

Investigating community formation through dense spatial and temporal sampling of 5-6th century cemeteries in Pannonia

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Author Contributions: Conceptualization, P.J.G., K.R.V., D.N.V., and I.K.; DNA preparation and sequencing A.M., R.R., and S.V.; Formal Analysis, D.N.V. and I.K.; Investigation: D.N.V., I.K., B.G.M., T.S., and T.H.; Resources, B.G.M., Y.T., M.L., P.S., Z.G., T.S., T.H., S.É., and Z.B.; Visualization, D.N.V. and I.K.; Writing, D.N.V. and I.K.; Supervision, W.P., Z.H., D.C, T.V., P.J.G., and K.R.V.

Competing Interest Statement: We do not have any competing interests.

Classification: Biological, Health, and Medical Sciences/ Genetics & Anthropology

Keywords: paleogenomics, kinship, burial archaeology, community formation, Early Medieval

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Abstract

As the collapse of the Western Roman Empire accelerated during the 4th and 5th centuries, arriving “barbarian” groups began to establish new communities in the border provinces of the declining (and eventually former) empire. This was a time of significant cultural and political change throughout not only these border regions but Europe as a whole. To better understand post-Roman community formation in one of these key frontier zones after the collapse of the Hunnic movement, we generated new paleogenomic data for a set of 38 burials from a time series of three 5th century cemeteries at Lake Balaton, Hungary. We utilized a comprehensive sampling approach to characterize these cemeteries, and analyzed them within a careful interdisciplinary framework along with data from 38 additional burials from a previously published mid-6th century site. Despite many commonalities in burial representation and demography, we find striking differences in how close genealogical relationships were recognized and expressed in burial customs amongst these small, rural communities. In addition, each site demonstrated a unique genomic ancestry profile, though interestingly a significant relationship was inferred between genetic variation and the presence of a 5th century “female package” of dress accessories and grave goods. Notably, the range of genetic diversity in all four of these local burial communities is extensive and wider ranging than contemporaneous Europeans sequenced to date. Our analysis shows that the formation of early Medieval communities was a multifarious process even at a local level, consisting of genetically heterogeneous groups demonstrating a variety of social systems.

Significance

The decline of the Western Roman Empire resulted in a significant cultural and political transformation of Europe during the early Medieval, and many modern nations claim to trace their origins to this period. We conducted an interdisciplinary paleogenomic study using a novel comprehensive, time-series approach, studying three mid-to-late 5th century sites and one mid 6th century site all from a localized region south of Lake Balaton (Hungary). We find very high levels of genetic diversity in this small region as well as significant variability in how genealogical relationships were reflected in cemetery organization. By carefully combining genetic, archaeological, and historical data, we demonstrate that post-Roman communities were formed by not only culturally, but also genetically diverse groups.

Introduction

With the dissolution of the Western Roman Empire, the 5th century was a period of great political, cultural, and demographic change in Europe (1, 2). This was particularly true in the Middle Danube Region, which long served as a frontier zone of the Roman Empire and also became a border zone after its division into the Western and Eastern Empires. By 433 CE, with the abandonment of the Pannonian provinces by the Roman civil and military administration, it had already lost its former political and military importance. The region's subsequent development was first determined by the period of Hunnic rule in the first half of the 5th century, after which it came under the influence of various "barbarian" groups (Goths, Heruls, Langobards, etc.). These changes resulted in the transformation of settlement structures and patterns, the appearance of new material culture, and the emergence of new communities that founded small burial sites compared to large late Roman cemeteries of the 4th century, which could sometimes contain thousands of graves (1, 2).

In this study, we combine the results of archaeological, paleogenomic, osteological, and historical analyses in order to understand the formation of three new post-Roman communities near the southern shore of Lake Balaton (in present-day Hungary): Fonyód-Mérnöki telep, Hács-Béndekpuszta, and Balatonszemes-Szemesi berek (henceforth Fonyód, Hács, and Balatonszemes). These cemeteries have been dated to the middle and second half of the 5th century CE (**Fig. 1**) (3). This interdisciplinary approach, when based on a comprehensive paleogenomic sampling of whole cemeteries, can provide a framework for answering questions and making insights that could not be addressed by each field individually (4, 5). One major research question of relevance to the process of group formation is whether biological relatedness played a role in the construction of social kinship and in the social organization of these post-Roman communities (6, 7). Another important question is whether these emerging archaeologically diverse communities were of similarly heterogeneous genetic backgrounds. In particular, we can now ask to what extent any genetic variation had significant social and cultural manifestations. Thus, in this study we focused in particular on how biological relatedness and genomic ancestry are related to burial customs and spatial organization at the three sites.

Previously, Amorim et al. (6) published a comprehensive paleogenomic analysis of Szólád, a Langobard-period cemetery dating to the middle of the 6th century also found on the southern shore of Lake Balaton. They showed that the community using the Szólád cemetery was organized primarily around a large, three-generation male kindred. It also proved possible to distinguish at least two groups of burials with different genomic ancestry that differed with regard to grave structure, the dress accessories and other grave goods. With the comprehensive analysis of the three 5th-century cemeteries in close vicinity to Szólád, we aim to determine whether the pattern observed in Amorim et al. (6) represented a long-standing regional tradition versus a recent development, capitalizing on our intensive local sampling to examine the extent to which there have been parallel changes over time between genomic and archaeological data.

Results

5th century Pannonia: Fonyód, Hács, & Balatonszemes

Fonyód, Hács, and Balatonszemes (*SI Appendix*, Section S1) are located on elevated loess ridges close to the southern shore of Lake Balaton, an area that became increasingly important for the Roman military and civil administration with the founding of a series of inner fortresses (*Innenbefestigungen*) in the 3rd-4th centuries (8). These inner fortresses remained in use until at least the middle of the 5th century, sometimes even after the Roman abandonment of the area (9–11). These three 5th century sites were chosen based on their geographical proximity to Szólád, are considered fairly typical of the region and period, and are found within 18km of each other. They chronologically cover the second part of the 5th century, with Fonyód dated to the middle third, Hács to the second half, and Balatonszemes to the end of the 5th century (**Fig. 1**). Their dating is based on certain jewelry types (brooches, pins, earrings, etc.) and tools ('nomadic mirrors' of the Čmi-Brigetio type, double-sided combs) (*SI Appendix*, Fig. S1) (12–16).

All three sites represent small (19, 29, and 19 graves respectively), probably short-lived rural communities. They show similarities in demographics with a very unbalanced sex ratio as a result of there being a major bias towards adult female burials. Hács and Balatonszemes are also very similar in terms

of burial representation, as artefacts are mostly found in female burials in contrast to male burials which are generally poorly furnished (12, 15, 16). Artificial cranial deformation (henceforth ACD) is also observable in all sites but it is only prevalent in Fonyód, while the other two sites only contained single examples. For our full analysis of ACD, please see *SI Appendix*, Section S2 and Fig. S2. The sites also show differences in terms of spatial organization of the burials, with Fonyód being unique (15) due to its six grave clusters lying at roughly equal distances of 50–60 m from each other (**Fig. 2**).

Genome sequencing and data set

Though our aim was to genomically characterize all individuals at all three sites, the archaeological preservation at these three sites and the availability of petrous bone material limited which individuals could undergo DNA extraction and sequencing. From Fonyód we were able to obtain genomic data from 13 of 14 graves with biological remains (Fonyod_493 lacked sufficient material). Several of the 29 graves from Hács were destroyed in construction or lost following excavation. Skeletal material was originally preserved from 27 graves but petrous bone was only available for 14 individuals (of whom all were sequenced). From Balatonszemes, 13 graves had preserved biological remains, while five other burials from Balatonszemes lacked any. From the graves with remains, the 11 with petrous remains were sequenced.

Ten samples (nine from Hács and one from Balatonszemes) underwent whole genome sequencing (WGS), and the remaining 28 (5, 10, and 13 from Hács, Balatonszemes, and Fonyod, respectively) underwent genome-wide SNP capture sequencing for ~1.2 million SNPs (1240K) (17–19). Mitochondrial contamination rate medians (20) were 1–3% for all individuals, while nuclear contamination point estimates (21) from the 14 males were between 0.4% and 2.2% (Table S1). Excluding Fonyod_305 (0.02×), SNP captured samples had 1240K coverages between 0.22× to 3.46×; WGS individuals had autosomal coverages between 5.15× and 11.07× (Table S1).

Sequenced adults were majority female with 20 females and 6 males, but sequenced children were the opposite with 4 females and 8 males (Table S1). When osteological sex determination was possible, genetic sex determination was consistent with the osteology (22). Mitochondrial haplogroups for individuals with sufficient read data and haplogroups for the non-recombining portion of the Y chromosome (NRY) for all males are provided in Table S1. Almost all identified mtDNA and NRY haplogroups were consistent with modern European populations. The sole exception was Bal_143 who had an mtDNA haplogroup R0a1a1, common in the Near East (23) (for further mtDNA analyses see *SI Appendix*, Section S3).

Biological kinship and spatial organization

IcMLkin (24) was used to identify close biological relatedness between the individuals studied from Fonyód, Hács, and Balatonszemes as well the 38 individuals from Szólád (6) (Table S2). We found one set of biological siblings from Fonyód (FONYOD1), one set of three biological relatives from Hács (HACS1), and two different sets of three biological relatives from Balatonszemes (BAL1 and BAL2) (**Fig. 3**). Data from uniparental markers (i.e., mtDNA and the NRY) and osteological data were also consulted alongside IcMLkin results to construct pedigrees. Additionally, when omitting likely spurious low-coverage individuals, no biological kinship ties below the 3rd degree ($\pi_{\text{hat}} \sim 0.125$) between individuals from different cemeteries were found (Table S3). When available, mtDNA and NRY haplogroup identifications always supported interpretations from IcMLkin. IcMLkin results from Szólád are consistent with the published findings (6) with the exception of the observation of an additional 2nd/3rd degree relationship between Sz_11 and Sz_20 (**Fig. 2d**; Table S3).

FONYOD1 consisted of two brothers (**Fig. 3**), Fonyod_304 and Fonyod_336. Fonyod_304 was a child, while Fonyod_336 was an adult male; both had minimal/no grave goods. They were buried next to each other along with Fonyod_305 (a young girl) as part of Burial group V. No relationships were detected between Fonyod_305 and the brothers; however, Fonyod_305 has very low coverage (0.02×), limiting power to infer any relationships or the lack thereof. The other three burial groups for which more than one individual was sequenced did not contain any relatives.

Biological kindred HACS1 consisted of Hacs_4, Hacs_20, and Hacs_24 (**Fig. 3**). Osteological aging information suggested that Hacs_4 was the mother of Hacs_20 who was in turn the mother of Hacs_24. At Hács, a strong archaeological connection between the closely biologically related individuals is not visible. Members of the grandmother-mother-son trio (Hacs_4, Hacs_20, Hacs_24) were buried in different parts of the site; the grandmother and her grandson were not far from each other in the northern part of the site, but the mother in the south was paired spatially with an unrelated child. A pair of bow brooches, silver polyhedral earrings, and amber beads were found in the mother's grave. The grandmother's burial is less richly furnished, with a double-sided comb and glass beads, while the grandson's grave did not contain any artefacts. This difference in grave goods could be a result of social difference, but also age as young adult females tended to be buried with more grave goods than older women, while grave goods are generally absent in child burials (25–27).

Kindred BAL1 consists of a parent-offspring trio between Bal_146 (son), Bal_149 (father), and Bal_150 (mother) (**Fig. 3**). In contrast to Hács but similar to the older Fonyód, all members of the two biological kindreds at Balatonszemes were buried in close proximity. BAL1 is situated in the irregular north to south row of graves with the two adults being buried next to each other. The mother was buried with a pair of gilded bow brooches, a girdle-hanger with gemstone pendant and silver shoe buckles, while the father's burial only contained a wheel-turned ceramic pot and their son's burial had no grave goods at all. Kindred BAL2 consists of three females with second-degree relationships (**Fig. 3**). Bal_267, Bal_268, and Bal_269 are three female individuals that were equally related to each other ($\pi_{\text{hat}} \sim 0.25$); this level of relatedness would correspond to half-sibling, aunt-niece, or grandmother-granddaughter relationships. Based on osteological estimates of age as well as sharing the same mtDNA haplogroup, it was inferred that Bal_267 and Bal_268 were maternal half-sisters (Table S3). We estimate based on the short occupation of the site that Bal_269 was either a grandmother or aunt of the half-sisters (though genetically we cannot rule out other relationships) (**Fig. 3**). This kindred shows an even stronger spatial and archaeological connection than BAL1; namely, they form a separate cluster in the cemetery and are all very richly furnished with gilded brooches, this is particularly interesting in case of Bal_267 and Bal_268, as children are generally buried without any grave goods in the 5th century.

Genomic ancestry and structure

In order to understand the distribution of genomic ancestry at Fonyód, Hács, Balatonszemes, and Szólád, PCAngsd was used to perform a principal component analysis (PCA) based on genotype likelihoods for the 1240K SNPs (28). Unlike previous studies that have used modern reference panels to structure ancient data (which may introduce certain biases), these data were analyzed exclusively alongside a penecontemporary set of 174 previously published individuals with date ranges overlapping the fourth to eighth centuries CE (6, 29–34).

A continuum of variation was found along PC1 (**Fig. 4**), with individuals from regions such as Scandinavia and the British Isles tending to fall on the one end of the distribution, while individuals from present-day Spain and Italy tending to fall on the other. PC2 demonstrated more noise, with great variation from run to run of PCAngsd. It is of note that while individuals from farther east tend to have higher PC2 values, individuals from further west do not consistently have lower values on PC2. For example, the Iberian and Italian individuals generally overlap with each other, though they can be distinguished in a supervised fastNGSadmix analysis using modern Europeans from these regions as well as PCAs using modern POPRES and AADR datasets (see below). This suggests that PCAngsd may not have enough power to discriminate a second PC for individuals with as low genetic differentiation as found between southern and western Europe when analyzing only low coverage ancient DNA (especially when we are analyzing so many low-coverage ancient individuals, which can introduce significant noise), and thus our focus is primarily on the distribution along PC1. A larger version of **Fig. 4** with all reference sites labeled as well as an analysis only using transversion SNPs are presented in *SI Appendix*, Figs. S3-4. PC1 and PC2 values for all analyzed individuals are also present in Table S4.

A wide range of PC1 values were found for the four target sites. In order to gain a more nuanced understanding of these four cemeteries and their similarities and differences, we opted to not create artificial genetic groupings for our dataset and instead chose to use PC1 values to define a gradient between two ancestries, Ancestry A and Ancestry B. More positive values (towards the right) denote more

Ancestry A, while more negative values (towards the left) denote more Ancestry B. PC1 values were normalized from -0.1686 to 0.0850 from the entire dataset (including penecontemporary reference data) to a range of 0 to 1 (Table S4). Individuals with the normalized PC1 values of 1 have the greatest Ancestry A, whereas those with normalized values of 0 have the greatest Ancestry B. We plotted the density distribution of ancestry for all four Balaton sites as well as the regions from the comparative dataset (**Fig. 5**). In contrast to the penecontemporary populations, which tend to be fairly unimodal and usually demonstrate an association between ancestry and geographical sampling location along a north/south axis, individuals from Hács fall on opposite sides of the distribution, with nine falling towards the Ancestry A extreme (0.635 to 0.795) and five towards the B extreme (0.200 to 0.260). A similar phenomenon is observed for Balatonszemes, but there are some individuals falling toward the middle of the distribution (e.g., Bal_111, Bal_143, Bal_268). At Szólád, this is continued as individuals are spread across the whole spectrum, ranging from 0.169 to 0.851. Fonyód, however, shows a different profile. While there are four individuals with extreme Ancestry B individuals (0.124 to 0.211), most individuals from Fonyód fall in the middle of the Ancestry A/B distribution from the offset. Additionally, with the exception of Fonyod_491 (0.694), there are no extreme Ancestry A individuals present at Fonyód. To test if ancestry distributions were changing between sites and becoming less extreme (i.e. bimodal Ancestry A/B profiles), we conducted a Hartigan's dip test (35) and found that increasingly younger sites demonstrated more unimodal (more mixed Ancestry A/B profiles) ancestry distributions (see *SI Appendix*, Section S4). These differences in bimodality/unimodality are clearly visible in **Fig. 5**.

We compared our PCAngsd results to results from unsupervised NGSadmixture (36) analyses and found a strong correlation between results from K=2 analyses and PC1 of our PCAngsd results ($R^2=0.97$) and highly consistent results from run to run, suggesting this result is highly robust. However, we observed considerable noise for K>2, similar to our observations for PC2 from PCAngsd (see *SI Appendix*, Section S5; Figs. S5-6).

Due to the limitations when running PCAngsd exclusively with ancient DNA described above, we also conducted a supervised ancestry clustering analysis using fastNGSadmix (37), which would have more statistical power in partitioning genomic ancestry but could also include a bias due to the use of modern reference populations [in this case 1000 Genomes Project European populations, YRI (Yoruba), and the Asian super-populations (EAS and SAS)(38)]. Results from fastNGSadmix for Fonyód, Hács, Balatonszemes, and Szólád are presented in **Fig. 6** and for all 253 analyzed individuals in *SI Appendix*, Fig. S7. Individuals with greater Ancestry A had significantly higher proportions of the CEU+GBR and FIN components ($R^2 = 0.88$, $p < 0.001$), while individuals with greater Ancestry B had significantly higher proportions of TSI and IBS components ($R^2 = 0.86$, $p < 0.001$) (*SI Appendix*, Section S5 and Fig. S8).

Similarly, in order to ensure our sequence data fit appropriately into known patterns of European genetic diversity, we also conducted fastNGSadmix analyses as well as pseudohaploid ADMIXTURE (39) analyses using 74 prehistoric individuals of Anatolian_Neolithic (n=26), Eastern Hunter-Gatherer (EHG) or Steppe_Eneolithic (n=2, n=18), Western Hunter-Gatherer (WHG) (n=15), Iran_Neolithic (n=9), and Morocco_Iberomaurusian (n=4) origin (Table S5) (18, 19, 40–48). Consistent with previous findings (6, 49), we found that individuals with greater TSI and/or IBS proportions tend to have greater Anatolia_Neolithic proportions (with Anatolia_Neolithic being a proxy for Early European Farmer ancestry) (*SI Appendix*, Section S5 and Figs. S9-10).

We also conducted Procrustes-transformed smartPCA analyses comparing the Balaton sites and penecontemporaneous reference individuals to modern European individuals from the POPRES dataset (50) as well as modern individuals genotyped on the Affymetrix Human Origins array from the Allen Ancient DNA Resource (AADR) v50.0 release (see Table S6 and *SI Appendix*, Section S5) (44, 45, 51–56). For the POPRES dataset, we conducted analyses with and without transversion SNPs (referred to as POPRES-Full and POPRES-Tv, respectively). For the latter dataset, we conducted two PCAs using only transversions, one using only western Eurasian reference populations (AADR WE) and one using a set that is pan-Eurasian (AADR PE); reference populations were chosen based on Gneecchi-Ruscione et al. (57). Unlike our PCAngsd analysis with penecontemporary data, these smartPCA approaches have the resolution to separate reference individuals along both north-south and east-west axes. In the POPRES and (to a lesser extent) AADR WE analyses, we found that most of the

variation in the Lake Balaton communities in this analysis was found predominantly along PC1 associated with a north/south axis (see *SI Appendix*, Section 6, Figs. S11-22). In these analyses, we can also see separation between Italian individuals from Collegno and Rome and the Iberian individuals, which was visible in our results from fastNGSadmix but not from PCAngsd. In the AADR PE analysis, we found that virtually all historic individuals fall within present-day European variation (*SI Appendix*, Figs. S23-26). Given the tight clustering, we do not use the AADR PE PCA in other analyses.

The position of Late Antique/Early Medieval individuals along PC1 for both POPRES and AADR WE PCAs were highly correlated with their Ancestry A/B values derived from the PCAngsd analysis (POPRES-Full: $R^2 = 0.93$, $p < 0.001$; POPRES-Tv: $R^2 = 0.92$, $p < 0.001$; AADR WE: $R^2 = 0.89$, $p < 0.001$), while PC2 values were substantially less correlated (POPRES-Full: $R^2 = 0.02$, $p = 0.03$; POPRES-Tv: $R^2 = 0.01$, $p = 0.08$; AADR WE: $R^2 = 0.18$, $p < 0.001$) (*SI Appendix*, Figs. S27). Thus the major population structuring in our dataset appears to be that captured by PC1 of our PCAngsd analysis for which we determined our Ancestry A/B gradient. PC1 and PC2 values for all ancient individuals from all smartPCA analyses also presented in Table S4.

Correlations between grave artefacts and genomic ancestry

To understand whether the identified biological connections (as determined by similar genomic ancestry) were acknowledged as meaningful social ties, we compared burial customs (i.e., structure and design of burials, appearance of dress accessories, and grave goods in the burials) with the results of the PCA (i.e., Ancestry A vs. Ancestry B). We note that very simple, shallow pit graves with variation only in grave size, which corresponds to the age of the deceased, are characteristic at all three sites. This lack of variation necessarily means that there is no clear difference in the structure of the graves based on genomic ancestry.

We examined to what extent genomic ancestry was related to exclusivity in grave goods, i.e., artefact types that are characteristic or only buried with members of a certain genomic background. This was only possible in case of the female burials, due to the low number of males in the three 5th century communities and the lower number of artefacts in those burials, especially in case of Hács and Balatonszemes. The sole exception is the heavily disturbed grave Hacs_5, that was found and destroyed before the excavation and its contents were only recollected later from the property owner. It contained both male (sax) and female (bow brooch) artefact types (**Fig. 2b**); the most parsimonious explanation is that it is the result of the mixture of a male and female burial (58), thus it was excluded from the comparative analysis (*SI Appendix*, Section S1).

No clear correlations between artefact types and ancestries were visible in Fonyód; both male and female burials were poorly furnished and the same artefact types (e.g., Čmi-Brigetio type mirrors and double-sided combs) are present in burials with different ancestries. In case of the female burials of Hács and Balatonszemes we were able to observe certain types of artefacts exclusively present in female burials with enriched Ancestry A, these include double-sided combs (Hacs_4, Hacs_13, Hacs_18), bow brooches of various types (Hacs_18, Hacs_19, Hacs_20, Bal_150, Bal_267, Bal_268, Bal_269), large, round amber (or other semi-precious stone) beads (Hacs_18, Hacs_20, Bal_150, Bal_267), and rings (Bal_267, Bal_268). The only exception is again the heavily disturbed male Hacs_5 that also included fragments of a brooch and comb and large amber beads (**Figs. 2b and 7**). These types form parts of a 'female package' (i.e. combination of amber beads, brooches, and double-side combs, rings, etc.) characteristic for the 5th century (59, 60). A jewelry type that is present in all three sites and were found in burials regardless of genetic background is polyhedral earrings (Fonyod_444, Hacs_1, Hacs_20, Bal_268).

We conducted logistic regressions to test for statistical associations between our PCA results and the presence or absence of amber beads, brooches, and double-sided combs from the female burials from Fonyód, Hács, and Balatonszemes. Regressions were conducted with all three sites separated and combined, including and excluding children. For PCAngsd PCAs, we conducted single logistic regressions using our Ancestry A/B results (i.e., normalized PC1 results) (*SI Appendix*, Figs. S28-30). For the POPRES and AADR WE PCAs, we conducted multiple logistic regressions using normalized results

from both PC1 and PC2, though we flipped the polarity of PC1 for POPRES-Full and AADR WE prior to normalization to better correspond with Ancestry A/B (*SI Appendix*, Section S6 and Fig. S27).

We found significant association between both amber beads and brooches with Ancestry A at Balatonszemes and in the multi-site analyses. Full results for all logistic regressions can be found in Table S7. For double-sided combs, given the rarity of these artefacts (as well as their complete absence at Balatonszemes), we do not find any significant associations with Ancestry A; however, at Fonyód, we find one spurious association with Ancestry B as only one female burial there contained a double-sided comb ($R^2 = 1$, $p = 0.0141$), which is replicated in POPRES and AADR WE results (Table S7). The lack of significance in many of these analyses (particularly, single site analyses) may be attributable to the lack of statistical power, due to lower sample sizes (and the number of unfurnished Ancestry A burials). For example, while at Hács brooches are only found with extreme Ancestry A females (**Fig. 2b**; *SI Appendix*, Fig. S29b), our analysis is not significant but also has low power (power=0.174, suggesting a low probability of detecting a true association). However, when we combined these artefacts and conducted association analyses between the 'female package' (i.e., individuals from graves containing one or more parts of it) and genetic ancestry, we found that there was a significant association between Ancestry A and the 'female package' in all analyses other than the analysis of Fonyód on its own (Table S7; *SI Appendix*, Fig. S31). Our multiple logistic regression results with the POPRES and AADR WE PCAs are generally consistent for tests involving individual artefact types (albeit with some minor deviations), but the POPRES-Full and AADR WE results are completely consistent when testing for associations regarding the 'female package' (Table S7).

We were also interested in testing associations between ancestry and artefacts from Szólád; however, due to limited published archaeological data, we focused on associations of ancestry with brooches from genetically female burials and with weapons from genetically male burials (based on the data published in Alt et al. (61)). Associations between ancestry and brooches among genetic female burials (including both adults and children) was not strong or significant at Szólád ($R^2 = 0.139$, $p = 0.157$) (*SI Appendix*, Fig. S32a). A very unique aspect of Szólád is that unlike other sites in this period, most male individuals (including male children) were buried with weapons (25, 61). We found a strong association between Ancestry A and weapons among adult males ($R^2 = 1$, $p = 0.00963$); however, all but one of the adult males (Sz_43) was buried with weapons, and Sz_43 was close to the Ancestry B extreme. When we performed a regression for ancestry and weapons using all genetic males, we found a non-significant association ($R^2 = 0.132$, $p = 0.0602$), despite the relatively high statistical power (0.810). Association between ancestry and weapons was weak and also non-significant among male children ($R^2 = 0.034$, $p = 0.499$) (*SI Appendix*, Fig. S32b-d). We have consistent results when we test for association with the POPRES-Full and AADR WE PCA results (Table S7).

Artificial cranial deformation

We also conducted a logistic regression comparing ACD to ancestry among the 10 sequenced individuals with well-preserved skulls and coverage greater than 0.1x from Fonyód. We found a strong association ($R^2 = 0.543$, $p = 0.00686$) between increased Ancestry A and ACD; we also found significant associations in our multiple logistic regressions using the POPRES and AADR WE PCAs further demonstrating an association between ancestry and ACD (Table S7; *SI Appendix*, Fig. S33). It is noteworthy that most of the individuals with ACD at Fonyód are mostly towards the middle of the Ancestry A/B spectrum as opposed to the extremes (**Fig. 5**; *SI Appendix*, Fig. S34).

Discussion

A major aim of this study was to examine to what extent genetic connections between individuals observed at these post-Roman 5th century Pannonian sites were known and acknowledged by members of their communities and thus could be understood as a component of social kinship. Biological relatedness might form the basis of or play an important role in the construction of social kinship, but social kinship can also be organized based on shared space, imagined or real descent, economical connection, social agreement, etc. as it is an outcome of social actions, developed and expressed through culturally defined social practices, including funerals (62–64). While there is a slight chronological difference between the three sites (with Fonyód being the earliest and Hács being a few decades older than Balatonszemes) the sites show similar characteristics in demography, funerary customs and burial

representation, and at all three sites, biological kindreds consisted of very close, first and second degree relatives, mostly on the mother's side. This is in stark contrast to Szólád where the cemetery was organized largely around male biological relatives with a large extended pedigree.

Interestingly, out of the six burial groups at Fonyód we do not find any relationships between individuals from Burial groups II, III, or VI, which also contained multiple sequenced graves. Burial groups such as these are generally interpreted as reflections of strong social ties between the deceased (15). In the case of Fonyód such connections are further supported by the distribution of ACD and the similarities in genetic ancestry (*SI Appendix*, Section S2). Burial Group V shows that biological relatedness likely had social value to the community, but the lack of such biological relations in the other burial groups - probably also influenced by the short occupation period of the site - demonstrates that other factors may also have had a major influence on group formation and the organization of the site.

A single biological kindred was recovered at Hács. The spatial organization of the graves does not suggest a clear expression of social kinship (**Fig. 2b**) and similarities are also nonexistent in terms of burial representation due to the generally different nature of adult female, adult male, and child burials in 5th-century Pannonia. Two members (Hacs_4 and Hacs_24) are buried in the same part of the site, while the middle link (Hacs_20) is separated and is located in the southern part of the cemetery. While the lack of spatial clustering may initially suggest a reduced role for biological kinship, it is important to note that all three are directly related along the maternal line, a connection that is especially hard to hide among members of a small community. Previous stable isotope results (65) suggest that Hacs_4 was most likely raised locally, but probably gave birth to her daughter elsewhere, as Hacs_20 lived in multiple locations during her childhood and youth (she also exhibits considerable shifts in her diet) and is the only individual in the Hács cemetery whose $^{87}\text{Sr}/^{86}\text{Sr}$ ratio falls completely outside of the regional values of the Balaton area. These relocations might have been results of marriage practices, and considering the isotopic data, her (Hacs_4) separation might have been a consequence of a unique lifepath. The fact that both women came back (together or separately) to this community later during their lives and were buried at Hács indicates that their connection to the community never ceased to exist and that their biological relatedness nevertheless reflected social ties as well.

The two biological kindreds found at Balatonszemes paint a very different picture. The clustering of three richly furnished female burials (Bal_267, Bal_268, and Bal_269) suggests strong social connection and could be understood as the expression of social kinship (**Fig. 2c**). Interestingly only one of the half siblings showed signs of ACD, which might indicate that the practice was associated with the father's side. The other three related individuals, a mother-father-son trio (Bal_150, Bal_149, and Bal_146), were also buried in close proximity to each other, however such a clear connection in the archaeological data is not observable among them. The differences in terms of grave goods could indicate a social difference, but it should rather be interpreted in the context of 5th century burial representation, where adult male and child burials contain very few if any artefacts (notable exceptions are Bal_267 and Bal_268), while social differences are more clearly expressed in female burials (59, 60).

The small burial groups at Fonyód were previously interpreted as signs of strong social ties (15), while Hács and Balatonszemes have been described as family cemeteries (12, 13, 65). We were able to identify biologically directly related individuals in all three sites, both members of nuclear families (FONYOD1, BAL1) and larger family groups (HACS1, BAL2). Connection in the funerary customs between biologically related individuals is mostly expressed through the position of the graves: related individuals are buried in close proximity to each other (FONYOD1, BAL1, BAL2). In case of BAL2 this is further emphasized by the elaborate burials, as this is the only case where the economical status of the family is similarly represented. While biological relationships indeed played their role in the formation of the analyzed 5th-century funerary communities, these sites (unlike Szólád) were not primarily organized around closely biologically related groups; more likely they can be understood as sites used by multiple social groups (including families) for a shorter period of time. Though more intense sampling of the period will provide a more nuanced understanding of the complex web of kinship relationships of the period, our work already hints at the power of paleogenomics to both directly and indirectly shed new light on the multitude of factors that likely played a role in the formation of these post-Roman communities, which clearly did not depend on simply genealogical ties.

The 5th and 6th centuries witnessed multiple political shifts in this region. Both historical and archaeological research have assumed the arrival and departure of various population groups from the beginning of the 5th century onwards as a result of the Hunnic movement (1, 2, 66). Fonyód, which dates to the peak of the Hunnic power in the region, differs markedly from the later two sites from both an archaeological and genetic perspective. The unique spatial structure of Fonyód suggests that it is a burial site of a short-lived coexistence of Hunnic period groups, part of them practicing the tradition of ACD that is less prevalent in the analyzed later sites. This change in traditions coincides with the lack of the extreme Ancestry A individuals at Fonyód, which we later find at Hács and Balatonszemes. The appearance of these Ancestry A individuals during the second half of the 5th century, after the collapse of the Hunnic Empire, could indicate the arrival of new groups in the region and is the only case in our series that would suggest larger scale mobility. Moreover, we also find that when we compare Hács and Balatonszemes to Szólád as part of a time series, we see a systematic increase in genetic diversity (i.e., wider arrays of prominent ancestry components, more individuals with mixtures of components) through time, with Szólád and Balatonszemes both being more genetically diverse than Hács, and in turn Szólád being more diverse than Balatonszemes. We find that extreme Ancestry A individuals are present at Hács, Balatonszemes, and Szólád, but we currently lack the analytical resolution to determine whether the Ancestry A individuals from these sites are consistent with the same geographic origins. Furthermore, the gradual appearance of individuals that fall towards the middle of the Ancestry A/B distribution could either be the result of the mixing of the extreme Ancestry A and B individuals, or could also mark the arrival of individuals with a new ancestry profile to the region. The continuous presence of extreme Ancestry B individuals from the middle of the 5th until the middle of the 6th century suggests a core population that stayed put and survived throughout this period, but we lack data from the earlier centuries to understand their origin. The wide genetic diversity of these small communities exceeds the diversity found from penecontemporary populations/communities and even from large swathes of late Antique/early Medieval Europe (**Fig. 5**). These findings clearly suggest that this heterogeneity was derived from the coexistence of groups of heterogeneous genomic origins and that the change in material culture does not necessarily need to reflect a change of genetic background.

We found a significant correlation between the presence of “female package” consisting of brooches, amber beads, double-sided combs, etc. (59, 60) and Ancestry A. Importantly, this correlation is replicated when testing with our more conventional POPRES and AADR PCA results. The most elaborate versions of this ‘female package’, including chip-carved buckles, plate brooches, and polyhedral earrings decorated with garnet inlays, appeared in the middle of the 5th century, and its dissemination over a wide area from western Europe to the Pontic Steppe is probably linked to the movements sparked off by the progression of the Huns and the extensive connections of the Hunnic period elites. However, this ‘female package’ was not restricted to the elites, but also appeared in less furnished burials in a reduced form (59). The fact that this ‘female package’ is linked to individuals enriched for Ancestry A could suggest that individuals with different genetic backgrounds were treated differently by the burying community that might indicate cultural and/or social differences between individuals of different ancestries. With the exception of Fonyod_444 we do not find relatively richly furnished burials (i.e., includes gilded jewelry, brooches, or multiple elements of the ‘female package’) among individuals enriched for Ancestry B, while they are common among individuals enriched for Ancestry A in Hács and Balatonszemes. We also conducted logistic regressions assessing the association between amber beads, brooches, and double-side combs individually with genetic ancestry. From multi-site analyses, we found significant associations between amber beads and brooches with Ancestry A (Table S7). The presence of brooches—an artefact that in various forms and types is present in the 5th-century sites as well as the 6th-century Szólád—loses its significant association with genetic ancestry by the 6th century (in the PCAngsd, POPRES-Full, and AADR WE regressions), where relatively richly furnished burials are also present among individuals enriched for Ancestry B. Interestingly, the weakening connection between genetic ancestry and burial customs appears as a parallel process to the gradual appearance of individuals that fall towards the middle of the Ancestry A/B distribution.

Conclusion

Early Medieval history has played a major role in the formation of modern Europe. Promotion of an ethnonationalist rhetoric in the 19th and 20th centuries allowed a link to be made between historical

peoples from various written sources and modern nation states (67, 68). The key to this linkage was the idea of a common ethnic and cultural heritage from these presumed ancestral and homogenous groups after the fall of the Roman Empire. More recent historical, archaeological, and anthropological studies have found this to be a vast oversimplification, with the material culture demonstrating significant complexity within and between communities across the early Middle Ages. However, the nationalist sentiment still remains in modern political discourse, with potentially unsettling consequences. With the development of the field of paleogenomics, a new, yet unexplored dimension could provide new insights into the formation of early Medieval communities. Perhaps the most striking results of our examination of three geographically proximate cemeteries representing post-Roman communities founded within a century of each other (four when including Szólád), are: A) These small sites within an area of less than 200 km² exhibit a striking genomic diversity in comparison to penecontemporaneous Europe; B) Differences and changes in material culture do not necessarily correspond to genetic shifts and differences; and C) The extent to which biological kinship impacts the organization of these cemeteries varied, thus presumably reflecting a wide range of thought on the importance of blood ties to social kinship and organization when founding these communities. Of course, our study represents just a regional vignette during the earliest stages of post-Roman Europe. However, given the immense complexity observed in just these four small cemeteries, it is clear that comprehensive fine-grained spatiotemporal genomic sampling will be critical to unpack the processes that underlie the subsequent development of modern Europe.

Material and Methods

Processing of novel genomic dataset

Petrous bone samples were collected from individuals from Fonyód, Hács, and Balatonszemes, with the exceptions of Fonyod_304 and Fonyod_305 where tibial samples were collected instead. To avoid contamination, the bone samples were powdered in ancient DNA clean rooms at the Department of Biology, Laboratory of Molecular Anthropology and Paleogenetics, University of Florence (for Hács and Balatonszemes) and at the Institute of Archaeogenomics, Eötvös Loránd Research Network in Budapest (for Fonyód). A silica-based protocol was used for DNA extraction (69). For detailed information about DNA extraction, see *SI Appendix*, Section S7.

For Hács and Balatonszemes, all 1240K library capture was conducted at the Max Planck Institute for the Science of Human History (MPI-SHH) in Jena, Germany, while libraries were prepared at the University of Florence. For Fonyód, all libraries and 1240K captures were prepared at the Max Planck Institute for Evolutionary Anthropology (MPI-EVA) Leipzig, Germany.

Libraries from Hács and Balatonszemes were sequenced in 2018, while the libraries from Fonyód were sequenced separately in 2021. For Hács, and Balatonszemes, all sequencing was performed at the New York Genome Center (NYGC). Initially, 25 double-stranded libraries were shotgun sequenced at NYGC using an Illumina benchtop sequencer (MiSeq). All 25 libraries passed screening and were chosen for further sequencing; the libraries that had the most reads mapping to the human reference genome (GRCh37) were chosen for whole genome sequencing (WGS), whereas the other libraries underwent 1240K capture sequencing for 1.24 million SNPs. In total, ten were chosen for WGS, nine from Hács and one from Balatonszemes. For 1240K sequencing from Hács (n=5) and Balatonszemes (n=10), double-stranded libraries were sequenced using paired-end 125bp sequencing on an Illumina HiSeq 2500 sequencer at NYGC. For WGS sequencing, double-stranded libraries were sequenced using single-end 100bp sequencing on an Illumina NovaSeq S4 sequencer at NYGC with each WGS library sequenced over four different flow cell lanes.

For Fonyód, all available genomic libraries underwent 1240K capture sequencing without any screening phase. These libraries were single-stranded (70, 71) and were UDG treated in a manner with results functionally similar to partial UDG treatment or UDG-half (72). These libraries were sequenced using single-end 76bp sequencing on an Illumina HiSeq 4000 sequencer at the MPI-EVA. Sequencing data was processed based on a pipeline from Kircher (73). Reads were trimmed and merged (when necessary), mapped to GRCh37 using samtools (74), duplicate reads were marked using Picard Tools (75), and

reads less than 30bp long were filtered out. All untrimmed reads were excluded from processing and analysis, as these reads are more prone to contain contaminant sequences. As WGS libraries were sequenced over four lanes, data from each lane were initially processed separately but BAM files were merged prior to marking duplicate reads. We used mapDamage 0.3.3 to assess postmortem DNA damage patterns (76).

BAM files from these three novel sites were called using in-house scripts (www.github.com/kveeramah). Non-UDG treated data was processed using a caller that incorporates damage patterns from mapDamage, while UDG treated data was instead processed using an indent caller that disregarded the first and last five bases of any given read. Output VCF files include diploid genotypes as well as genotype likelihoods

We calculated coverage for all individuals for the autosomal 1240K SNPs (as well as for the mitochondrial genome) using gatk v4.2 DepthOfCoverage; for novel WGS data, we also calculated full autosomal coverage using gatk v3.3 DepthOfCoverage in multi-threaded operation (75). We calculated the number of autosomal 1240K SNPs covered by at least one read with mapping and base quality scores greater than or equal to 30 in each BAM file using samtools depth (74). Individuals' genetic sex was identified using the Sex.DetERRmine pipeline to determine relative X and Y chromosome coverage (<https://github.com/TCLamnidis/Sex.DetERRmine>). Subsequently, we used Angsd to estimate nuclear contamination rates of genetic males based on the hemizygous X chromosome (21). We also used Schmutzi to estimate mitochondrial contamination rates for all individuals (20).

Mitochondrial and Y chromosome analyses

For mitochondrial analysis, BAM files from the four Balaton region sites were filtered down to reads aligning to the mitochondrial genome as fastq files using samtools. Mitochondrial genomes from individuals with >1500 mitochondrial reads were assembled using Mapping Iterative Assembler (77) (which was designed for ancient mitochondrial genome assembly) and then haplotype assignment was made using MitoTool 1.1.2 (78).

For the Y chromosome, we restricted the analysis to 1240K Y chromosome SNPs among 1240K-captured males but analyzed the whole chromosome for the four WGS males. Phylogenetic position of each Y chromosome SNP was ascertained based on published databases (<https://isogg.org/>) (38, 79–81) and used to identify NRY haplogroups.

Comparative dataset

We analyzed our newly sequenced data alongside comparative datasets (6, 29–34). All individuals analyzed had date ranges overlapping with the 4th to 8th centuries CE. For all comparative individuals, coverage of the autosomal 1240K SNPs was calculated using gatk DepthOfCoverage as well (75), and (with the exception of four individuals from Szólád) comparative individuals with <0.1× coverage were excluded from our dataset and thus all analyses. In most cases, we used the BAMs published in the study, but in some cases we used BAMs that were re-processed in the Veeramah lab from published fastq files using the same type of pipeline as our novel data. BAMs were called using in-house genotype callers consistent with our novel data. Data from the 1000 Genomes Project phase 3 release was also obtained and used for allele frequency data (38).

Biological relatedness assessment

We used the software package lcMLkin to identify close biological relatedness between the individuals from all four Balaton sites (24). All individuals were included regardless of level of coverage. We used a modified version of lcMLkin that included external allele frequency data, similar to Amorim et al. (6). Analyses were performed using genotype likelihood data from 1,079,996 autosomal 1240K sites as input. We used allele frequency data from the 1000 Genomes CEU and TSI populations (as well as the merger of the two, CEU_TSI) (38). We ignored all relationships between low-coverage individuals with minimal common SNP coverage (i.e., less than 5,000 shared SNPs in an lcMLkin analysis) as these are likely spurious relationships and do not reflect real relationships.

Principal component analysis and Model-based clustering analyses

We analyzed the data from the Balaton region sites alongside data from 182 penecontemporaneous comparative individuals. The list of autosomal 1240K sites was pruned for linkage disequilibrium using Plink 1.9 (82, 83) using data from the 1000 Genome Project EUR super-population using `--indep-pairwise 200 25 0.4` consistent many other studies (29, 44, 84–87). We generated Beagle PL files for the genotype likelihoods for all individuals for the 259,551 pruned-in sites using vcftools (88). We used these Beagle PL files for PCAs using PCAngsd and supervised model-based clustering analysis using fastNGSadmix (28, 37); individuals with coverage $<0.1\times$ were excluded from these analyses. As close biological relatives cannot be included together in a PCA, eight comparative individuals from Olalde et al. (32) and Margaryan et al. (31) were excluded from this analysis; due to more complicated relationships individuals from Collegno from Amorim et al. (6) were also transformed onto the PCA instead of being excluded due to relatedness. PCAs were conducted with individuals from Fonyód, Hács, Balatonszemes, and Szólád as well as Collegno dropped into the PCAngsd analysis one-by-one; each analysis was allowed up to 1000 iterations to ensure convergence. Custom Python and R scripts were used to automate all of these PCAngsd analyses and convert output to a consistent format and PC1/2 polarity; a Procrustes transformation was then used to merge the PCAs into one. An alternative version of the PCAngsd analysis was also run where all transitions were removed from the set of 259,551 SNPs, leaving only 49,692 transversions; individuals with fewer than 10,000 of these sites were excluded from this analysis. The results were qualitatively similar to the overall analysis (*SI Appendix*, Fig. S4).

Logistic regressions testing association of ancestry with grave goods and ACD were calculated using R v4.1.2 (89) and plotted using the ggplot2 (90) and cowplot packages; power analyses were conducted using the WebPower package (91). Hosmer and Lemeshow's R^2 and chi-square p values for each regression were calculated within R.

FastNGSadmix analyses were run for each individual one-by-one using the same 259,551 sites, and related individuals omitted from the previous analysis were included here. Each individual analysis was run 50 times and the run with the greatest (i.e., least negative) likelihood was used. Allele frequency data from the 1000 Genomes Project were used to supervise the analyses (38). As per Amorim et al. (6), we used CEU+GBR, FIN, IBS, TSI, YRI, EAS, and SAS; we merged the CEU and GBR populations into a single population, as the two populations are not properly distinguishable by these types of analyses (6).

Data availability

Our newly generated sequence data from 38 individuals are available from the NCBI Sequence Read Archive (SRA) database under accession PRJNA811958 for 1240K data and accession PRJNA812074 for WGS data. We accessed the POPRES (Population Reference Sample) dataset collected and published by Nelson et al. (50) from dbGaP (accession [phs000145.v4.p2](#)).

Acknowledgements

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement n° 856453 ERC-2019-SyG), from the Anneliese Maier Research Award of the Alexander von Humboldt Foundation, from the Max Planck Society, from the German Federal Ministry for Education and Research, from the Swedish Riksbankens Jubileumfond, by the Czech Grant Agency (GACR 21-17092X), from the Gerard B. Lambert Foundation, from the Institute for Advanced Study Director's Office, and from the Italian Ministry of Education, University and Research (project "Dipartimenti di Eccellenza 2018–2022" and PRIN2017 grants no. 20177PJ9XF). We thank Szilvia Honti, Péter Németh, and the Rippl-Rónai Museum, Kaposvár for providing access to the material from Balatonszemes, and the Hungarian Natural History Museum, Budapest for providing access to the anthropological material from Fonyód and Hács. We also thank Johannes Krause for funding and performing all SNP capture and sequencing.

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Figure Legends

Figure 1: Location and chronological position of the four investigated sites within the (Western) Roman Empire. Roman roads are indicated with red lines, while late Roman inner fortresses of the Lake Balaton area are marked with black. Map created with QGIS v3.22.1 and resources used from Ancient World Mapping Center. “Coastline, River, Inland water, Open water, Roman road”. <<http://awmc.unc.edu/wordpress/map-files/>> [Accessed: January 07, 2022] (92).

Figure 2: Cemetery maps of the four sites. Graves are identified based on age, sex, ancestry, and sampling status as well as the presence of amber beads, brooches, double-sided combs, and weapons. Dotted outlines indicate biological kindred and dotted lines connect kindred members who were buried separately. **a.** Cemetery map of Fonyód. Due to the size of the cemetery, graves sizes are not to scale with the size of the cemetery. This figure was modified from Gallina and Straub (15). **b.** Cemetery map of Hács. This figure was modified from Kiss 1995 (12). **c.** Cemetery map of Balatonszemes. Bal_26 is not included in this figure, as this individual was buried 200 meters west of the main site. This figure was modified from Mihácz-Pálfi (16). **d.** Cemetery map of Szólád. Sz_44 is not included in this figure, as this individual was buried at the southern extreme of the site. This figure was modified from Alt et al. (61).

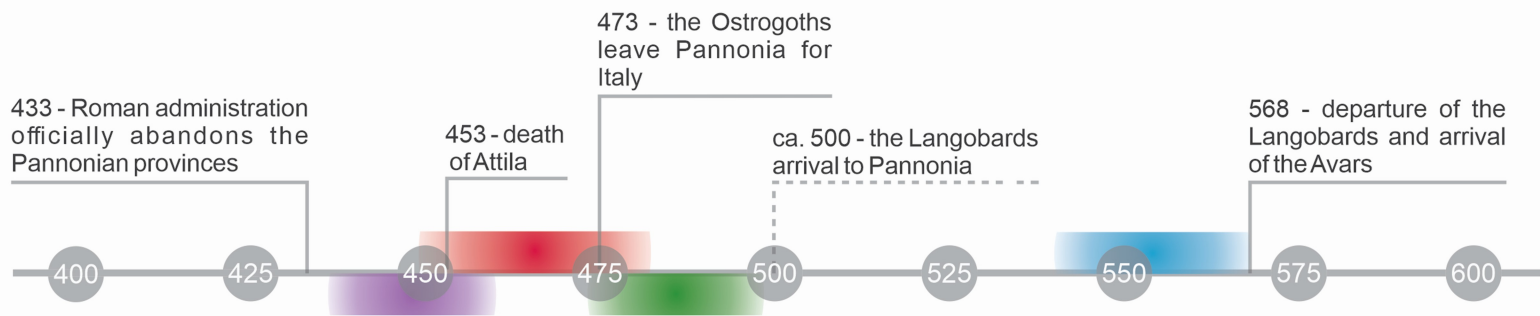
Figure 3: Pedigrees of the biological kindreds found at Fonyód, Hács, and Balatonszemes. Circles represent female individuals and squares males. Individuals are colored based on their Ancestry A/B values consistent with Figure 2. Dashed lines are used in the BAL2 pedigree as the exact placement of Bal_269 is unknown.

Figure 4: Procrustes PCA of individuals from Fonyód, Hács, Balatonszemes, and Szólád transformed on to a PCA with 174 reference individuals (6, 29–34) dating to around the 4th to 8th centuries CE using genotype likelihoods from 259,551 SNPs. References have been grouped by geographical regions. Individuals with <0.1× coverage were excluded from this analysis.

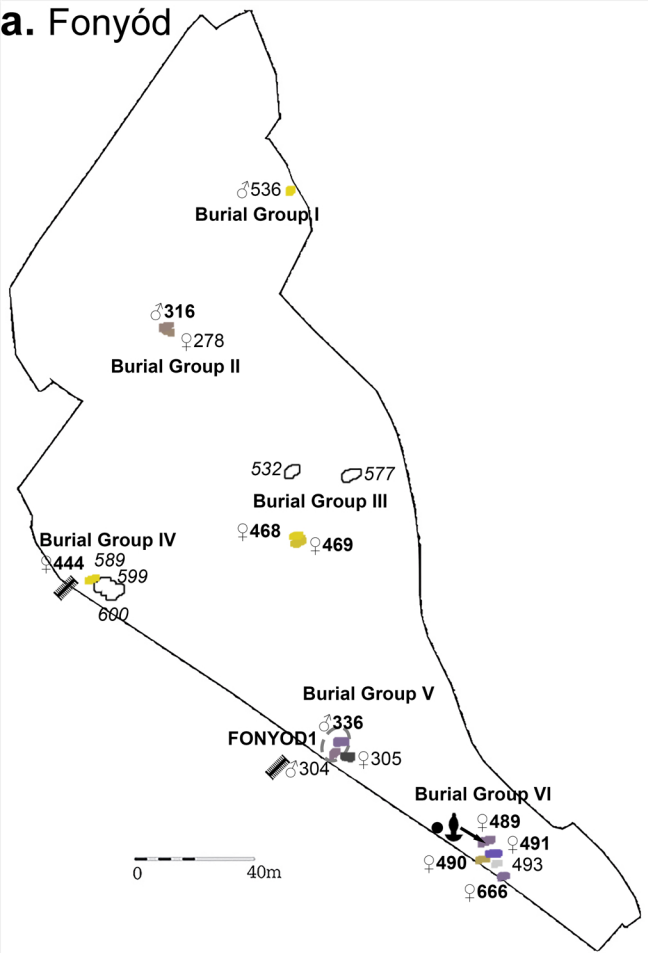
Figure 5: Distribution of Ancestry A percentages from the individuals from Fonyód, Hács, Balatonszemes, and Szólád as well as from the regions from the comparative reference dataset. Colors correspond to the colors used in Figure 4. Crimea and Serbia are excluded from this figure due to low sample size (n=1 each). Individuals from Collegno from Amorim et al. (6) are plotted separately from individuals from Roman sites from Antonio et al. (29) due to the differences between these penecontemporary communities. Similarly, Bavarians from Veeramah et al. (34) were plotted separately based on whether or not they had ACD.

Figure 6: Supervised ancestry proportions from individuals from Fonyód, Hács, Balatonszemes, and Szólád. Proportions were estimated using fastNGSadmix using 1000 Genomes populations as references (38); CEU+GBR refers to a merger of “Northern Europeans from Utah” (CEU) and “British in England and Scotland” (GBR), FIN refers to “Finnish in Finland”, IBS refers to “Iberian populations in Spain”, and TSI refers to “Tuscans from Italy”; EAS refers to the East Asian super-population, SAS refers to the South Asian super-population, and YRI refers to “Yoruba in Ibadan, Nigeria”. Individuals are sorted based on decreasing CEU+GBR. Individuals with <0.1× coverage were excluded from this analysis.

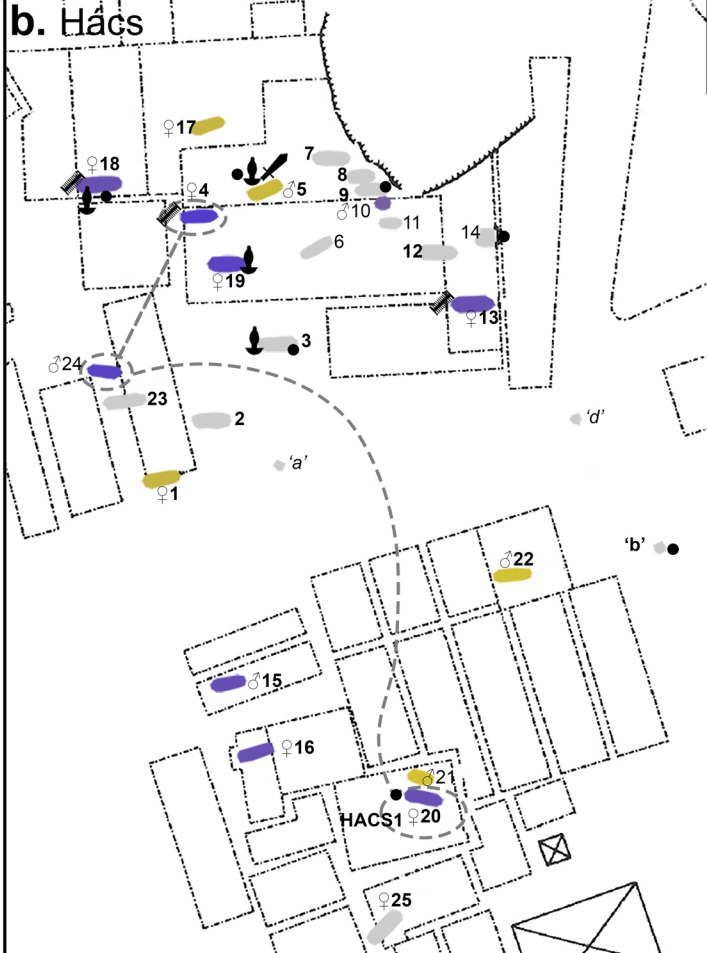
Figure 7: Procrustes PCA from Figure 4 with individuals from Fonyód, Hács, and Balatonszemes in color and all others in grey. The presence of amber beads (black circle), bow brooches (brooch symbol), double-sided combs (comb symbol) and weapons (sword symbol) are indicated. The heavily disturbed and possibly admixed context of Hács_5 that we had to exclude from archaeological analyses is marked with a question mark.



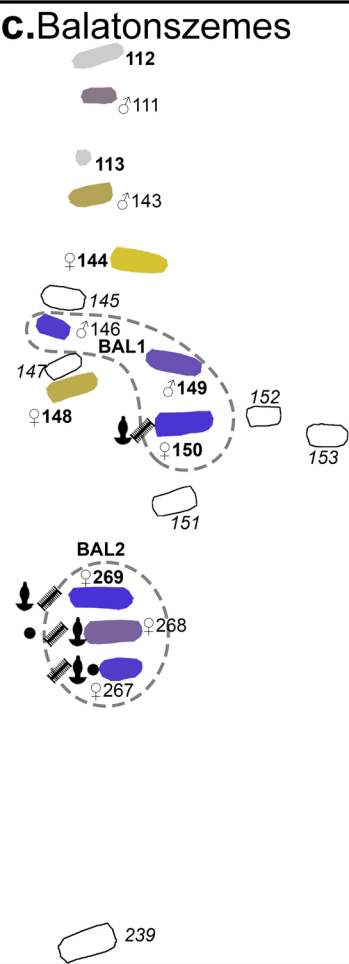
a. Fonyód



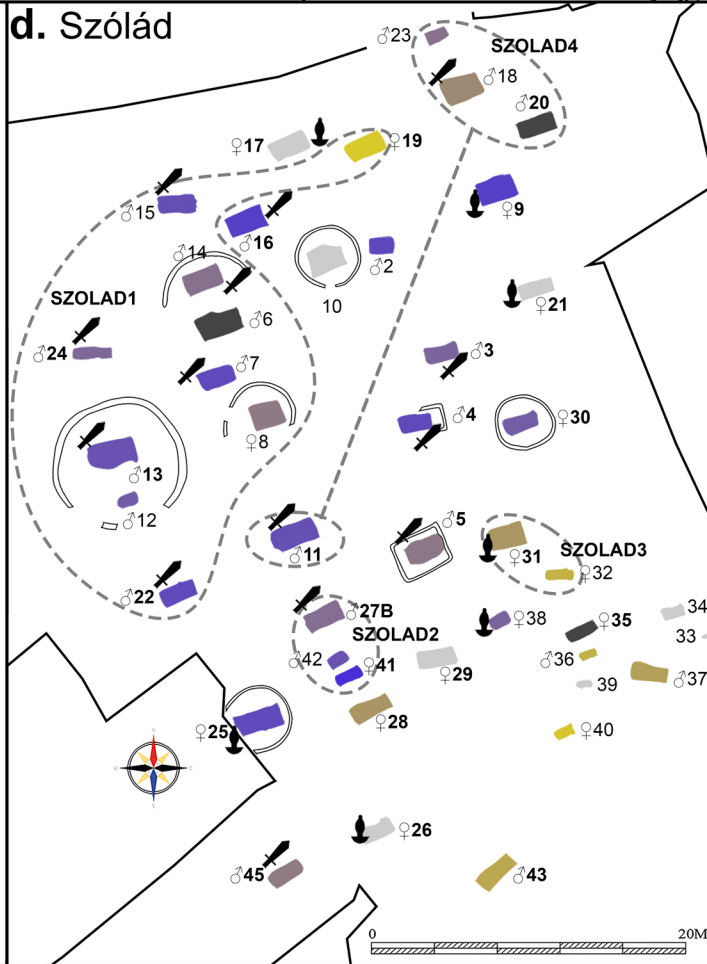
b. Hács



c. Balatonszemes



d. Szólád



Legend

Osteological Age:
Bold: Adult
Regular: Child
Italics: Unidentified

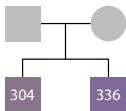
Sex:
♂: Male
♀: Female

Ancestry:
 Ancestry A/B
 Low coverage (<0.1×)
 Grave with bad preservation
 Empty grave

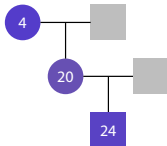
Archaeology:
 Amber beads
 Brooch
 Comb
 Weapon

Biological Kindreds:

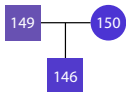
Kindred FONYOD1



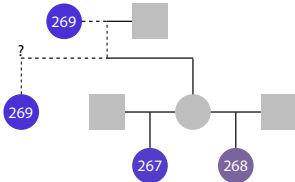
Kindred HACS1



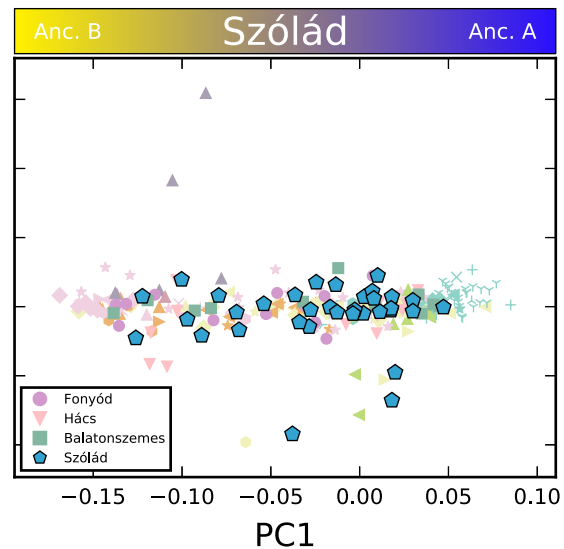
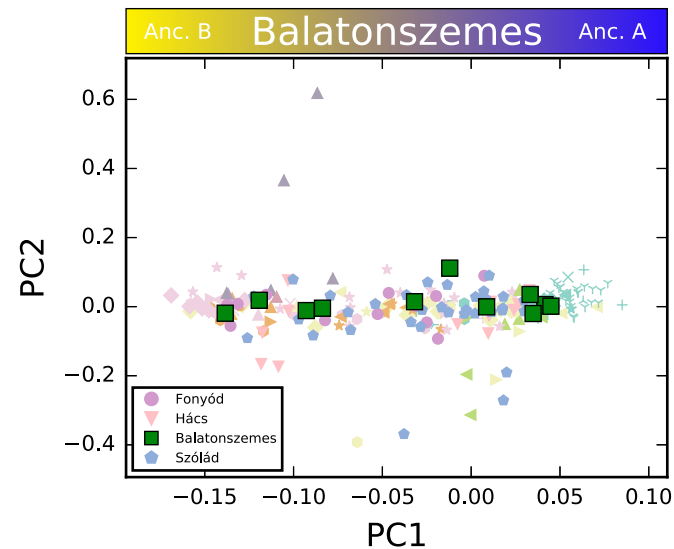
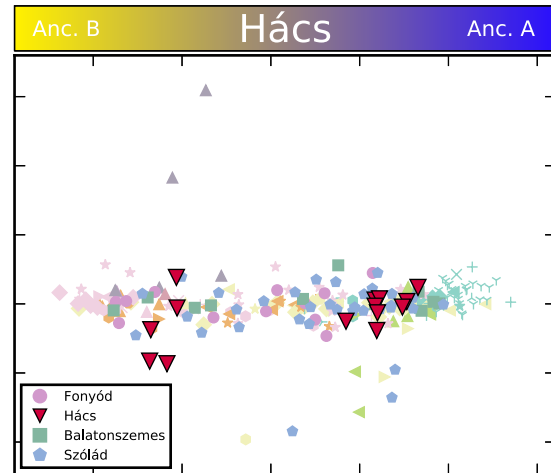
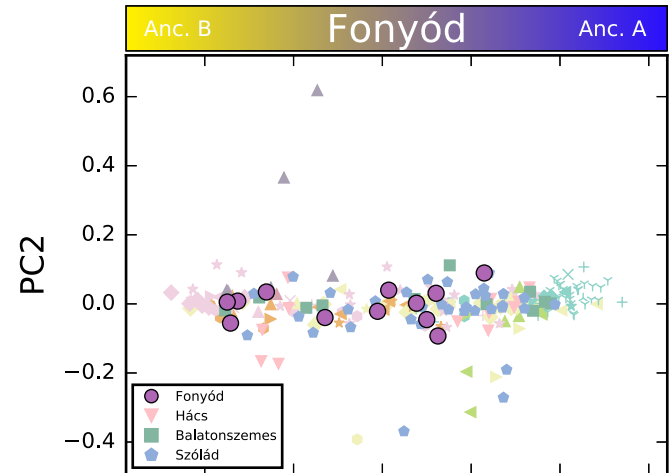
Kindred BAL1

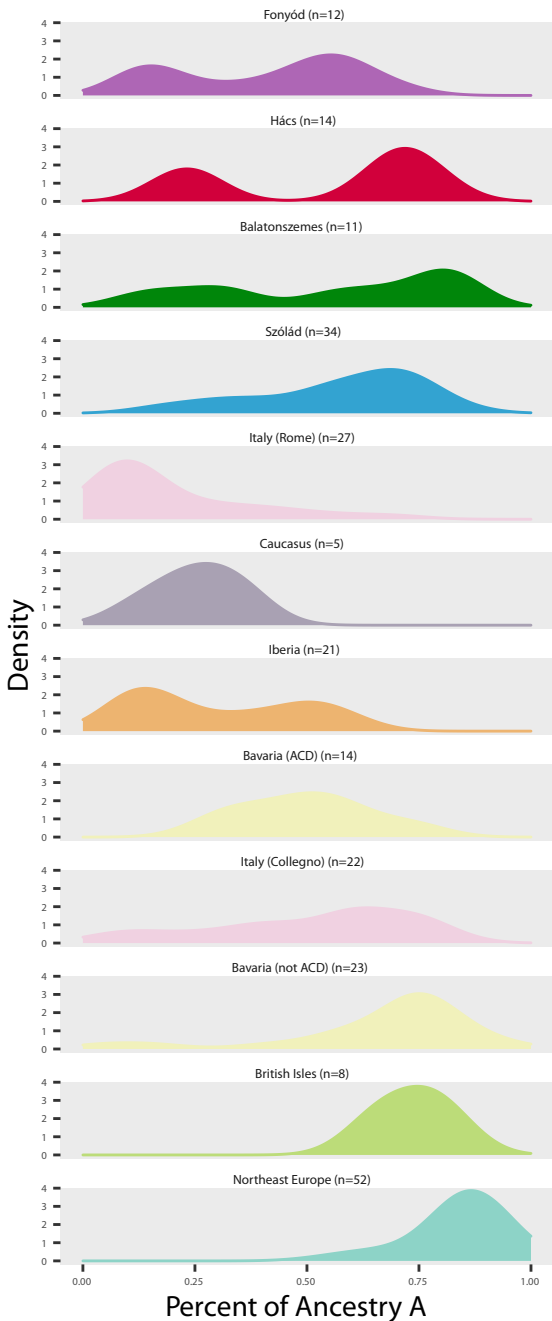


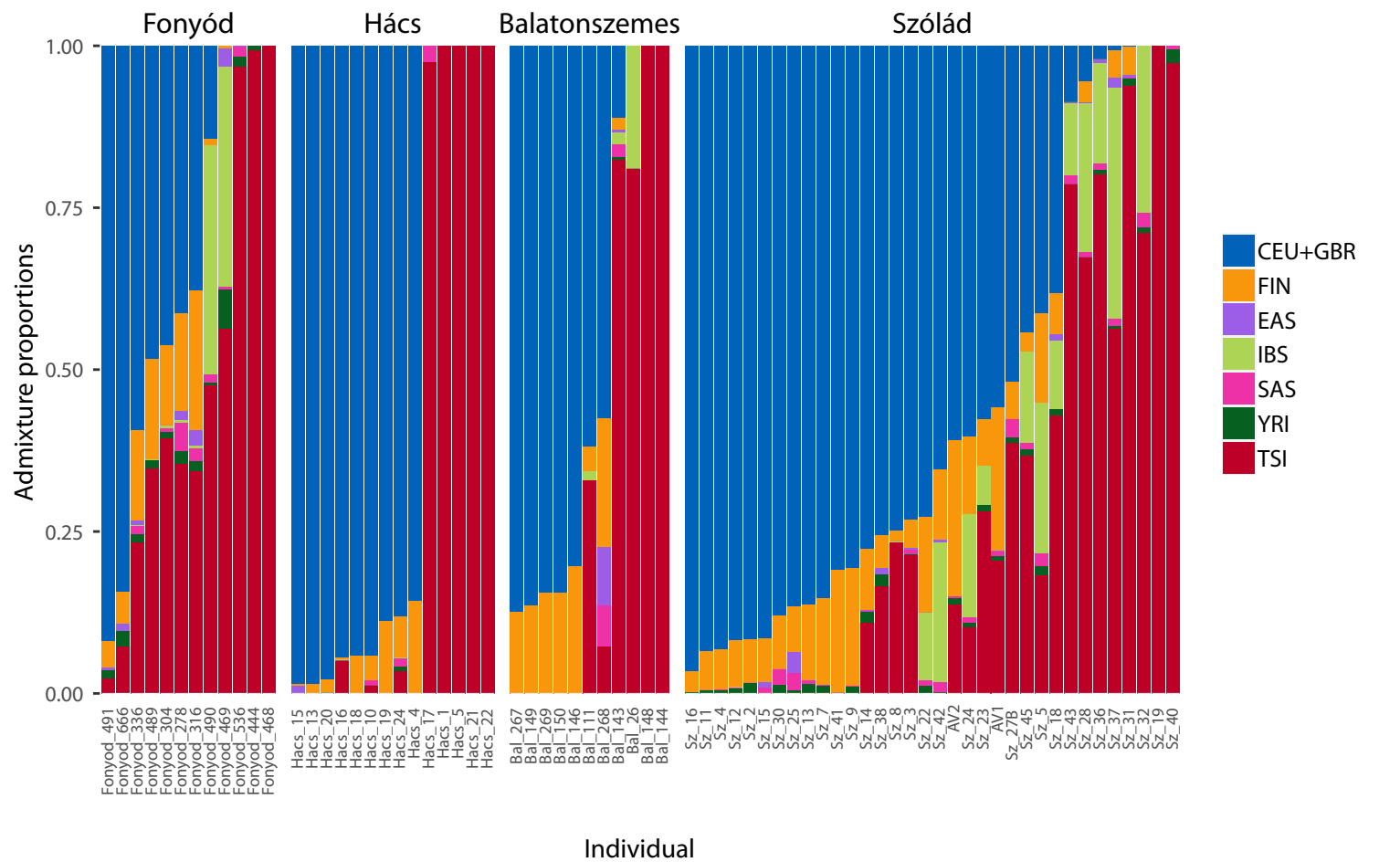
Kindred BAL2



PC1 vs. 2







PC1 vs. 2

