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valentina.coia@eurac.edu

## Highlights

High genetic complexity in individuals from a single small medieval alpine cemetery

Ancestry primarily from south Europe, with only few possible non-local individuals

Cultural hybridization and complex genetic admixture in South Tyrol

A multiple burial hosts a father-son couple who belonged to a high-ranking *familia*

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# Ancestry and kinship in a Late Antiquity-Early Middle Ages cemetery in the Eastern Italian Alps

Valentina Coia,[1,2,*] Alice Paladin,[1] Stefania Zingale,[1] Christina Wurst,[1] Myriam Croze,[1] Frank Maixner,[1] and Albert Zink[1]

## SUMMARY

**In South Tyrol (Eastern Italian Alps), during Late Antiquity-Early Middle Ages, archeological records indicate cultural hybridization among alpine groups and peoples of various origin. Using paleogenomics, we reconstructed the ancestry of 20 individuals (4th–7th cent. AD) from a cemetery to analyze whether they had heterogeneous or homogeneous ancestry and to study their social organization. The results revealed a primary genetic ancestry from southern Europe and additional ancestries from south-western, western, and northern Europe, suggesting that cultural hybridization was accompanied by complex genetic admixture. Kinship analyses found no genetic relatedness between the only two individuals buried with grave goods. Instead, a father-son pair was discovered in one multiple grave, together with unrelated individuals and one possible non-local female. These genetic findings indicate the presence of a high social status *familia*, which is supported by the cultural materials and the proximity of the grave to the most sacred area of the church.**

## INTRODUCTION

Since prehistory and particularly from the Roman Period to the Early Middle Ages (3rd–11th cent. AD), the Eastern Italian Alps, including South Tyrol (Figure 1A), played a crucial role as a bridge between northern and southern Europe. Thanks to the wide valleys (*Isarco/Eisack* and *Adige/Etsch*) and the Alpine passes (e.g., *Resia/Reschen* and *Brennero/Brenner*), the territory provided paths for the north-south flow of people, objects, and ideas.[1]

In South Tyrol, following a long period of political stabilization and socio-cultural homogeneity under the Romans ("Romanization" process[2]), local archaeological findings suggest that mostly from the 6th cent. AD, the society changed and different people of Northern and Eastern origins (e.g., Bavarians, Franks, Langobards, Slavs) reached this territory. Thus, a complex process of cultural hybridization[3–5] took place in this area, as attested by the local funerary contexts that show a mixture of different material culture (e.g., grave goods) and social status within the cemeteries.[6,7]

Recent whole mitochondrial (mtDNA) and stable isotope ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$) results from several Late Antiquity-Early Middle Age (LA-EMA) individuals from various cemeteries in South Tyrol[8,9] suggested there had been genetic exchanges with allochthonous people, although with different intensity in the territory, probably linked to differences in mobility patterns and geomorphological and historical factors.[8] On the other hand, ancient nuclear genomic data from alpine samples were not available before the present study, with the exception of three prehistoric individuals.[10–12]

In general, little is known about the genomic structure and kinship (used here as biological relatedness) of LA-EMA people, particularly from southern Europe and Italy. Furthermore, there are few ancient studies that have focused on inhumated individuals from a single cemetery, even though this type of investigation can help to better interpret paleogenetic data in light of local archaeological information (e.g.,[13]).

A paleogenomic study conducted in two early medieval cemeteries from southern (*Collegno*, north Italy) and central Europe (*Szólád*, Hungary) associated to the Langobard culture has made an essential contribution in this regard.[14] This study revealed that the two cemeteries were organized around male-dominated biological kinship groups and that the individuals from both graveyards had different ancestries but with a primary ancestry from central-north Europe. Another cemetery-based study from a different cultural context was conducted on the Alemannic graveyard in southern Germany (Niederstotzingen), which dates back to the early 7th cent. AD.[15] Although based on a small number of individuals, this study clearly revealed their different ancestries, with some individuals being genetically more related to northern and eastern European populations, whereas others to southern Europeans. Furthermore, the study suggested that kinship and fellowship were held in equal regard.[15]

In the current study, we analyzed the genomes of 21 specimens (shotgun data plus nuclear capture data in four samples) that were buried in the LA-EMA cemetery of *Malles Burgusio Santo Stefano/Mals St. Stephan ob Burgeis* (BSS) located in the *Venosta/Vinschgau valley* in the

[1]Institute for Mummy Studies, Eurac Research, Viale Druso 1, 39100 Bolzano, Italy
[2]Lead contact
*Correspondence: valentina.coia@eurac.edu
https://doi.org/10.1016/j.isci.2023.108215

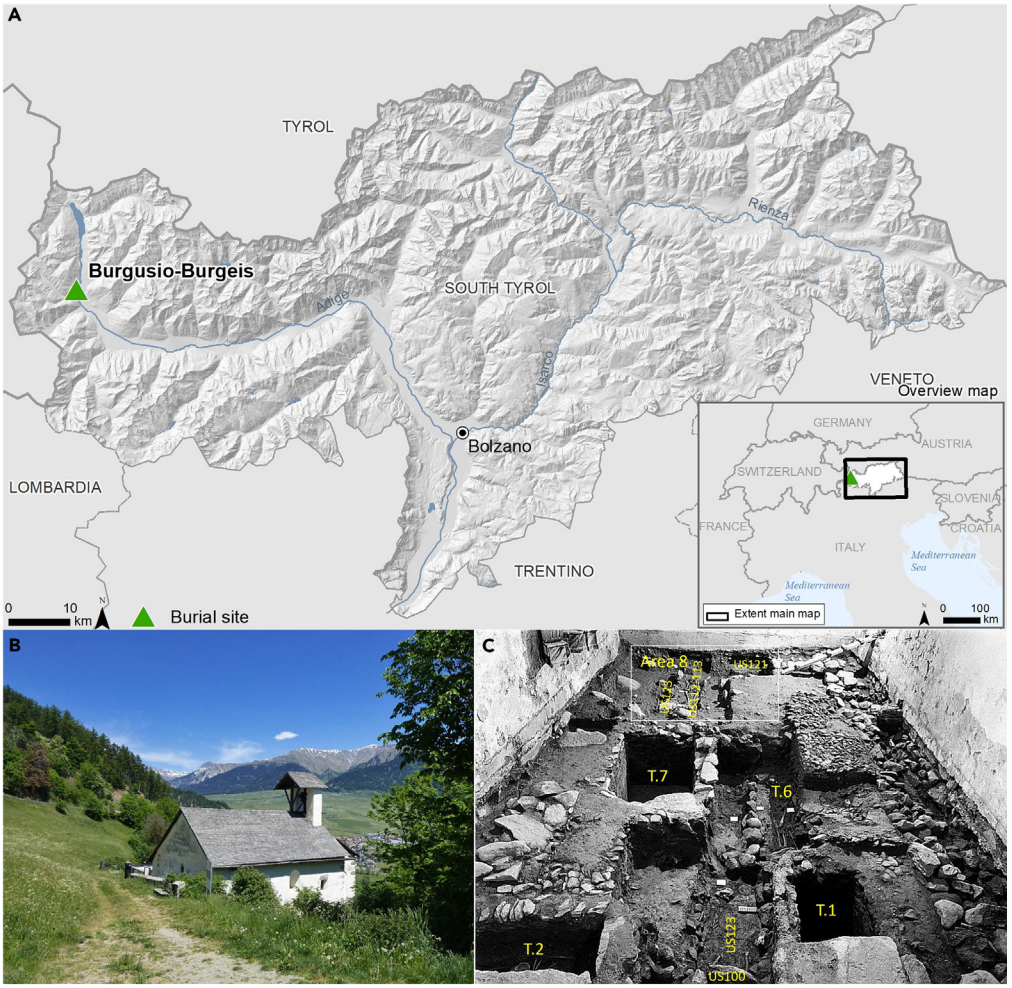



**Figure 1. Location and distribution of graves in the cemetery**

(A) Map of South Tyrol showing the location of the cemetery of *Burgusio Santo Stefano–St. Stephan ob Burgeis* (BSS).

(B) The church of BSS as it appears today.

(C) Graves' distribution in the church (archive photos of the Archaeological Office of the Autonomous Province of Bolzano in Reuβ 2016, modified).

north-western area of South Tyrol (1,364 m.a.s.l). Archaeological records suggest that the burial site underwent more construction phases, from phase 1 to phase 3 (see STAR methods, Table 1, Figures 1A–1C and SM1). The interred individuals, dated to the 4$^{th}$–7$^{th}$ cent. AD, were found in tombs of various typologies, with single or more skeletons and mostly buried without any associated cultural materials. Only two tombs contained grave goods, including multiple belts of German style.[16,17] Additionally, it has been suggested that one multiple burial (T.2) hosted biologically related high-ranking individuals.[16,18]

We used paleogenetic data to reconstruct the ancestry of the alpine individuals from the BSS cemetery and their biological relationships, contributing to the understanding of their social organization. More specific research questions were as follows: (1) do the individuals show homogeneous or more mixed genetic ancestry? (2) does the genomic data indicate the presence of kinships in the cemetery as suggested by the archeological records? Are the two individuals with grave goods related to each other? (3) are there any differences and/or similarities between our results and those obtained from individuals from other cemeteries of the same period from Italy and central Europe?

We integrated the nuclear ancient DNA (aDNA) results generated in this study with ancient mitogenomic data, and we discuss genetic data also taking into consideration available stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$) and anthropological and archaeological information of the same individuals.[8,9,19]

## RESULTS

### Kinship, molecular sex, and unilinear transmitted markers

Following criteria for aDNA authentication, we restricted the analyses to 20 samples that showed typical damage pattern for aDNA, highly fragmented reads, and low contamination from modern human DNA estimated using mtDNA and X chromosome data (STAR Methods,

**Table 1. The twenty-one samples from the BSS cemetery analyzed in this study sorted by grave number**

| EURAC ID | Chronological phases | $^{14}$C AD (95.4%) | Tomb, SU | Mol. sex | Age at death (years) | mtDNA hg | Y chrom. hg |
|---|---|---|---|---|---|---|---|
| 2277 | Phase 3 | — | T.2, US117 | ND | 35–40 | ND | N.D. |
| 2417 | Phase 3 | — | T.2, US119 | XY | 50+ | J1c3c | R1b1a2a1a2b1c2b1a |
| 2418 | Phase 3 | 428–567 | T.2, US119 | XX | 25–30 | H | — |
| 2419 | Phase 3 | — | T.2, US119 | XY | 40–50 | H1e | J |
| 2422 | Phase 3 | — | T.2, US117 | XX | 30–35 | H1 | — |
| 2423* | Phase 3 | 557–634 | T.2, US118 | XY | 35–40 | H | J |
| 2067* | Phase 1 (skeleton) Phase 2 (T.3) | 429–564 | T.3, US100 | XY | 35–40 | H3b+16129 | I2a2a1b1b2 |
| 2404 | Phase 1 | 387–532 | T.5, US137 | XY | 35–40 | N1a1a1a1 | R1b1a2a1a2b1c2 |
| 2420 | Phase 2 | — | T.6, US126 | XX | 35–40 | K1a4 | — |
| 2068 | Phase 2 | — | T.7, US105 | XY | 40–45 | H27+16093 | I |
| 2424 | Phase 2 | — | T.7, US105 | XY | 20–25 | H1 | J |
| 2069 | Phase 2 | — | Area 8, US113 | XY | 40–45 | I2 | E1b1b1 |
| 2405 | Phase 1 | — | Area 8, US121 | XX | 4–6 | T2k | — |
| 2425 | Phase 2 | — | Area 8, US112 | XY | 35–40 | H1e | E1b1b1a1b1a |
| 2426 | Phase 1 | — | Area 8, US120 | XY | 25–35 | H1q | R1a |
| 2427 | Phase 1 | — | Area 8, US125 | XY | 35–40 | H3ap | G2a2b2a1a1b1a |
| 1895 | Phase 2 | — | US163, outside | XY | 30–35 | U8a1a1a1 | R1b1a2a1a2b1a1 |
| 2429 | Phase 2 | — | US163, outside | XX | 60+ | J2a1a1a | — |
| 2428 | Phase 2 | — | US123 | XY | 20+ | H5 | R1 |
| 2324 | Phase 3 | — | US158, outside | XY | 5–7 | H39 | G2a2b2a1a1b1a |
| 2430 | Phase 2 | — | US167, outside | XY | 50–60 | U5b2a3 | R1b1a2 |

EURAC ID, molecular identification number; phase 1 (AD 640), phase 2 (AD 640–660), phase 3 (AD 660–700), chronological phases based on the church construction phases (Reuß 2016); $^{14}$C AD, radiocarbon dating; SU, stratigraphic unit to which the skeleton has been assigned; Mol. sex, molecular sex assignment; mtDNA hg, mitochondrial DNA haplogroups (data from Coia et al. 2022); Y chrom. hg, Y chromosome haplogroups; asterisk, individual found with grave goods (part of multiple belts).

Tables SM1 and SM2). The percentage of human reads ranged from 7% to 73%, whereas mean genome coverage ranged from 0.021 to 1.97 (Table SM1).

The molecular sex analysis revealed 15 males (XY) and 5 females (XX), including two infants for whom sex could not be determined based on anthropological inspection (Table SM1).

Kinship analyses performed with three different methods[20–22] estimated three pairs of related individuals in the cemetery, involving four out of the five samples successfully analyzed from one multiple grave (T.2) and one from a single tomb (T.5) (Tables SM1 and SM3). Indeed, two adult males from T.2 (2419 and 2423 buried with parts of belts) resulted related at first-degree level, and one method (KIN) specifically detected a parent-child relatedness. In addition, individual 2423 showed a second- or third-degree relatedness (according to TKGWV2 and KIN methods, respectively) with one adult female from the same grave (2418). The last kinship was found between another adult male from T.2 (2417) who was related at second-degree level (i.e., nephew/niece-uncle/aunt, grandparent-grandchild, or half-siblings) to the male (2404) from the graveyard T.5. All the remaining individuals from the cemetery, including the two males buried with the grave goods (2423 and 2069), were genetically not closely related, at least up to the third degree, which is the maximum level that can be detected by the applied methods.

The analyses of the paternal Y chromosome haplogroups based on the data generated in this study and those available for the maternal mtDNA[8] added further information on the parental relationships among the BSS individuals. In fact, Y chromosome haplogroup assignment (Tables 1 and SM4) found haplogroup J (same last derived mutation J-M304 G>A) in the first pair of related males (2419/2423) and haplogroup R1b1a2a1a2b1c2* for the other couple (2417/2404). Nevertheless, the latter pair differs for the last mutation, which is R-A1168 T>C (defining R1b1a2a1a2b1c2b1a) for sample 2417 and R-S8183 G>T (R1b1a2a1a2b1c2) for the other sample. However, the SNPs that define more derived R sub-lineages than R-S8183 found in sample 2404 were not covered, due to the absence of reads at this position or low-quality data, so we cannot exclude that this individual carried the same haplogroup as 2417. Additionally, mtDNA data showed that the two pairs of males unambiguously carried different mtDNA haplogroups (2419 haplogroup H and 2423 H1e; 2404 haplogroup N1a1a1a1 and 2417, J1c3c). On the other hand, the couple (male 2423 and female 2418) from multiple grave T.2 shared the same mtDNA haplogroup H with an identical haplotype (mutation at positions 263G, 310.1C, 750G, 1438G, 4080C, 4769G, 8860G, 15326G, and 16519C[8]). The remaining unrelated males,

according to our kinship analyses, carried Y chromosome haplogroup J*, E1b*, G2a*, I*, R1*, R1b*, and R1a* (Tables 1 and SM4). They also carried different mtDNA haplogroups or diverse haplotypes (with rare mutations) of the same haplogroups (e.g., H1 for 2422 and 2424 or H1e for 2419 and 2425; Table SM1A in[8]).

Moreover, analyses of the distribution of mtDNA lineages[8] in BSS individuals revealed some differences between the sexes. In fact, the maternal haplogroups found in females (K1a4, T2k, and J2a1a) were never found in males (H1e, H1q, H3ap, H3b+16129, H5, H27+16093, H39, I2, J1c3c, N1a1a1, U5b2a3, U8a1a1) and vice versa, except for the basal H and H1 lineages (Table SM1). According to the Dataset available at the David Reich Lab webpage (v.54.1, Nov 2022)[23] and considering ancient individuals dating from around the same period as the alpine samples analyzed, mtDNA lineages found in BSS females were observed in only a few (7) other individuals, mostly from Northern Europe (Vikings), Italy (Imperial), and in a Late Medieval sample from Germany.[24–26] On the other hand, maternal lineages found in BSS males have been detected in many other ancient individuals (45) from northern (e.g.,[24,25,27]), south-western (e.g.,[28]), central (e.g.,[14,29,30]), and southern Europe (Italy[26,31]), which include several early Medieval samples from Germany, Hungary, and Italy.[14,25,29,31]

## Genomic structure of individuals from the BSS cemetery

The genomic relationships among 18 non-related individuals from the BSS cemetery and present-day populations from Europe and the Middle East (Table SM5) were explored by principal-component analysis (PCA). Two samples (2417 and 2423), which showed lower coverage among the related individuals, were excluded for comparative analyses (Table SM1).

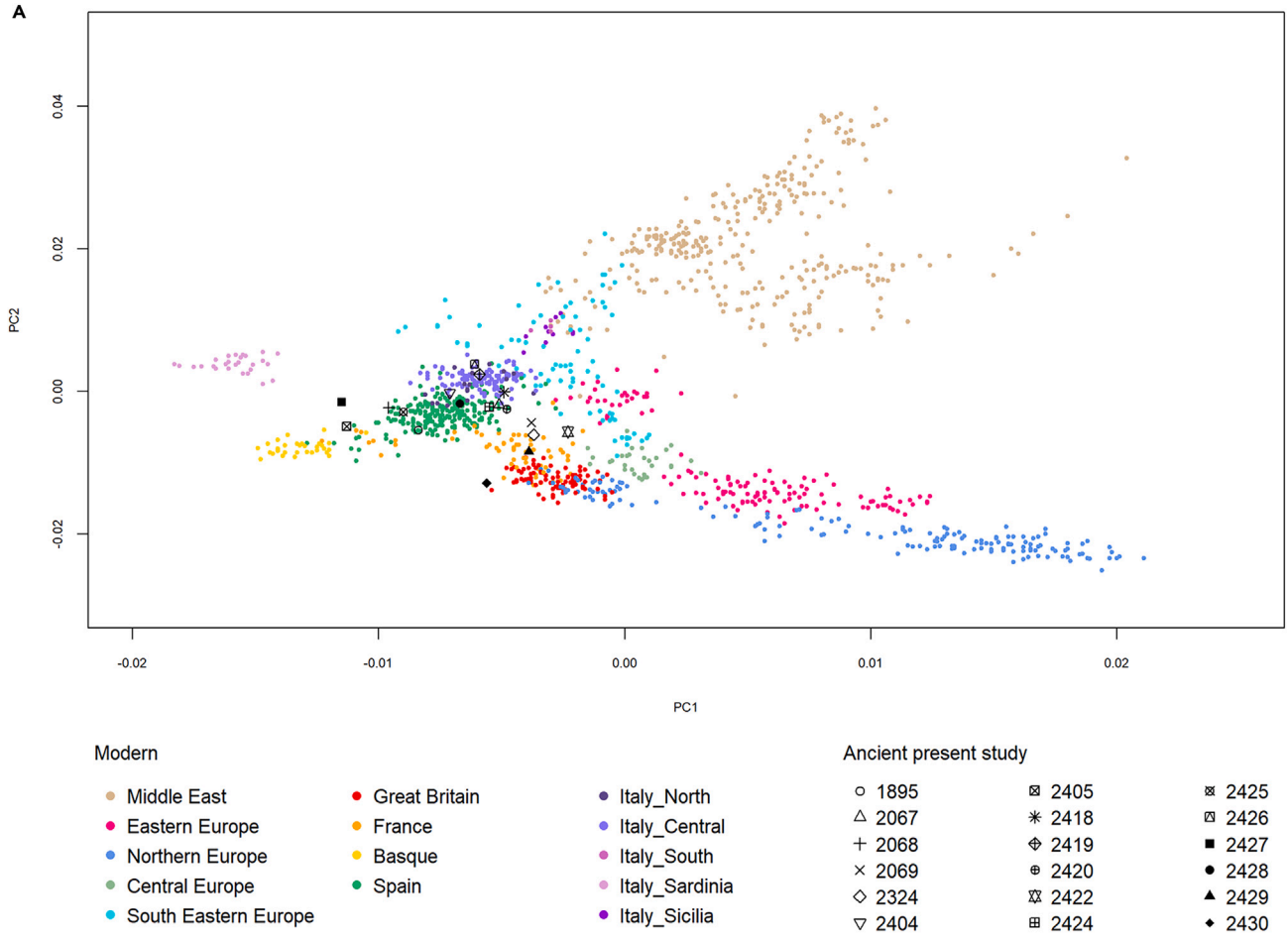
The genomic diversity of most of the alpine individuals overlaps that of present-day populations from south (north and central Italy from Tuscany) and south-west Europe (Iberia) with one individual that is further apart (2427). However, five samples (2069, 2422, 2429, 2430, and 2324) shift in the plot more toward the genomic diversity of modern individuals from western and northern Europe (France and Great Britain, respectively) (Figure 2A).

Unsupervised clustering analysis by ADMIXTURE was computed using genomic data from present-day populations, which can be used as surrogates for ancestral populations of the analyzed medieval samples (Table SM5 and Figure SM2). The analysis showed that the genetic structure of the alpine individuals was composed of European genomic components especially from TSI (Central Italy, Tuscans) and IBS (Iberians), with a small component from FIN (Finnish) (K = 5 and K = 6 with equal lowest cross-validation error) (Table SM6). To further investigate the European ancestry of ancient BSS samples, a supervised model-based clustering analysis (K = 4; Figure 2B) was performed with genomic data from five modern European reference populations (Table SM5): TSI (Tuscans), IBS (Iberians), FIN (Finnish), CEU (Utah residents with ancestry from Northern and Western Europe), and GBR (British). The latter two were considered as one group named GBR (see STAR methods). Consistent with unsupervised clustering and PCA analyses, the major genetic component in our LA-EMA individuals from the BSS cemetery is TSI, which is present in all samples [with an average across our samples of 66.6% (31.1%–98.1%)], followed by IBS [18.3% (0%–51.4%)], GBR, and FIN components (12.8% and 2.3%, respectively) (Table SM7 and Figure 2B).We made a rough grouping of the BSS individuals into four differently colored groups based on the relative amounts of ancestry found (Figure 2B). Three BSS specimens (2069, 2418, and 2419) showed more than 90% of the TSI component (~93%–98% across our samples, named TSI-like), six individuals a combination of mainly TSI and IBS ancestry (TSI+IBS-like, samples 2068, 2404, 2424, 2425, 2426 and 2428), four a combination of TSI and GBR (TSI+GBR-like, samples 1895, 2405, 2420, and 2422), and the remaining individuals with more mixed ancestry with TSI in different combinations and proportions with the other components named TSI+GBR/IBS/FIN-like (samples 2067, 2427, 2430, 2324, and 2429) (Table SM7 and Figure 2B).

Overall, our results, based on comparison with the genomic diversity of present-day populations, differ from those obtained for the individuals found in two other contemporary cemeteries in northern Italy (*Collegno*) and Hungary (*Szólád*). In fact, based on supervised clustering analysis, the major genetic component that has been found in the specimens from both cemeteries was CEU+GBR followed by TSI (64%–57% and 25%–33% across samples, respectively[14]).

To better compare our results with those from this previous study, we performed again the supervised clustering analysis (K = 7) by running the alpine samples (without and with related individuals) together with the ones from *Collegno* and *Szólád* (unrelated individuals only) and using the same dataset of Amorim and collaborators,[14] which included genomic modern data from Eurasia and Africa in addition to the five modern European reference populations (Tables SM5 and SM8, Figure SM3). The results confirmed those obtained with our dataset as described earlier, with the exceptions of samples 2404 and 1895, who were found to be of TSI-like and TSI+IBS/GBR-like ancestry instead of TSI+IBS-like and TSI+GBR-like, respectively (Figure 2B). Additionally, the results showed the ancestry of the two excluded related BSS individuals from our previous analysis (2417 and 2423), who had TSI+GBR and TSI-like ancestry, respectively (Figure SM3). The cluster analyses further highlighted that the alpine BSS individuals have a similar pattern of ancestry to only few individuals from the cemetery in northern Italy (*Collegno*), which showed southern (e.g., CL38) or more mixed ancestry (e.g., CL94, CL23[14]). This differs from the other individuals from *Collegno*, who mostly had primarily central-northern ancestry.

To formally test genetic relationship between our BSS individuals, we carried out an Outgroup F3-statistics analysis in the form (YRI; BSS1, BSS2) by using shotgun data only (STAR Methods,Table SM9). The obtained values were visualized with a heatmap (Figure SM4). In accordance with model-based clustering analysis, the individuals who retain more mixed ancestry (TSI+GBR/IBS/FIN-like; Figure 2B) tend to form a cluster in more reddish color in the heatmap graph. Indeed, samples 2430, 2429, 2067, 2427, and 2420 show more genetic affinity between themselves and less compared with the remaining samples, which in turn are not clearly genetically differentiated. Except for one (2420), the other four samples shared the same three different ancestry that could explain their highest genomic affinity. However, F3 statistics only shows the major differences between the BSS samples and do not allow to highlight more subtle differences, possibly due to the small number of SNPs available for this analysis.



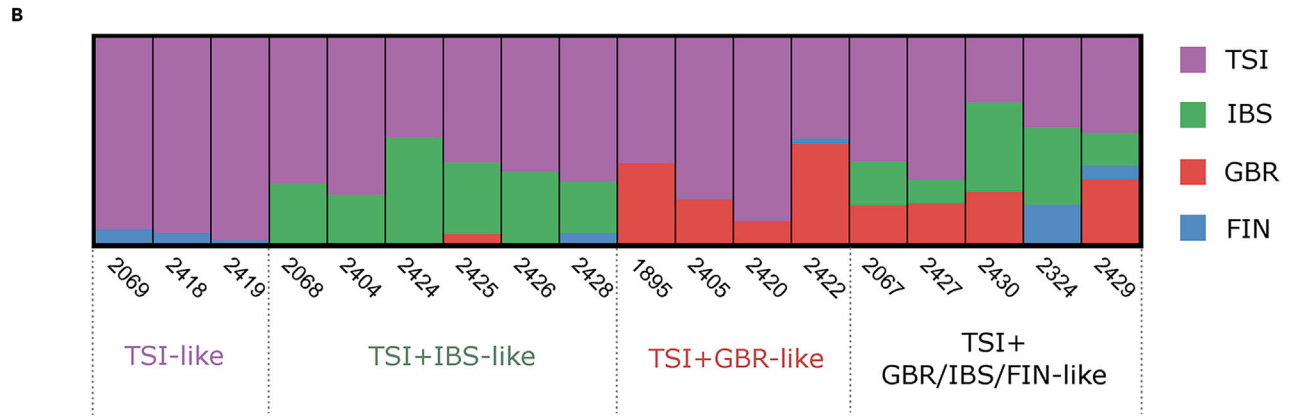




**Figure 2. Principal-component (PCA) and clustering analyses**

(A) PCA performed using genomic data from 18 unrelated individuals from the BSS cemetery and present-day populations. For more details refer to Table SM5.

(B) Supervised clustering analysis (K = 4) performed using data from 18 unrelated individuals from the BSS cemetery and present-day samples from five groups: Tuscans = TSI, violet; Iberians = IBS, green; Utah residents with ancestry from Northern and Western Europe (CEU) and British = GBR, red; Finns = FIN, blue.


**Figure 3. Schematic representation of the location of the studied graves within the BSS cemetery and relative genetic results**
Ancestry is marked by four different colors as presented in Figure 2B.

Interpretations of the results that follow are mainly based on the groupings of the BSS samples according to the clustering analysis.

Additional indications on the genomic ancestry of individuals can be provided by the Y chromosome lineages distribution in some males. For instance, haplogroup E1b1b1, which is distributed today in southern Europe, particularly in the Iberian and Italian peninsulas (e.g., [32,33]), and haplogroup J-M304, which is found in the Arabian Peninsula, Mediterranean, and south Europe, including central and southern Italy, [34] are present only in individuals with TSI-like or TSI+IBS-like ancestries. On the other hand, the I2a2a1b* or R1b1a* haplogroups, which are more frequent nowadays in Central and Western Europe, or the G2a* lineage, which is distributed mostly in the Caucasus, central Europe, and Italy (e.g., [10,35,36]), were found in the individuals with more mixed ancestry (Table 1, Table SM4).

Our study highlighted that there is no clear relation between the ancestry of the individuals and their distribution in the different graves or chronological phases that refer to the different construction phases of the burial site (Figure 3).

Multiple grave T.2 (phase 3) hosted most of the individuals with primarily southern ancestry, who were also genetically related to each other. Additionally, grave T.2 also contained two individuals with more mixed ancestry (TSI+ GBR-like), one adult female (2422, phase 3) and a male (2417, phase 3), neither of whom had any biological relationship with the others (Figure 3). The only other individual (2069) with more southern ancestry was buried in Area 8, which, however, shows approx. 7% of relative ancestry from north Europe, which could explain its intermediate position in the PCA plot of Figure 2A.

Additional information on the genomic structure of alpine BSS samples is provided by unsupervised clustering analysis (Figure SM5A–SM5D) performed using data from other ancient individuals, including those from the major prehistoric groups that have contributed to the genetic makeup of present-day Europeans [37] (Mesolithic Hunter-Gatherers, Neolithic farmers from Anatolia and Iran, and herders from the Pontic Steppe; Tables SM5 and SM10 and references therein). Overall, the analysis (K = 4) showed that BSS samples retain a high percentage of the Neolithic component related to farmers from Anatolia (average across our samples, 55.9%), suggesting a more southern ancestry in agreement with results on comparative analyses using modern data. The Neolithic component is followed by that related to Bronze Age herders from the Pontic Steppe (Yamnaya, average 36.2%), Iranian farmers (3.5%), and, finally, the component related to hunter-gatherer groups (4.4%) (Table SM11 and Figure SM5A–SM5D).

Finally, we projected our BSS samples together with other ancient genomes from Europe onto the PCA performed using genomic data from present-day populations (Figure 4; Table SM10).

Few samples (2068, 2425, 2405, and especially 2427) shift more toward the genomic diversity of prehistoric individuals (Bronze Age and Iron Age) from Italy and south-western Europe, including ancient Sardinians (Figure 4), probably as a result of the high percentage of Neolithic component exclusively from farmers from Anatolia (~62.5%–66.3%) in the genomes of these BSS samples. This can also explain the position of 2427 in the PCA plot of Figure 2A, which is more toward present-day Sardinians, who retain the highest percentage of that component among Europeans. However, all the remaining BSS samples show genomic affinity (Figure 4) with individuals of different time periods and origins. These include the Langobard groups from Central Europe and north Italy, who show mixed ancestry (Hungary_Langobard_o1 and Hungary_Langobard_o2; Italy_North_EarlyMed_Langobards_2; Table SM10) and more recent samples from Medieval-Early Modern periods from central Italy with central European origin, [26] as well as prehistoric (Bronze Age, Iron Age) samples from central and western Europe. Additionally, our results show that the BSS

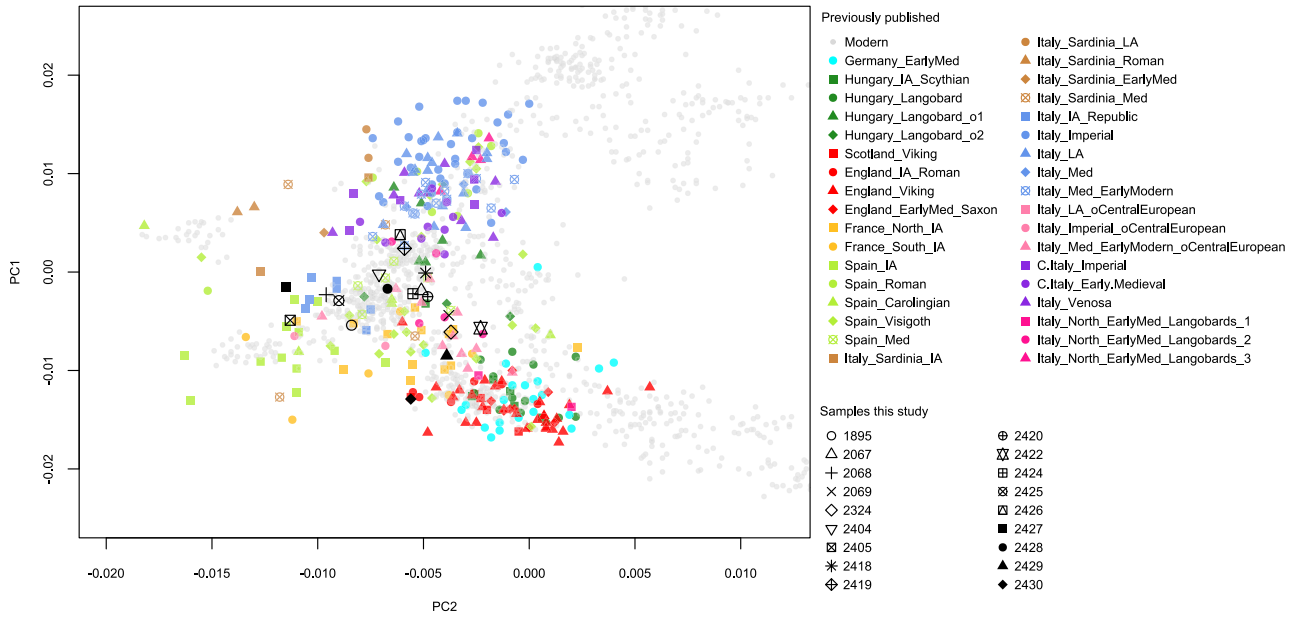



**Figure 4. Principal-component analysis (PCA) executed by projecting the 18 unrelated BSS individuals and other ancient genomes from Europe onto the PCA performed using genomic data from the present-day populations (Figure 2A)**

For more details refer to Table SM10.

individuals (2324, 2069, 2422, 2429, and 2430), which shift in the plot more toward the genetic diversity of central and northern ancient Europeans, consistently retain higher percentages of the steppe-related component (approx. 44%–60%), with the highest value in the male 2430, which shows more genetic affinity to ancient and modern samples from Great Britain (Figures 2A, 4, and SM5D). Finally, few BSS individuals (2419 and 2426) show significant percentages of Iranian-Neolithic-related component (11.4% and 8.6%), which can explain their shift in the plot toward the genomic variation of Imperial Italy (1–400 CE). In fact, individuals from central Imperial Italy retain high levels of Iranian-Neolithic-related ancestry explained by admixture with groups from the Mediterranean area during the Roman period[26] (Table SM11 and Figure SM5D).

## DISCUSSION

Through paleogenetic investigation, we reconstructed the ancestry and kinships of individuals from the LA-EMA cemetery of *Malles Burgusio Santo Stefano–St. Stephan ob Burgeis* in the Eastern Italian Alps (South Tyrol). Genomic analysis focused on 20 individuals, which represented approx. 60% of the total minimum number of inhumated individuals excavated from the cemetery.

Molecular sex assignment determined the biological sex of two infants, and, except in one case, it confirmed the anthropological estimations and thus a demographic profile with a higher prevalence of males (15) compared with females (5). The underrepresentation of females has already been documented for other European funerary contexts[38] and in several LA-EMA Italian cemeteries, including in South Tyrol.[19] Indeed, our recent anthropological study in the Venosta valley, where BSS cemetery is located, revealed that among adults, only 3.5% were females and 17.3% males (for a total minimum number of 52 individuals[19]). However, the reasons behind the distortion of this sex ratio are still under debate, and these include the hypothesis of intentional funerary practices, like the presence of dedicated areas for women or a social-status-based selection.[39–41]

Interestingly, our study indicated some differences between the sexes in the BSS individuals at the mtDNA level, suggesting a different maternal genetic history in males and females.

An important outcome of our study concerns the discovery of high heterogeneous ancestry in the alpine genomes, suggesting a possible different origin of some individuals and genetic exchanges.

Indeed, all BSS show a primary ancestral component from present-day southern Europeans in varying proportions but with the addition, for most of them, of ancestry from populations distributed in different geographic areas especially from south-west, west, and north Europe.

Unfortunately, no comparable nuclear data from present-day alpine individuals are available, which prevents any possible comparison between ancient and modern alpine groups on a more local level. However, high genetic diversity of populations from present-day Eastern Italian Alps has been detected by autosomal and mtDNA low-resolution data.[42,43]

Comparative analyses with other ancient genomes, including those from prehistoric groups, also indicated primarily genomic ancestry from southern Europe. Additionally, they evidenced differences in the genomic affinity of alpine individuals from the BSS cemetery with samples from different time periods (from the Bronze Age to Modern Period), geographic origin (south/south-west, west, and north), and cultural groups, such as Langobards from north Italy, which have mixed ancestry, or those from Hungary or Vikings from Britain.

Further indications were offered through stable isotope values ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) in the analyzed alpine individuals.[8,9] The $\delta^{13}C$ and $\delta^{15}N$ ratios suggested a terrestrial-based diet with a great consumption of animal protein, and a comparison of data with other ancient individuals of the Venosta valley did not indicate the presence of any outliers in the cemetery. Information on mobility was obtained from the analysis of the $\delta^{34}S$ stable isotope, which presented a single outlier (2324; 5–7 years). One other case might be the woman 2422 (30–35 years) from multiple grave T.2 who showed a sulfur value (+5.56‰), which slightly deviates from the faunal baselines of the BSS site (+6.86 ± 0.8‰), indicating her possible different origin.[8,9] Interestingly, she also retains the highest percentage of relative ancestry from northern Europe (51.5%). These results would suggest that most of the inhumated individuals from BSS were likely local rather than migrants from outside the investigated alpine area.

Furthermore, our findings indicate that complex genetic exchanges with other groups of various origin occurred in South Tyrol. These observations confirmed the Italian Eastern Alps as a contact area between populations. Nevertheless, it is noteworthy that such genomic complexity is found in individuals from a small graveyard situated in a relatively isolated alpine valley (1364 m.a.s.l.) and also considering that previous studies on isotopic and mitogenomic data indicated lower mobility and genetic exchange in the Venosta valley compared with other locations of South Tyrol, as for example in the Isarco valley.[8]

An additional outcome of our study regards the general low level of close genetic relatedness among all inhumated individuals from the cemetery. However, in agreement with the archaeological interpretation, kinship was found to involve mainly individuals from multiple grave T.2, which can be traced back to the advanced phase of the cemetery construction (phase 3; Figure 3). Notably, in this multiple burial, our investigations also revealed the presence of a high-ranking *familia*, which is a social group of both related and unrelated members.[44] Indeed, kinship and unilinear transmitted marker analyses (same Y chromosome but different mtDNA haplogroups) supported a father-son relationship between the male with the belt (2423, age at death: 35–40 years old; AD 557–634) and another male (2419, 40–50 years; AD 428–567) from the same grave T.2. Since, according to the archaeological records, individual 2423 was one of the last deceased to be buried in the grave, we can speculate that he was the son of 2419. Furthermore, he was also maternally related, at a second-degree or third-degree level (same mtDNA haplotype), with the woman of the same burial T.2 (2418, 25–30 years) whose skeletal remains were found, in secondary deposition, at the bottom of the grave (Figure SM6). In grave T.2, there were also two individuals with mixed ancestry, who were not genetically related. The first (2417, 50+ years) shows a second-degree paternal kinship with another one (2404, 35–40 years, AD 387–532) interred in single grave T.5 referable to the earlier phase of cemetery construction (phase 1). The second one is a woman of possible external origin (2422), who was buried next to the man with the belt (2423) whom we speculate was the son of 2419. The two skeletons were found in a supine position, next to each other. Based on the archaeological records, the woman was most likely buried last as her shoulder was positioned on top of the man's shoulder (Figure SM6), suggesting a close relationship between them. Kinship results also implied that already by the 4$^{th}$ cent. AD, members of this privileged *familia* were buried in the graveyard, because man 2404 was buried in the first phases (4$^{th}$–5$^{th}$ cent. AD), whereas the others in the later phases of the church (7$^{th}$ cent. AD, Figure 3).

The prestige of the individuals buried in T.2 is suggested by the presence of the multiple belt that accompanied the man 2423 and by the location of the grave near the altar, the most prestigious area of the church. In fact, the structure of the church itself was modified to create space for the tomb. The only other interred individual 2067 found with part of a multiple belt was recovered in T.3, also near the altar. Intriguingly, the skeletal remains of this male were intentionally moved into grave T.3 during phase 2, whereas T.2 was located in the same zone during the later phase 3. Therefore, the individual of T.3 might have belonged to an élite class as well, without having any kinship with the *familia*. Concerning the belts found in both tombs, the archaeological interpretation suggests that these objects were introduced by high socially ranked individuals possibly from southern Germany or they could have been culturally acquired by local Romanized individuals[17,18,45] (Figure SM7). Although it is difficult to verify, overall, our results may suggest the cultural acquisition of such grave goods.

Finally, our study strengthens our knowledge of the social organization of southern European communities in the Early Middle Ages. It highlighted some interesting differences and similarities compared with the previous study based on a single cemetery and from the same period.[14] In fact, in the other two cemeteries in northern Italy (*Collegno*) and Hungary (*Szólád*), the authors found that most of the individuals had primarily central/northern European ancestry and were probably migrants from central Europe and showed extended kinship.[14] Instead, compared with the early medieval site of *Niederstotzingen* (southern Germany), individuals excavated from twelve graves exhibit genetic affinity with groups of different origins and a similar *familial* structure to BSS.[15] Nevertheless, the different resolution of the genomic data as well as the differences in funerary contexts (e.g., graveyard in and surrounding a church as is the case in BSS versus tombs close to Roman crossroads as is the case in *Niederstotzingen*) together with the cultural group diversity must be taken into account. In fact, the archaeological data alone cannot define a clear cultural group in the BSS cemetery. Instead, this was possible for both *Collegno* (Langobard graveyard[14]), thanks to the finding of Langobardian funerary practices and material culture, as well as *Niederstotzingen* (Alemannic tombs[46,47]), thanks to the discovery of horses and military equipment attributable to the Alemannic groups as well as the clear support of historical evidence regarding the Alemannic presence at the time in that geographical area. However, our study, similarly to the others, confirms a high genetic complexity among individuals within the same cemetery in the Early Middle Ages, showing the importance of studying ancient genomic variations at a finer scale.

## Limitations of the study

Overall, our findings indicate complex genetic exchanges between the LA-EMA alpine individuals and other groups of different origin. However, the extent and timing of these exchanges cannot be ascertained in this study. Furthermore, the low number of available SNPs and the type of data (shotgun) do not allow the detection of subtle differences between BSS samples by more quantitative analyses. These points will need to be further investigated with higher resolution genomic data, including those of older samples from more sites and from present-day individuals from the same alpine area.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108215.

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## AUTHOR CONTRIBUTIONS

Conceptualization, V.C. and A.P.; Formal Analysis, M.C. and V.C.; Investigation, A.P., V.C., C.W., and S.Z.; Resources, V.C, F.M., and A.Z.; Writing—Original Draft, V.C. and A.P.; Writing—Review & Editing, V.C., A.P., S.Z., C.W., M.C., F.M., and A.Z.; Visualization, M.C., A.P., and C.W.; Supervision, V.C.; Project Administration, V.C. and A.Z., Funding Acquisition, V.C. and A.Z.

## 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 | | |
| bone sample from Pars petrosa | This study | 1895 |
| bone sample from Pars petrosa | This study | 2067 |
| bone sample from Pars petrosa | This study | 2068 |
| bone sample from Pars petrosa | This study | 2069 |
| bone sample from Pars petrosa | This study | 2277 |
| bone sample from Pars petrosa | This study | 2324 |
| bone sample from Pars petrosa | This study | 2404 |
| bone sample from Pars petrosa | This study | 2405 |
| bone sample from Pars petrosa | This study | 2417 |
| bone sample from Pars petrosa | This study | 2418 |
| bone sample from Pars petrosa | This study | 2419 |
| bone sample from Pars petrosa | This study | 2420 |
| bone sample from Pars petrosa | This study | 2422 |
| bone sample from Pars petrosa | This study | 2423 |
| bone sample from Pars petrosa | This study | 2424 |
| bone sample from Pars petrosa | This study | 2425 |
| bone sample from Pars petrosa | This study | 2426 |
| bone sample from Pars petrosa | This study | 2427 |
| bone sample from Pars petrosa | This study | 2428 |
| bone sample from Pars petrosa | This study | 2429 |
| bone sample from Pars petrosa | This study | 2430 |
| Chemicals, peptides, and recombinant proteins | | |
| EDTA disodium salt dihydrate | ROTH | Cat # 8043.2 |
| Proteinase K | Qiagen | Cat #19131 |
| QuantiFluor® ONE dsDNA System (500rxn) | Promega | Cat #E4870 |
| BSA - Bovino Serum Albumina | Biolabs | Cat #B9001S |
| NEBNext End Repair Enzyme | Biolabs | Cat # BE6050L |
| T4 DNA Ligase (5 U/µL) | LifeTechnologies | Cat # 15224-041 |
| dNTPs (2.5 mM each) | LifeTechnologies | Cat # 46-0344 |
| Bst polymerase, large fragment (8 U/µL) | Biolabs | Cat #M0275S |
| Adapter Mix (5 µMol each) | LifeTechnologies | ND |
| AccuPrime™ Pfx SuperMix | LifeTechnologies | Cat # 12344-040 |
| Critical commercial assays | | |
| myBaits ® Expert Human Affinities – Prime Plus | Arbor Bioscince Biodiscovery LLC | Cat #311008 |
| MinElute PCR Purification Kit | Qiagen | Cat #28006 |
| Bioanalyzer High sensitivity DNA Analysis | Agilent | Cat # 5067-4626 |
| Magnetic Beads (DNA & RNA Purification) | NucleusBiotech | Cat # 40051M |
| Deposited data | | |
| Shotgun | This study | ENA: PRJEB60362 |
| Nuclear Capture | This study | ENA: PRJEB60362 |
| mtDNA capture | Coia et al. 2022 | ENA: PRJEB60362 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Shotgun and nuclear capture data. Dataset v50.0 including data from 1240K to 1000 Genomes Project, Phase 3 | Mallick et al. 2023; The 1000 Genomes Project Consortium 2015 | https://reich.hms.harvard.edu/ |
| Software and algorithms | | |
| PEAR | Zhang et al.[48] | https://github.com/tseemann/PEAR |
| QualityFilterFastQ.py script | Kircher et al.[49] | https://bioinf.eva.mpg.de/fastqProcessing/ |
| BWA | Li et al.[50] | http://bio-bwa.sourceforge.net/ |
| DeDup | Peltzer et al.[51] | https://github.com/apeltzer/DeDup |
| mapDamage | Jónsson et al.[52] | https://ginolhac.github.io/mapDamage/ |
| schmutzi | Renaud et al.[53] | https://github.com/grenaud/schmutzi |
| ANGSDTool | Rasmussen et al.[54] | https://github.com/ANGSD/angsd |
| Bcftools | Danecek et al.[55] | https://samtools.github.io/bcftools/bcftools.html |
| PMDtools | Skoglund et al.[56] | https://github.com/pontussk/PMDtools |
| pileupCaller Sequence Tools | N.A | https://github.com/stschiff/sequenceTools |
| Yhaplo | Poznik et al.[57] | https://github.com/23andMe/yhaplo |
| ISOGG | ISOGG; Version: 15.73 Date: 11 July 2020 | https://isogg.org/tree/ |
| READ | Kuhn et al.[20] | https://bitbucket.org/tguenther/read/src/master/ |
| TKGWV2 | Fernandes et al.[21] | https://github.com/danimfernandes/tkgwv2 |
| KIN | Popli et al.[22] | https://github.com/DivyaratanPopli/Kinship_Inference |
| EIGENSOFT package (v16000) | Price et al.[58] | https://github.com/DReichLab/EIG |
| ADMIXTURE | Alexander et al.[59] | http://dalexander.github.io/admixture/download.html |
| Plink | Chang et al.[60] | https://github.com/chrchang/plink-ng |
| Pong | Behr et al.[61] | https://github.com/ramachandran-lab/pong/ |
| AdmixTools | Patterson et al.[62] | https://github.com/DReichLab/AdmixTools |
| gplots package (v. 3.1.3) | Warnes et al.[63] | http://CRAN.R-project.org/package=gplots |
| SAMtools v.1.3 | Li et al.[64]; Danecek et al.[55] | http://www.htslib.org/doc/samtools.html |
| Molecular sex determination | Skoglund et al.[56] | https://github.com/pontussk/ry_compute |

## RESOURCE AVAILABILITY

### Lead contact

Further information on materials, datasets, and protocols should be directed to and will be fulfilled by the lead contact, Valentina Coia (valentina.coia@eurac.edu).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

The raw data (FASTQ files) for each library generated and used in this study have been submitted at the European Nucleotide Archive (ENA) with the accession number PRJEB60362 and are publicly available as of the date of publication.
All codes used in this study and other previously published genomic data is available at the sources referenced in the key resources table.
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## EXPERIMENTAL MODEL AND STUDY PARTICPANT DETAILS

The church of *Burgusio Santo Stefano – St. Stephan ob Burgeis*[18,65] is under the protection of the Autonomous Province of Bolzano. This is located on a steep slope near the village of *Burgusio-Burgeis* (1364 m a.s.l.) in the Upper *Venosta* valley-*Vinschgau* (Figure 1). The relative chronologies[17] suggest that this burial site underwent three main construction phases, from a more rural phase (phase 1) to the building and transformation into the church (phase 2–3) during the Early Middle Ages (Figure SM1).

Our previous anthropological study reconstructed a total of 35 individuals from the cemetery, which consisted of 20 males, 6 females and 9 individuals with undetermined sex, including 8 subadults (Tab.2A and 3A in[19]).

Twenty-one individuals with a well-preserved Pars Petrosa (PP), were selected for paleogenomic analysis (Table SM1). They were recovered from single [(T.3, T.5, T.6 and Area 8 (samples 2426, 2405 and 2427)], dual (Area 8, samples 2069 and 2425) and multiple burials (T.2, T.7), while four more individuals (1895, 2324, 2429, 2430) were found in stratigraphic units (US) (Figure 3; Table SM1). The grave typologies included simple earthen pits, stone-lined pits or chamber tombs, and the skeletons were found in primary, North-South or West-East cardinal oriented, or in secondary deposition (Table SM1).

Multiple grave T.2 had a privileged position inside the church as it was located near the altar, the most sacred area of the church, and it has been suggested that it hosted biologically related individuals who were part of a high-ranking family.[16,18] Additionally, archaeological records revealed that only one individual from T.2 and the one from T.3, which was also buried near the altar, were accompanied by parts of multiple belts. These belts, which have decorative-manufacturing styles typical of southern Germany,[16,17] were generally used for holding weapons and are social status indicators.[66,67]

The sampling and the scientific analyses performed in this study were authorized by the competent authority (*13.2 Ufficio Beni archeologici, Provincia Autonoma di Bolzano-Alto Adige*).

## METHOD DETAILS

### Molecular analyses

For each selected sample, approximately 150 mg of powdered bone was collected from the inner part of the PP, isolated or still anatomically connected to the skull, by using a drill[68] in a dedicated pre-PCR area of the ancient DNA laboratory in Bolzano and following all the strict rules required for ancient DNA analyses. DNA samples were extracted using a purification method based on silica columns[69] and double-stranded genomic libraries were constructed.[70] These were then sent to an external company (Macrogen) for shotgun sequencing [100 bp paired-end (PE), Hiseq2500 and 150 bp HiSeq-X systems, Illumina]. Additionally, four samples (2418, 2419, 2423 and 2430) were further analyzed for more than 2 million SNPs in the human genome, using the in-solution target capture kit myBaits Expert Human Affinities – Prime Plus (Arbor Bioscience). The protocol was modified by increasing the hybridization time from 16 to 40 h (Human Affinities, Version 1.0, March 2021 - Daicel Arbor Bioscience) and sent for sequencing as reported below.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Bioinformatic analyses and aDNA authentication

FASTQ files from different libraries and sequencing runs (data from this study plus capture mitochondrial data from[8]; see Table SM12 for more details) of the same sample were concatenated in a single FASTQ file. The reads were then trimmed and merged (PEAR)[48] if they overlapped by at least 25 bp and with a minimum length of the assembled sequences of 25. The QualityFilterFastQ.py script[49] was applied to eliminate reads with 5 bases below the quality threshold of 15. The reads were then aligned to the Genome Reference Consortium Human Build 37 (hg19) and to the revised Cambridge Reference Sequence (rCRS)[71] with BWA[50] with minimum mapping quality set at 25. Duplicates were removed by using DeDup.[51] Damage patterns among the ancient reads were tracked and quantified (fragmentation and misincorporation patterns) by using mapDamage.[52]

Contamination estimates based on mtDNA data were inferred for all samples by using schmutzi.[53] Only for male individuals, contamination estimates were also made based on X chromosome data, using the method implemented in ANGSD (Analysis of next generation Sequencing Data[54]) (not applicable to females). However, for one male (sample 2430), contamination estimate was not possible due to the low number of reads on X chromosome. To identify degraded DNA sequences that are unlikely originated from modern contamination, human reads of contaminated samples based on estimates on mtDNA data (2277 and 2429) were filtered by using the PMDtools method implemented in.[56] As a result, sample 2277 was excluded from the study (due to the very low number of reads left after filtering) while the reads after filtering for sample 2429 were used for the analyses (Table SM1).

The individuals were genotyped at each SNP using the reads with a minimum base and mapping quality of 30 with the *bcftools mpileup* program.[55] Then, a pseudo-haploid genotype for each sample was reconstructed with *pileupCaller* (https://github.com/stschiff/sequenceTools) by performing a random allele call between bases from each site. Transition sites were removed to avoid taking possible false polymorphisms due to deamination linked to postmortem degradation processes into account in the analyses.

### Sex determination and Y Chromosome assignment

Biological sex was estimated by calculating the ratio of sequences aligning to the X and Y chromosomes.[72]

In order to determine the Y chromosome haplogroups of males, genotypes of individuals were called using *bcftools mpileup*[64] with mapping and base quality $\geq$30 overlapping Y chromosome SNPs (Human reference genome hg19). Y chromosome haplogroups were then assigned using the yhaplo software (Identifying Y Chromosome Haplogroups: https://github.com/23andMe/yhaplo;[57]). Mutations in samples 2404 and 2417 were checked in the vcf files and compared to the most up to date phylogenetic tree of human Y chromosome lineages.[73]

### Kinship analyses

Relatedness among all early medieval individuals from the BSS cemetery were inferred using two different methods, based on pseudo-haploid approach, which are specifically designed for low coverage genomic data and can determine kinship up to 2nd degree level. The READ method (Relationship Estimation from Ancient DNA[20]) applies a non-parametric approach by calculating and normalizing a mismatch rate across the whole genome on pseudo-haploid data to infer the degree of relationship for each pair of individuals. Instead, the TKGWV2 method (Thomas Kent Genome-Wide Variants 2[21]) uses genome-wide variants and allele frequencies to infer the relationship between a pair of individuals. This software was run using population allele frequencies from current European populations (CEU from 1000 Genomes, phase 3), which were provided with the software. The third method used in this study (KIN), infers relatedness of pair of individuals based on the identical-by-descent segments they share, and it can classify up to 3rd-degree relatives and differentiates siblings from parent-child pairs.[22] Results are considered as significant for a Log Likelihood Ratio >1. Input files for KIN from bam files were generated with the script KINgaroo provided by the author. The default parameters were used, and no contamination correction was applied.

### Comparative datasets

The pseudo-haploid data reconstructed for BSS samples were then merged with previously modern and ancient published genomic data reported in the dataset of David Reich's lab (v50.0, 1240K),[23] which include data from the 1000 Genomes Project, Phase 3.[74] All duplicates and possible related individuals were removed from the dataset. Moreover, only data from individuals classified as 'PASS', with a minimum coverage $\geq$0.01 and with more than 5000 SNPs were kept for comparative analyses. Different datasets were assembled for bioinformatic analyses, and more details are provided below and in the Tables SM5 and SM10.

### Principal component analysis

Principal Component Analysis (PCA) was performed using the *smartpca* software from the EIGENSOFT package (v16000[58]). Genomic data from 1294 present-day individuals from West Eurasia were merged, which resulted in a total number of 110,416 SNPs. Ancient comparative data from 503 individuals (dated from Bronze Age to Early Modern Period) and the 18 BSS samples, with the exclusion of relatives, were then projected onto PC1 and PC2 using the "lsqproject:YES" option (Table SM10). The "shrinkmode:YES" parameter was selected to correct the shrinkage effect when predicting PC scores.

### Model-based clustering analyses

Supervised clustering analysis using ADMIXTURE[59] was performed to estimate the ancestry components of modern European populations from five groups from Europe (TSI = Tuscans; IBS = Iberians, CEU = Utah residents with ancestry from Northern and Western Europe, GBR = British and FIN = Finns; 493 individuals) in the alpine BSS samples. The data was retrieved from the 1000 genome project phase 3[74] and those from GBR and CEU were merged due to their very similar ancestry, as shown in our unsupervised analysis (Figure SM2) and reported in a previous study,[14] so that the analysis was executed setting K = 4 (TSI, IBS, GBR-CEU and FIN). Supervised clustering analysis was also performed to compare the BSS samples to those from Amorim and collaborators[14] using data from the four above-mentioned European groups, plus data from Africa (YRI), East Asia (EAS) and South-west Asia (SAS) (a total of 1603 individuals), which were retrieved from the 1000 Genome Project phase 3, and setting K = 7 (YRI, EAS, SAS, TSI, IBS, GBR-CEU and FIN) (Table SM5).

Unsupervised model-based clustering analyses by ADMIXTURE were executed using genomic data from 1603 individuals from 16 present-day populations and 909 ancient individuals (dated from Mesolithic to the Medieval Period), which were retrieved from the Reich laboratory dataset (v50.0, 1240K) (Tables SM5 and SM10).[23] These included major European prehistorical ancestral groups such as Mesolithic Western hunter-gatherers, Neolithic Anatolian farmers, Neolithic Iranian and Yamnaya groups from the Russian steppe. The data was filtered by pruning variants for linkage disequilibrium using plink[60] with the parameters ''–indep-pairwise 200 25 0.2'' and for missing genotypes with ''-geno 0.99''. The unsupervised analyses were carried out for each value of K, from 2 to 8, and repeated five times with different seed values for each repetition. Runs with the highest likelihood were taken as the final results. These results were visualized using the pong software.[61]

### Outgroup F3-statistics

To measure the shared genetic drift among couples of BSS samples from an outgroup,[62] Outgroup F3 statistics was performed on the 18 unrelated BSS samples in the form (YRI; BSS1, BSS2) with YRI (Yoruba) as outgroup using qp3Pop from AdmixTools.[62] Only shotgun data were used for this analysis. We plotted F3-statistics results in a heatmap generated using *heatmap.2* function of the R-package gplots (v. 3.1.3).[63]