap |
| mapDamage v2.0.9    | Jönsson et al. | https://github.com/ginolhac/mapDamage |
| MultiVCFAnalyzer v0.0.87 | Bos et al. | https://github.com/alexherbig/MultiVCFAnalyzer |
| SNPEvaluation       | Keller et al. | https://github.com/andreasKroepelin/SNP_Evaluation |
| raxml-ng v0.9.0     | Kozlov et al. | https://github.com/amkozlov/raxml-ng |
| FigTree v1.4.4      | http://tree.bio.ed.ac.uk/software/figtree | http://tree.bio.ed.ac.uk/software/figtree |
| MEGAX v10.1.8       | Kumar et al. | https://www.megasoftware.net |
| bedtools v2.25.0    | Quinlan and Hall | https://bedtools.readthedocs.io/en/latest/ |
| R v3.5.3            | R Core Team | https://www.r-project.org |
| R studio v1.1.423   | RStudio | https://www.rstudio.com |
| Ggplot 2 package    | Wickham | https://cran.r-project.org/web/packages/ggplot2/index.html |
| IGV v2.4.8          | Robinson et al. | https://software.broadinstitute.org/software/igv/ |
| EAGER v1.92.59      | Peltzer et al. | https://eager.readthedocs.io/en/latest/ |
| Dedup v0.12.2       | Peltzer et al. | https://github.com/apeltzer/DeDup |
| trimBam             | https://genome.sph.umich.edu/wiki/BamUtil_trimBam | https://genome.sph.umich.edu/wiki/BamUtil_trimBam |
| Schmutzi            | Renaud et al. | https://github.com/grenaud/schmutzi |
| ANGSD v0.910        | Korneliussen et al. | http://www.popgen.dk/angsd/index.php/ANGSD |
| pileupCaller        | https://github.com/stschiff/sequenceTools/tree/master/src/SequenceTools | https://github.com/stschiff/sequenceTools/tree/master/src/SequenceTools |
| EIGENSOFT package v7.2.1 | Patterson et al. and Price et al. | https://github.com/DRreichLab/EIG |
| qpAdm ADMIXTOOLS v5.1 | Patterson et al. | https://github.com/DRreichLab/AdmixTools |
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Archaeological information

The Hagios Charalambos cave (latitude 35.1772505, longitude 25.4410963) is situated 835 m above sea level on the Lasithi Plain on Crete, Greece (Figure 1). The cave was discovered accidentally in 1976 when road building destroyed the roofs of two of the outer chambers brimming with human remains. A rescue excavation was directed by Costis Davaras in 1982–1983 and many important findings were moved to the Archaeological Museum of Heraklion, Crete. One room, though, was intentionally left intact for future studies with more advanced technology and resources, and the cave was sealed with iron bars and concrete to prevent looting. When looters did break into the cave during 2000, a new team of excavators was assembled under the direction of Philip Betancourt, Costis Davaras and Eleni Stavropodi, and the auspices of the American School of Classical Studies. The excavations lasted from 2002 to 2003 and were published in two volumes.\(^{14,16}\)

The human remains recovered from all campaigns were exceptionally preserved owing to low and stable temperatures of the cave. The disarticulated condition of the bones indicates that the cavern was used as an ossuary. There was little actual soil deposit in the cave and post-excavation, it was determined that the secondary deposition was a unique event and that there was no meaningful stratigraphy. The pottery found alongside the commingled human remains provides a date range for the primary burials spanning the Late Neolithic II (ca. 4\(^{th}\) millennium BCE) to the Middle Minoan IIB (18\(^{th}\) century BCE). The primary burials must have been re-deposited in the cave without concern for their original contexts, but with careful transportation of original grave goods. Stones intrusive to the cave suggest that the primary interments might be from built tombs, but no traces of tombs have been found so far. However, the Psychro Cave, only 1 kilometer distant, could have been the original location of the primary burials, which needed to be removed to purify it when Psychro became the focus of cult worship in the Middle Minoan period, coinciding chronologically with the use of Hagios Charalambos for secondary burials.\(^{15}\)

In the Neolithic and Early Minoan periods primary burials in caves were common, but the Hagios Charalambos cave stands out as an ossuary that represents one of the largest and best-preserved corpora of human remains from this early period. Currently, 32,000 bone fragments have been catalogued and their study has shown a low percentage of immature individuals. This poor representation could be partly due to the greater fragility of immature remains. Some sub-adult remains present repetitive injuries to articulations that suggest involvement of the very young in arduous day-to-day tasks. There is evidence for a range of pathologies: traumas, arthropathies, spondyloarthropathies, neoplasm\(^{21}\) endocrine and genetic disorders. Furthermore, there is evidence for procedures to treat head traumas performed by surgeons who laid down the foundations for head surgery long before the Hippocratic treatises were written.\(^{20}\)

The teeth for the ancient pathogen screening were all collected from Room 5, several centimeters below the surface of the deposit and were loose, commingled with other skeletal elements. Since no direct association with other bones or artefacts could be established, the teeth of ten individuals were radiocarbondated at the CEZ Archaeometry gGmbH, Mannheim, Germany, using a MICADAS-type AMS (Table S1). Of these, teeth which represent individuals HGC004, HG009 and HGC040 were \(^{14}C\)-dated to 2196-2034 (MAMS-37429), 2036-1909 calBCE (MAMS-45081) and 2280-2039 (MAMS-49762), respectively, confirming the proposed range suggested by pottery fragments.

Permits

Necessary permits for sample export and analyses were obtained from Ephorate of Paleoanthropology – Speleology, Greece, under protocol number 2397.

METHOD DETAILS

Sampling, library preparation, and sequencing

All laboratory work was performed at the cleanroom facilities dedicated to the work with ancient DNA (aDNA) of the Max Planck Institute for the Science of Human History in Jena, Germany.

Teeth were irradiated with UV light for 15-30 min from two sides and cut with an electric saw at the cemento-enamel junction to separate the crown and roots. With a dentist drill, 30-50 mg of powder were drilled out from the inner pulp chamber and the root canals [https://doi.org/10.17504/protocols.io.bqebrmtan], and for samples HGC001–HGC056 DNA was extracted with a protocol modified after Dabney et al.\(^{84}\) [https://doi.org/10.17504/protocols.io.baksicwe]. For sample HGC009 an additional extract was prepared. From all extracts of samples HGC001–HGC036, 25 \(\mu l\) were turned into double-indexed, double-stranded (ds) Illumina libraries with previous partial USER enzyme treatment based on Rohland et al.\(^{85}\) [https://doi.org/10.17504/protocols.io.bvmh6k39e, https://doi.org/10.17504/protocols.io.bvt8n6w6], which partially repairs the deamination from damaged ancient DNA molecules by maintaining the signal only at the terminal 3’ and 5’ positions. For HGC009 two ds libraries were prepared from each extract. From samples HGC037–HGC056 as well as HGC004 and HGC009 double-indexed single-stranded (ss) libraries (no USER treatment) were prepared from 30 \(\mu l\) DNA extracts using a protocol that included an automated liquid-handling system.\(^{86}\) The same protocol including automated DNA extraction\(^{86,87}\) was used for samples HGC057–HGC068 starting from bone powder directly without manual DNA extraction.

All libraries except ss libraries from HGC004 and HGC009 were initially sequenced on an Illumina HiSeq4000 with a single-end setting (1 x 76+8+8 cycles) on average to five million reads each, and were screened for the presence of endogenous host (human)
and pathogenic DNA (see below). After in-solution capture (see below) for Y. pestis, the two ss and two ds libraries of HGC009 were sequenced on an Illumina HiSeq4000, and two ds libraries on an Illumina NextSeq500 with paired-end settings (2 × 76+8+8 cycles, all at ten million reads, and the captured ss library of HGC068 was sequenced on an Illumina HiSeq4000 to ten million reads in single-end setting. The ds library of HGC004 enriched for S. enterica was sequenced to ten million reads with paired-end settings and the ss libraries of both HGC004 and HGC040 with single-end setting to ten million reads and 50 million reads, respectively, all on an Illumina HiSeq4000. Libraries from all four individuals enriched for c. 1.2 million SNP targets across the human genome were also sequenced on an Illumina HiSeq4000 with single-end setting for c. 23–60 million reads.

### In-solution capture

HGC009 and HGC068 fulfilled the screening criteria to be positive for Y. pestis and all libraries were in-solution captured for the whole genome of Y. pestis with a probe set designed as described before based on the following sequences as templates: CO92 chromosome (NC_003143.1), CO92 plasmid pMT1 (NC_003134.1), CO92 plasmid pCD1 (NC_003131.1), Pestoides F chromosome (NC_009381.1), KIM10 chromosome (NC_004088.1), and Y. pseudotuberculosis IP 32953 chromosome (NC_006155.1). As positively identified by pathogen screening, HGC004 and HGC040 were in-solution captured for S. enterica. The probe set was generated from 67 chromosomal and 45 plasmid sequences/assemblies representing the modern S. enterica diversity. Additionally, libraries from all four individuals were enriched for 1,237,207 ancestry-informative positions across the human genome (1240K). All libraries were amplified to a DNA concentration of 200–400 ng/μl with IS5 and IS6 primers and then captured according to a previously published and well-established protocol. Capture was performed in 96-well plates in two rounds to enrich for pathogen DNA and one round for 1240K, except the library of HGC009 which was captured two rounds as well.

### QUANTIFICATION AND STATISTICAL ANALYSIS

#### Pathogen DNA screening

All samples were screened for the presence of pathogen DNA using the screening pipeline HOPS v0.2. Adapters from reads of shotgun sequencing were clipped with AdapterRemoval v2 within the nf-core/eager v2.2.1 pipeline and reads were then used as input in the first step of HOPS. Reads were mapped with MALT v.0.4.0 to a RefSeq Genome database which includes all complete bacterial and viral genomes as of 2017 as well as additional eukaryotic pathogens as identified by pathogen screening. Mapped reads were then filtered with a custom-made list of pathogens and aDNA damage pattern. Here, the edit distance distribution is expected to show a negative correlation in the number of reads with the number of mismatches to the reference for positive candidates, i.e. there should be more reads with no or one mismatch compared to reads with 3 or more mismatches. In addition, the MALT generated ram6-files of putatively positive samples were visually inspected in MEGAN-LR v6.21.16 and top percent value was set to 1. In the second step of HOPS, reads were clipped and overlapping paired-end reads merged with AdapterRemoval v2. Reads from USER enzyme treated ds libraries were trimmed one base pair from both 5’ and 3’ ends to remove possible aDNA damage with fastx_trimmer v0.0.14 from the FASTX-Toolkit and quality filtered using the -q 30 flag. With the Borrows wheelier aligner bwa v0.7.17 reads were mapped against the Y. pestis reference genome CO92 chromosome (NC_003143.1) and plasmids pCD1 (NC_003131.1), pMT1 (NC_003134.1) and pPCP1 (NC_003132.1). First, they were mapped with relaxed parameters with a seed length set to 16 (–bwaalnl 16) and mismatches to 0.01 (–bwaalnn 0.01) using a quality filter of 37 (–bam_mapping_quality_threshold 37). Duplicates were removed with MarkDuplicates of GATK v4.1.7.0.

Because contamination of the samples with closely related organisms can lead to falsely called SNPs in later analysis, mapped reads from HGC009 were extracted from the bam-files with samtools v1.3 bam2fq and the resulting fastq-files used as input for the MEGAN Alignment Tool MALT v0.4.0. The MALT run was performed in BlastN.
mode and with the SemiGlobal alignment type. The top percent value and minimum support for a node were set to 1, and the maximum number of alignments to 100. No minimum percent identity was defined (-m BlastN -at SemiGlobal -top 1 -sup 1 -mq 100). The fastq files were run against a previously costum-built MALT database, which is based on the NCBI nucleotide (nt) database (October 2017, uploaded to Zenodo: https://doi.org/10.5281/zenodo.4382154) and had been indexed with MALT using the mlt-build command. The resulting rma6-files were then loaded in MEGAN-LR v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and rooted with IP32953. The pairwise genetic distance between HGC009 and 6POST was calculated within MEGAX v10.1.873 using pairwise deletion for both positions of SNPs on the CO92 reference genome which were excluded: 47,939, 234,331, 362,683, 481,841, 481,875, 916,388, 1,011,354, 1,035,698, 1,306,826, 1,587,091, 2,122,841, 2,732,783, 2,897,244, 3,985,662, 4,621,067. From the resulting snpAlignment.fasta file, all SNPs that were covered by at least 98 % of the analysed genomes were extracted (98 % partial deletion) with an in-house python script.

A maximum likelihood (ML) phylogenetic tree was calculated from the generated 98 % partial deletion SNP-alignment with raxml-ng v0.9.0 using the –all function under the GTR+G substitution model. Bootstrap values were calculated with the Felsenstein method under the autoMRE option, i.e. bootstrapping was stopped when the test reached convergence (after 900 replicates here). The tree was finally visualized in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and rooted with Y. pseudotuberculosis. The genome from Goekhmen12 was excluded from this analysis because of its low coverage, but was added by hand afterwards and placed according to its publication due its important position in the phylogeny (Figure 2B).

The pairwise genetic distance between HGC009 and 6POST was calculated within MEGAX v10.1.8 using pairwise deletion for missing data.

Y. pestis virulence factor analysis
In order to analyse for the presence and absence of previously reported virulence factors of Y. pestis,28 data from HGC009 (before MALT-processing) were mapped to the Y. pestis reference genome CO92 (chromosome and all three plasmids) as described above but without quality filtering (–b_mapping_quality_threshold 0). The coverage of 115 chromosomal genes as well as 37 genes on the pCD1, 6 genes on the pMT1 and the pla gene on the pPCP1 plasmids was calculated using bedtools v2.25.0.77 Visualisation was performed in R studio v1.1.42375,76 using the ggplot2 package77 (Figure S3).

Pseudogenization of the virulence associated genes PDE-2, PDE-3, ureD, rscA and flhD through mutation or single-nucleotide deletions/insertions was one of the key steps in Y. pestis evolution for flea-adapted transmission.29,30 Reads mapping to these genes were inspected visually in IGV v2.4.84 after the mlted reads were additionally mapped to Y. pestis KIM (NC_004088.1) and Y. pseudotuberculosis IP32953 (Figure S4). PDE-2 and PDE-3 are phosphodiesterases involved in biofilm degradation. PDE-2 is inactivated by a frameshift through the insertion of a T at position 1,434,044 in CO92. PDE-3 is pseudogenized by a C to T change in the promoter region and nonsense G to A substitutions at positions 3,944,166 and 3,944,543 in activated by a frameshift through the insertion of a T at position 1,434,044 in CO92. PDE-3 is pseudogenized by a C to T change in the promoter region and nonsense G to A substitutions at positions 3,944,166 and 3,944,543 in CO92.

FlhD is involved in flagella biosynthesis and is pseudogenized through a T insertion at position 1,892,659 in CO92.32 While these genes are still active in all LNBA strains, they are inactivated in the Bronze strains RT5 from the Baikal region in Russia.85

Genetic analysis of Salmonella enterica data
Sequence data of samples HGC004 and HGC040 after Salmonella whole genome enriched were processed similar to HGC009 in the Y. pestis analysis. With the nf-core/eager pipeline,96 reads were mapped to the S. enterica Paratyphi C str. RKS4594 reference
genome (NC_012125) using strict parameters for ds and relaxed parameters for ss libraries. Bam-files were merged for each sample and genotyped with the UnifiedGenotyper of the GATK tool kit v3.5. \(^{69}\) Variance calling was performed with MultiVCFanalyzer v0.0.87 \(^{71}\) using the same parameters as for Y. pestis, with nine ancient \(^{25,42,43,98}\) and eighteen modern S. enterica genomes of the ParaC lineage. The S. enterica Paratypi B SPB7 was added with the outgroup-function. Repeatitive, phage- and recombination-related regions were excluded. \(^{43}\) A ML phylogenetic tree was constructed from the resulting SNP alignment which comprised all 51,358 SNPs with raxml-ng v0.9.0 \(^{72}\) using the—all function and a GTR-G substitution model. Bootstraps were calculated with 1,000 iterations. The ML tree was visualized in FigTree and rooted with the outgroup (Figure 2A).

Using MEGAX v10.1.8 \(^{73}\) the pairwise genetic distance between HGC004 and HGC040 was calculated with pairwise deletion for missing data.

To check for the presence and absence of Salmonella pathogenicity islands SPI-1 to SPI-5 and SPI-7 to SPI-10, reads were mapped against the S. enterica Typhi CT18 strain (NC_003198.1) with the nf-core/eager v2.3.5 pipeline \(^{66}\) as described before for the Y. pestis virulence analysis. Coverage of the genomic regions were calculated with bedtools v2.29.2 \(^{74}\) within the pipeline (–run_bedtools_coverage). Coordinates of the different SPIs on the Typhi CT18 chromosome for the annotated input bed-file were taken from PAIDB. \(^{104}\) The reference for SPI-6 was taken from PAIDB for mapping as described before, and coordinates for selected genes were taken from the annotation of the Para C lineage. \(^{65}\) Coverage data were all SPIs were combined and visualised in R studio v1.1.423 \(^{75,76}\) using the ggplot2 package \(^{77}\) (Figure S2).

**Genetic analyses on human 1240K data**

To assess human DNA preservation, shotgun sequencing data of all samples were mapped with the Bowser wheelier aligner bwa v0.7.17 \(^{68}\) against the human reference genome hs37d5 with–bwaalnn 0.01 and a quality filter of 25 (–bam_mapping_quality_threshold 25) within the nf-core/eager v2.3.5 pipeline. \(^{66}\) Duplicates were removed with MarkDuplicates of GATK v3.5 \(^{69}\) (Table S2).

Sequencing data of samples enriched for human DNA were demultiplexed and processed through EAGER v1.92.5 \(^{79}\) for FastQC, clipping of Illumina adapters with AdapterRemoval v2, \(^{67}\) mapping against the hs37d5 human reference with the Bowser wheelier aligner bwa v0.7.17 \(^{68}\) and a quality filter of 30, and then removal of PCR duplicates with dedup v0.12.2. \(^{79}\) Sequencing data from different runs were merged at the level of bam files, and dedup was run again. A version of bam masked on the two (HGC009 and ten (HGC040) terminal bp was created with trimBam (https://genome.sph.umich.edu/wiki/BamUtil: trimBam) to remove damage. The sex of HGC009 and HGC040 was estimated by comparing the coverage on X and Y chromosomes (both numbers normalized with the autosomal coverage), and determined a male and female individual, respectively. Contamination was estimated both on the mitochondrion (original bam file) with Schmutzi, \(^{80}\) and on the Y chromosome of HGC009 (masked bam file) with the tool from ANGSD v0.910. \(^{81}\) Both methods agreed to a low average contamination of c. 1%. A pileup on the 1240K array positions was generated and genotypes were called with pileupCaller (https://github.com/stschiff/sequenceTools/tree/master/src/SequenceTools) and the option–randomHaploid which represents a genotype by randomly choosing one read at each site. The final genotype file of HGC009 included transitions that were retained from the masked bam file and transversions from the original. The genotype file of HGC040 was generated from the original bam file with the option from pileupCaller–singleStrandMode, which effectively removes residual damage from ss libraries. The two genotype files were then merged with publicly available datasets from ancient and modern individuals. \(^{19,33,90,91,105–132}\) These included data produced with the 1240K capture array, shotgun-sequencing or whole-genome sequencing data that were subsequently pulled down to 1240K markers, and modern populations genotyped on the Illumina ‘Human Origins’ Affymetrix array. A subset of the latter (West Eurasian populations only) were used to perform a principal component analysis with the tool smartpc from the EIGENSOFT package v7.2.1 \(^{122,83}\) and the ancient datasets were projected on the PCs using the option ‘lsqproject’ (Figure 3A). We modeled admixture proportions using qpAdm from the package ADMIXTOOLS v5.1 \(^{133}\) (Figure 3B). A detailed explanation of the method is given in Haak et al. \(^{91}\) For the setting of the right (reference) populations we used worldwide modern populations from the Simons Genome Diversity Project (SGDP) (Mbuti, Ami, Mixe, Onge) and ancient populations grouped as follows:

1. Western European hunter-gatherers (WEHG; n=14) \(^{90,108,116,119,121}\)
2. Eastern European hunter-gatherers (EEHG; n=6: I0124, I0211, I0963, Popovo2, Uzo077) \(^{90,123}\)
3. Caucasus hunter-gatherers (CHG; n=2: KK1, SATP) \(^{76}\)
4. Ancestral North Eurasian (ANE; n=2: MA1 and Afontovo Gora2) \(^{128}\)
5. Iran Neolithic from Ganj Darej (n=8) \(^{117}\)
6. Israel Natufian (n=6) \(^{117}\)

For the tested admixing sources, ancient individuals were grouped as follows:

1. Anatolia_N (Neolithic Anatolia; n=30), from Barcin, Menteşe and Boncuklu sites \(^{90,108}\)
2. C. Anatolia_LC (Late Chalcolithic Central Anatolian Çamlıbel Tarlas; n=9) \(^{130}\)
3. Germany_LNBA (Corded Ware; n=11) \(^{91}\)
4. Mountain Caucasus, EnBA (Eneolithic and Bronze Age Caucasus from mountain range, n=32; I1634, I1632, I1631, I1635, I1633, I1658, I1656, I1409, I1407, RISE396.SG, RISE397.SG, RISE407.SG, RISE408.SG, RISE412.SG, RISE413.SG,
RISE416.SG, RISE423.SG, ARM001, ARM002, I1720, I2051, I2056, I6266, I6267, I6268, I6272, KDC001, MK5004, MK5008, OSS001, SA6002, VEK007\textsuperscript{105,107,117,132}

5. Caucasus-Samara Steppe EnBA (Eneolithic and Bronze Age Eurasian Steppe from Samara to the steppe environment of Caucasus, n=28; I0370, I0441, I0444, I0439, I0357, I0429, I0438, I0443, I7489, BU2001, GW1001, I1723, KBD001, LYG001, MK3003, MK5009, PG2001, PG2002, PG2004, RK1001, RK1003, RK1007, RK4001, RK4002, SA6003, SA6010, VJ1001, ZO2002\textsuperscript{30,132}}

6. Iran_C (Chalcolithic Iran, n=20), from Seh Gabi, Hajji Firuz and Tepe Hissar\textsuperscript{117,124}