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Short title: mtDNA haplogroup prediction for medieval Bosnian population

The First Insight into Mitochondrial DNA Haplogroup Prediction for the Population Originating from Medieval Bosnia

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Abstract

Population genetic studies have shown that the Bosnian-Herzegovinian (B&H) population is a part of the European gene pool, but until now there is limited information about the genetic structure of ancient B&H populations. In this regard, the aim of our study was to determine the frequency and distribution of mitochondrial DNA (mtDNA) haplogroups for medieval Bosnian population. Thirty-four samples, excavated from medieval necropolises located in the B&H according to the borders of medieval Bosnia, were subjected to analysis in this study. Sequencing of the mtDNA HVS1 region and RFLP analysis were performed for haplogroup determination. In our study, all 32 samples were identified as haplogroup H, with subhaplogroups H2a and H5 determined in 30 and 2 samples, respectively. The frequency of H haplogroup was significantly different between the studied samples and previous studies of contemporary populations in B&H, where the H haplogroup frequency was approximately half of that determined in the current study. A significant difference in H haplogroup frequency was also observed in comparison with other medieval populations outside of B&H, where the ancient B&H population is most similar to ancient

Italians. Our results provide insight into the mitochondrial landscape of populations that inhabited the territory of present day B&H in the Middle Ages. Our study reveals that inhabitants of Medieval Bosnia carried genetic lineages that exist today in B&H populations suggesting continuity of mtDNA haplogroup over a long period of time, regardless of various historical demographic events that shaped the genetic structure of the modern B&H population.

Introduction

In the Upper Palaeolithic, approximately 45000 yBP, the first modern humans arrived in Europe from the Near East and replaced the resident Neandertals (Hajdinjak et al., 2021; Andreeva et al., 2022). After the Last Glacial Maximum (~20000–25000 yBP), Europe was recolonized from southern European refugia (~10000–15000 yBP). Then, about 10000 years ago (during the early Neolithic), demographic growth led to a dispersal of Near Eastern farmers across the continent (Hernández et al., 2017; Lazaridis, 2018; Moutsouri et al., 2024). As a result of these three demographic events, the European gene pool was founded. The European gene pool was further shaped by migrations from the eastern Mediterranean and Near East during the Bronze and Iron Ages. The arrival of nomadic tribes from Russian steppe in southeastern Europe during the Bronze Age (3rd to 4th century BP) was particularly important since intermixing between Proto-Indo-Europeans and autochthonous populations resulted in Proto-Illyrians, the ancestors of Illyrians which were the oldest known Indo-European population in Balkan Peninsula (Sarac et al., 2014). With the Roman domination and later arrival of Slavs in the 6th century, Illyrians have mostly assimilated (Stavros & Stoianovic, 2000).

Located in the Western Balkans, Bosnia and Herzegovina shares borders with Croatia, Serbia, and Montenegro. Its dual name has existed since the Austro-Hungarian occupation in 1878. Due to its geopolitical position, it has long been a magnet for various colonizers, conquerors, and traders, all of whom have contributed to shaping the genetic structure of the modern B&H population. Throughout its dynamic history, numerous cultures have left their mark on the genetic structure of the modern B&H population. Among these, the Illyrians stand out as one of the most significant. Over time, the Illyrians were influenced by Greeks and Romans, followed by Celts, Goths, Avars, and finally the Slavs (Lasic et al., 2016; Ahmic et al., 2019a). During the medieval period (from the 8th century until the Ottoman occupation in 1463), Bosnia experienced significant growth, evolving from a small territory into one of the strongest countries, both territorially and politically, in the Balkans.

In the medieval period, Bosnia enjoyed various forms of state independence and had an

established legal system, as evidenced by numerous charters and contracts. Ban Kulin's charter stands out as one of the oldest and most significant documents in Bosnian history, shedding light on the state-legal and socio-economic conditions of its time (Imamović, 1998). Medieval Bosnia boasted its rulers (initially bans, later kings), flag, army, currency, script (Bosančica), language (Bosnian), religion (Bogumil), defined borders and territory, and its populace (Bosniaks or Bosnians), encompassing all the characteristics of a sovereign nation (Klaić, 1989; Imamović, 1998; Jaliman, 1999; Đozić, 2012). Additionally, other ethnic groups, albeit in small numbers, coexisted within the medieval Bosnian state: Vlachs (settled herders), Saxons (Germans attracted by mining prospects), Latins and Jews, the latter seeking refuge in Bosnia during the late 15th century after being persecuted by the Inquisition following Spain's fall (Malcolm, 1996; Ibraković, 2008).

During subsequent periods, the Ottoman Empire left its mark on the Bosnian population primarily in terms of culture and religion. Similarly, the Austro-Hungarian occupation (1878–1918), Second World War (1941–1945), and the Yugoslav Wars (1991–2001) each significantly influenced Bosnia and Herzegovina's historical and cultural landscape. Migration patterns during these periods were predominantly characterized by emigration and internal displacement (Stavros & Stoianovic, 2000).

Regarding the preservation of genetic information in bone cells for thousands of years, DNA analysis of archaeological human remains has become the preferred method for studying the genetic diversity of past human populations (Shinoda & Doi, 2008). While bones and teeth are the most common materials for DNA extraction from archaeological samples, the quality of these aged specimens is often very low, making their handling more challenging compared to contemporary samples (Daskalaki, 2014). Additionally, it is more likely to obtain information from mitochondrial DNA (mtDNA) than from nuclear DNA when working with ancient samples, making mtDNA analysis the primary and often the only viable choice. Moreover, uniparental markers such as mitochondrial DNA and the Y chromosome have proven to be effective for studying the genealogy and geodemography of populations, which is why they are widely utilized in population genetic studies. Analysis of mtDNA has emerged as a powerful tool for investigating intra- and inter-population diversity, the genetic history of human populations, migration routes, and the settlement patterns of various geographical regions worldwide (Shinoda & Doi, 2008; Li et al., 2015; Poma et al., 2019; Sehrawat et al., 2022).

To date, no studies have investigated the mitochondrial DNA markers of ancient populations that inhabited the territory of present-day Bosnia and Herzegovina. As previously mentioned, the genetic makeup of the modern Bosnian and Herzegovinian population is the result of complex interactions between local and foreign genetic pools. Analyzing mtDNA haplogroups provides a means to chronologically trace the flow of genes within a population, with ancient genetic information serving as direct evidence of past events. Therefore, the objective of our study was to analyze the mtDNA haplogroups of ancient skeletal remains from the medieval Bosnia period to gain insight into the genetic structure of the population during that time.

Materials and methods

Samples

A total of 34 skeletal remains were included in this study. These samples were excavated from various medieval (pre-Ottoman) necropolises (Figures 1 and 2): 1) Biskup Glavaticevo (14th century)—1 sample, 2) Bobovac—Crkvica (14th-15th century)—6 samples, 3) Celebici (14th-15th century)—3 samples, 4) Tuzla (15th century)—8 samples, 5) Klisa-Guca Gora (Travnik) (14th-15th century)—6 samples, 6) Alihodze (Travnik) (14th-15th century)—2 samples, 7) Arnautovici-Mili (14th-15th century)—4 samples, 8) Metaljica (Hadzici) (14th-15th century)—4 samples. The excavations were conducted in strict adherence to regulatory frameworks, with permission granted from the Cantonal Institute for the Protection of Cultural-Historical and Natural Heritage (Phase 1—Z-04—Z-05—1406/19—1 and Phase 2 01—814/20-Z05). This project is funded by a public research grant from the Ministry of Science, Higher Education, and Youth of Canton Sarajevo, subject to scrutiny and approval by designated international reviewers. Despite the challenges posed by the antiquity of the samples, their lineage remains indeterminate. Nevertheless, all procedures were meticulously executed in accordance with legal statutes governing archaeological endeavors. Consensus was attained from the local community, the landowner hosting the archaeological site, and the respective municipality overseeing the necropolises.

{~?~IM: insert Figure 1 here.}

Figure 1. Approximate location of medieval necropolises where the samples were excavated: 1. Biskup Glavaticevo; 2. Bobovac—Crkvica; 3. Celebici; 4. Tuzla; 5. Klisa-Guca Gora (Travnik);

6. Alihodze (Travnik); 7. Arnautovici-Mili; 8. Metaljica (Hadzici)

{~?~IM: insert Figure 2 here.}

Figure 2. Aerial footage capturing the Metaljica necropolis (post-excavation)

DNA extraction and purification

To mitigate the risk of contamination with contemporary DNA, extraction procedures were conducted in a dedicated pre-PCR laboratory specialized for ancient DNA analysis at the Laboratory for Forensic Genetics, University of Sarajevo-Institute for Genetic Engineering and Biotechnology, Bosnia and Herzegovina. These procedures were carried out in accordance with recommendations for ancient DNA authentication (Willerslev & Cooper, 2005). The laboratory hood specifically designated for ancient DNA work, as well as all working areas and laboratory equipment, underwent thorough cleaning with sodium-hypochlorite and 70% ethanol, followed by overnight irradiation with UV light. Prior to DNA extraction, samples were immersed in a 5% w/v sodium-hypochlorite solution, rinsed three times with distilled water, soaked in absolute ethanol (Sigma Aldrich, USA), and then placed in a clean paper towel for five days to air dry. Subsequently, the dried samples were ground into a fine powder using an IKA Tube mill (IKA® -WerkeGmbH&Co.KG, Germany). Approximately 1g of dental powder per sample was used for DNA extraction.

Optimized phenol-chloroform-isoamyl alcohol protocol was used for DNA extraction, preceding decalcification with 0.5 M EDTA (Sigma Aldrich, USA) for seven days. DNA extracts were then purified using DNA-free water and Amicon Ultra 0.5mL centrifugal filter units (Merck, Millipore, Carrigtwohill, Co. Cork, IRL). To ensure the reliability of our results, DNA extraction procedure was done in two temporally and spatially separated times for each sample by different laboratory researchers. Additionally, negative extraction controls, for each isolation procedure, were included to rule out contamination during the extraction process. These controls, lacking the target DNA, played a crucial role in identifying any potential contamination during the extraction phase. The reliable absence of DNA amplification in these negative extraction controls offered additional confidence in the purity of our samples right from the start. For the additional authentication of isolated ancient DNA (aDNA), DNA profiles were generated using autosomal STR markers for 32 analyzed samples. This analysis utilized either PowerPlex® Fusion System (Promega Corporation, Madison, WI, USA) or Investigator® 24plex QS kit

(Qiagen, Hilden, Germany), following the manufacturers' recommendations (Promega, 2020; Qiagen, 2021).

Amplification, sequencing of hypervariable segment 1 (HVS1) and RFLP analysis of mtDNA coding region

Hypervariable segment 1 (HVS1) of the mtDNA control region (CR) was amplified and sequenced for all tested samples. The amplification of the total HVS1 CR mtDNA was performed using the primers L15971 (TTA ACT CCA CCA TTA GCA CC) and H16410 (GAG GAT GGT GGT CAA GGG AC) under the following conditions: initial denaturation at 94°C for 2 min, followed by 32 cycles of denaturation at 94°C for 20s, annealing at 56°C for 25s, and elongation at 72°C for 30s, with a final extension at 72°C for 5 min. The total volume of the PCR reaction was 30 µl, containing 1× PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 0.05 U/µl Taq polymerase, and primers at final concentrations of 0.2 µM. PCR products were analyzed on a 1.5% agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe). To mitigate PCR-based contamination, two independent replications were conducted in a separate PCR laboratory that was cleaned with sodium-hypochlorite and 70% ethanol and UV irradiated overnight. A blank PCR control was included in each reaction. To ensure the integrity of our PCR process, we used a positive PCR control containing DNA from one of the rarest haplogroups in Bosnia, haplogroup M. This control served as a benchmark for successful DNA amplification. To monitor the possibility of contamination during PCR, we also included negative PCR controls lacking template DNA in each PCR run. The consistent absence of DNA amplification in all negative control samples strongly indicated the absence of contamination during PCR setup and execution. This rigorous approach significantly minimized the risk of false-positive results, ensuring the reliability of our experimental outcomes.

Moreover, the genetic background of the researchers involved in the PCR setup was taken into account. It's worth noting that neither of the two researchers involved in the PCR setup possesses haplogroup H, eliminating the possibility of inadvertent contamination from the personnel directly involved in the experimental procedures. Sequencing of PCR products was performed in collaboration with Eurofins Genomics (Ebersberg, Germany). Restriction Fragment Length Polymorphism (RFLP) analysis of haplogroup-specific markers of the mtDNA coding region was conducted following the protocol by Torroni et al. (1996).

Analysis of sequences

BioEdit software 7.2 (Hall, 1999) was used for sequence alignment, in which all of the sequences were aligned against revised Cambridge Reference Sequence (rCRS) (NC_012920.1; Andrews et al., 1999). Each nucleotide position in the corresponding electropherograms was manually verified. For haplogroup prediction, we utilized MITOMASTER (www.mitomap.com) and HaploGrep2 (Weissensteiner et al., 2016) with PhyloTree 17 (van Oven & Kayser, 2009).

Statistical analysis

Given that the percentage frequency of the H haplogroup was 100% in our study, χ^2 test of percentage was used to compare proportion of mtDNA haplogroups between populations. Above mentioned calculation was done using MedCalc software v. 20.115 (MedCalc® Statistical Software, 2022). For the purpose of comparing our results with other European medieval human populations, we constructed a neighbor-joining dendrogram using PAST software version 4.12 (Hammer, 2001).

Results and discussion

The analysis of DNA from ancient skeletal remains dating back to the medieval period has provided the first comprehensive database of mitochondrial DNA variations among populations living in the area of Bosnia and Herzegovina during this historical era.

In this study, we examined thirty-four skeletal remains excavated from eight necropolises dating from the medieval Bosnia period to determine their mitochondrial DNA haplogroup. Autosomal ancient DNA profiles were successfully generated for 32 samples, confirming authenticity, and ruling out cross-contamination (Supplementary Table 1). Ancient DNA profiles could not be obtained for two samples, likely due to insufficient DNA quantity, resulting in their exclusion from further analysis. Furthermore, all laboratory personnel at the Institute maintain STR DNA profiles to facilitate the identification of any contamination. Subsequent STR profiling of medieval samples revealed that none of the profiles corresponded to those of the project participants (Supplementary Table 1). For each of the 32 aDNA samples, amplification of the hypervariable segment 1 (HVS1) of the mtDNA control region was conducted in duplicate. Negative PCR controls were included in each reaction to exclude contamination with

contemporary DNA. In order to maintain the accuracy of our PCR procedure, we utilized a positive PCR control containing DNA from haplogroup M, an uncommon haplogroup in Bosnia and Herzegovina. All negative PCR control samples showed no amplification of DNA, and both researchers participating in the PCR setup do not carry haplogroup H. Sequencing of the HVS1 region was successful for all samples, and the results were further confirmed by RFLP analysis. In summary, our meticulous implementation of quality control measures, including both positive and negative PCR controls, consideration of researchers' genetic backgrounds, and the utilization of laboratory personnel STR profiles, upheld the rigorous integrity of our experimental process. These measures collectively safeguarded against contamination, reinforcing the accuracy and validity of our research findings.

Our study reveals that the inhabitants of medieval Bosnia predominantly carried mitochondrial haplogroup H (Table 1, Figure 3). Haplogroup H is the most common and diverse mitochondrial DNA lineage in present-day western Europe, with frequencies ranging from 30% to 50% (Richards et al., 2000; Hernández et al., 2017). In contemporary populations of Bosnia and Herzegovina, haplogroup H also exhibits a high frequency, approximately 50% (Malyarchuk et al., 2003; Cvjetan et al., 2004; Ahmic et al., 2014). This haplogroup is believed to have originated in the Near East before migrating to Southwest Europe, where it survived the Last Glacial Maximum around 22,000 years ago, followed by a post-glacial re-expansion (Torroni et al., 1996; Richards et al., 2000; Achilli et al., 2004; Pereira et al., 2005). According to the study by Brotherton et al. 2013, haplogroup H was established in Europe by the Mid Neolithic populations around 4000 BC, with significant genetic contributions from various pan-European cultures during the Late Neolithic period around 2800 BC.

{~?~IM: insert Table 1 here.}

Table 1. Haplogroup/subhaplogroups determined in our study

{~?~IM: insert Figure 3 here.}

Figure 3. H haplogroup percentage by studies and populations

Several studies have reported varying frequencies of haplogroup H in contemporary populations of Bosnia and Herzegovina, ranging from 47% according to Cvjetan et al. (2004) to approximately 50% in populations of North-Eastern Bosnia as reported by Ahmic et al. (2012, 2013, 2014, 2019). Haplogroup H is similarly prevalent in neighboring Serbian and Montenegrin populations (Sarac et al., 2017; Scorrano et al., 2017) as well as in the Croatian population (Sarac

et al., 2012; Barbaric et al., 2020). Kovacevic et al. (2014) further noted that haplogroup H exhibits the highest frequency among all analyzed western Balkan populations, ranging from 31.9% to 46.3%. The dominance of haplogroup H in contemporary populations has led many authors to hypothesize that its high frequencies have persisted for a considerable period, potentially from ancient times to the present. Indeed, studies have indicated that haplogroup H was prevalent in populations inhabiting the area of Italy since the Metal Age (Brisighelli et al., 2008; Ottoni et al., 2009; Poma et al., 2019; Modi et al., 2020; Fontani et al., 2021; Aneli et al., 2022). Similar observations have been made for Hungarian (Semino et al., 2000; Nadası et al., 2007; Cs6sz et al., 2016) and Romanian populations (Turchi et al., 2016; Cocos et al., 2017; Gıngua et al., 2021), where haplogroup H remains the most frequent both in contemporary and ancient populations.

The apparent uniformity of haplogroup distribution among samples from medieval Bosnia was unexpected despite the high frequency of haplogroup H in modern Bosnia and Herzegovina populations (Malyarchuk et al., 2003; Cvjetan et al., 2004; Ahmic et al., 2013). This consistency may suggest a pattern of sustained high frequencies of haplogroup H from the medieval period to the present day. However, a comparison of haplogroup proportions between medieval and contemporary Bosnia-Herzegovina populations using a χ^2 test (MedCalc software v. 20.115, 2022) revealed a statistically significant difference in the frequency distribution of haplogroup H.

Comparing the percentage representation of haplogroup H in the population of medieval Bosnia with previous studies of contemporary populations in Bosnia (Table 2), it is evident that there is a statistically significant difference ($p < 0.0001$). In the medieval population of Bosnia, the percentage of representation of haplogroup H was 100%. However, in contemporary populations, this percentage generally hovered around 50%, varying across geographical regions where most of the samples were taken. The smallest difference was observed in the studies by Primorac (2016) and Ahmic et al. (2013), around twice as much, respectively.

{~?~IM: insert Table 2 here.}

Table 2. χ^2 test of percentage comparison between medieval and contemporary populations from Bosnia and Herzegovina

Numerous studies on mtDNA variations in Southeastern European populations, which encompass Bosnia and Herzegovina, have revealed the presence of characteristic Western-

Eurasian mtDNA haplogroups, including H, I, J, K, T, U, V, W, and X. These studies also highlight a smaller proportion of Asian and African lineages, as well as the significant impact of historical population migrations in shaping the genetic structure of these populations (Malyarchuk et al., 2003; Cvjetan et al., 2004; Ahmic et al., 2013, 2014; Kovacevic et al., 2014; Kapur-Pojksic et al., 2014; Davidović et al., 2015; Šarac et al., 2014, 2017).

Previous studies on mtDNA polymorphisms in the contemporary population of Bosnia and Herzegovina have demonstrated high variability in mtDNA markers without significant genetic differences within the population (Malyarchuk et al., 2003; Cvjetan et al., 2004; Ahmic et al., 2012, 2013, 2014, 2019; Kapur-Pojksic et al., 2014; Kovacevic et al., 2014). Haplogroup H, considered a major determinant of all Paleolithic European settlers (Torroni et al., 1998; Richards et al., 2000; Achilli et al., 2004; Pereira et al., 2005), is the most common mtDNA haplogroup in the Bosnian-Herzegovinan population, with a frequency of approximately 50%. The second most frequent haplogroup is U (~10%), which appeared among the first European settlers in the early Upper Paleolithic (Richards et al., 2000; Malyarchuk et al., 2003), followed by haplogroup J (~8%), associated with gene flow brought to Europe by Neolithic expansion from the Near East (Pala et al., 2012). The remaining 32% belong to other typical Western-Eurasian haplogroups (I, K, T, V, W, X). Furthermore, previous studies of variations of the mtDNA in Bosnia and Herzegovina (Ahmic et al., 2013; Ahmic et al., 2019) have identified the presence of rare European lineages, such as the HV* clade (HVO, HV4, HV12b), which are informative in the study of prehistoric movements after the Last Glacial Maximum (LGM) from the Middle East to Europe and South Asia (Shamoon-Pour et al., 2019). Additionally, the Africa/Southern Asia Neolithic N1a haplogroup with variant 16147G (Palanichamy et al., 2010), almost absent in Europe, has been observed in the Bosnian-Herzegovinan population. These findings suggest an early settlement of the Bosnia territory by Early Neolithic populations from the Middle East.

Comparison with the other medieval populations (Table 3) also indicates a statistically significant difference in the frequency of haplogroup H due to the fact that the percentage representation of the populations in the mentioned studies was from 0% to 75% ($p=0.0047$; $p=0.0004$; $p<0.0001$).

{~?~IM: insert Table 3 here.}

Table 3. χ^2 test of percentage comparison between medieval populations

The construction of the neighbor joining dendrogram (Figure 4), based on the Euclidean distance using the percentage prevalence of the H haplogroup, reveals a grouping of the population of medieval Bosnia with Italian and Spanish medieval populations, distinct from Asian and Central European populations. This finding is novel, as contemporary populations from South-Eastern Europe typically exhibit closer genetic affinities. There is no historical data about rapid migration processes and admixture between Bosnian and Mediterranean population from Italy and Spain to justify results which we obtained about similarities based on the highest frequencies of haplogroup H. Furthermore, the absence of other haplogroups in the samples from different regions of medieval Bosnia suggests a high probability of low frequency of other haplogroups in the overall population of that period. This somewhat surprising result, indicating the presence of the same mtDNA haplogroup in all samples from different localities, may suggest that the population of medieval Bosnia was relatively closed, with limited gene flow within medieval populations from today's neighboring countries and the wider region.

{~?~IM: insert Figure 4 here.}

Figure 4. Neighbor joining tree of medieval populations

At the subhaplogroup level, all samples from the ancient medieval Bosnia population belonged either to the H5 or H2a sub-haplogroup of haplogroup H (Table 1). Subhaplogroup H2a, one of the most common subclades of haplogroup H in Europe, with the highest frequencies observed in eastern Europe (Brotherton et al., 2013), was predominant in our study. This lineage is also prevalent in contemporary populations of Bosnia and Herzegovina (Kovačević et al., 2014), as well as in populations from neighboring regions such as Serbia (Davidović et al., 2015), Croatia, and Slovenia (Sarac et al., 2014). The prevalence of subhaplogroup H2a in the autochthonous medieval Bosnian population suggests its long-standing presence in the region since the period of the Last Glacial Maximum. It is proposed that H2a entered Europe from the Near East around the Last Glacial Maximum and underwent partial redistribution across Europe during the late-glacial re-expansion (Pereira et al., 2005)

Subclade H5, predominant among contemporary populations of Western and Eastern Slavs, is believed to have originated approximately 11,000 to 16,000 years ago in southern Europe and subsequently re-expanded across Europe from southern European refugia after the Last Glacial Maximum (Mielnik-Sikorska et al., 2013). In our study, we identified two samples with the standard motif T16304C of haplogroup H5 and the rare mutation C16192T, which,

according to a recent global mtDNA phylogenetic tree (van Oven & Kayser, 2009), is only recorded in the study by Behar et al. (2012) and defines haplogroup H5p. Additionally, research on ancient mitochondrial profiles of Neolithic origin (approximately 6000 years ago) suggests that European farmers were carriers of haplogroup H5 (Fernández et al., 2008). Although the lineage H5 is represented by a reduced frequency in samples from ancient medieval Bosnia, it is noteworthy that they have also been observed in contemporary samples from Bosnia (2.53%) in the study by Ahmic et al. 2022. Furthermore, this haplogroup is observed in neighboring populations from Serbia (Davidovic et al., 2015), Croatia and Slovenia (Sarac et al., 2014). These findings may potentially be attributed to the Slavic migrations to the Balkans, including the territory of present-day Bosnia and Herzegovina, during the Early Middle Ages (Koder, 2020), leaving a lasting genetic imprint until today.

Our findings offer the first insights into the mitochondrial gene pool of the medieval Bosnian population, revealing the persistent presence of the H haplogroup at high frequencies over an extended period. Furthermore, our results suggest that sub-haplogroup H2a was the prevalent sub-haplogroup within the haplogroup H lineage of the medieval Bosnian population. These findings contribute to our understanding of the chronology of genetic structure shaping in Bosnia and Herzegovina, spanning from the Middle Ages to the present day. Additionally, they support previous research indicating the enduring dominance of haplogroup H in southeastern Europe since the arrival of the first humans in the region.

The genetic structure of modern Bosnia and Herzegovina (B&H) population has been continuously influenced by numerous migrations, driven by its specific geographical position and historical socio-economic circumstances (Lasic et al., 2016). In comparison to contemporary populations, where higher frequencies of haplogroups U, J, and K have been detected alongside haplogroup H, these four haplogroups collectively constitute 84% of the frequency in present-day Bosnia and Herzegovina, with haplogroup H alone representing over 50% of that.

It is noteworthy that the homogeneous nature of the medieval Bosnia population, characterized by identical haplotypes with 93.75% belonging to haplogroup H2a, may be attributed to factors such as population size, demographic history, and the influence of different micro-evolutionary processes during this period.

The remarkably high frequency of haplogroup H in the Bosnian population, particularly in comparison to contemporary populations, may be attributed to various factors. Mezzavilla et

al. 2023, studying the variation of 1000 European genomes, concluded that the reduction in the effective size of human populations between the 13th and 16th centuries coincided with the onset and conclusion of the Black Death epidemic in Europe during the medieval period, particularly in the 14th century. We assume that the Bosnian population experienced a similar pattern during this time. Our findings suggest the enduring dominance of haplogroup H over a long period, from the Middle Ages to the present day, despite the various historical and demographic events that have influenced the genetic structure of the Bosnian and Herzegovinian (B&H) population. Unfortunately, archival data regarding the Bosnian authorities' response to the outbreak of the plague and subsequent migration are scarce, as many records were destroyed during the Ottoman invasion. However, indirect information can be gleaned from documents in the Ragusa Republic archive (now Dubrovnik), with which Bosnia maintained close relations.

The historical data reveal a series of waves of the Black Plague during the medieval period, with specific outbreaks documented in 1348, 1371, 1401, 1416, 1421, and 1456 (Ravančić, G. 2006; Hrabar, B. 2008). Additionally, Hrabar mentions a recorded great famine in Bosnia during the period 1454–1455, which undoubtedly resulted in numerous fatalities. These events would have undoubtedly had a significant impact on the demographic landscape and genetic composition of the Bosnian population during that time.

The most probable transmission route of the plague was from the Ragusa Republic, which had extensive trade relations with Bosnia. Since the Ragusa Republic archives also encompassed records of medieval Bosnia, it can be inferred that the population loss during the first wave of the plague in 1348–1349 in Dubrovnik amounted to 60%, mirroring the devastation seen in other Mediterranean seaports and trading centers. As the plague spread to the continental part, the proportion of population loss ranged between 25–40%. Historical estimates suggest that the population loss in medieval Bosnia fell within this range for the continental part. All the locations from which samples in this study were obtained represent areas of higher population density, some of which were designated as royal cities. It is therefore assumed that the percentage of population loss due to the plague in these areas corresponded to the higher end of the range, approximately 40%.

The emigration processes until 1463 should be taken into account as a factor of possible genetic drift. Migrations were recorded in the direction from Bosnia towards Dubrovnik, driven by economic reasons as well as wars during the period of Ottoman arrivals in 1390, 1416, and

1463 (Dinić Knežević, 1995). In 1459, according to information from Ćosiković, 1990, King Tomas persecuted members of the Bosnian Church, labelling them as heretics, and most of them escaped to the outskirts of Dubrovnik. A study by Živković (1990) states that migrations mainly occurred from Bosnia to Dubrovnik, while traders from Dubrovnik brought ores and other goods, mostly to ore-rich areas. Studies indicate that they typically did not settle in Bosnia but were only tied to it by work. Besides them, merchants from Venice, Genoa, Florence, and others could be found, but they stayed for a limited period without the intention of settlement. The only historically recorded migrations, on a small scale, were those of the Vlachs, mostly in border areas, where many of them became slavicized.

All these factors could have a significant impact on the fluctuation of the human population within medieval Bosnia. However, determining the population size in the latter part of the 14th century and the initial half of the 15th century proves challenging based on archival records. Nevertheless, the Ottomans compiled a defter (tax list) in 1468, as noted by Malcom in 1996, relying on the count of families, where he assumed an average family size of five. This implies a population slightly surpassing no more than 200,000. It's essential to approach this figure cautiously, considering fluctuations due to the impact of black plague epidemics during that period. The result of bottleneck analysis based on 22 autosomal STR markers from the medieval Bosnian human population (Džehverović, 2022) supports the claim about a notable size reduction. The study showed a bottleneck process within the medieval Bosnian population, as statistically significant heterozygosity excess was noticed ($p < 0.05$) when IAM and TPM models were considered. Simulation of the SMM model showed that this excess was at a statistically significant value limit ($p = 0.05$). Due to all the aforementioned factors and the limitation in the number of samples, genetic homogeneity is observed in the medieval population concerning mtDNA.

Conclusion

The detected genetic homogeneity in the medieval Bosnian population is 100% haplogroup H, a frequency that is twice as high as in contemporary populations. Histogrammic data indicate a reduction in the size of the population, possible due to reduced gene flow (pronounced emigration processes) and increased mortality due to Black Death and famine, thus increasing the probability of genetic drift. The greatest similarity according to the frequency of haplogroup

H is with the populations of Italy and Spain, although there are no historically justified reasons, given that no mixing of the mentioned populations has been recorded.

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Conflict of interest statement

The authors report no conflict of interest.

Data availability statement

All data that support the findings of this study are available in the tables and figures contained within this article. Additionally, relevant genetic sequences of the HVS1 region have been deposited in the NCBI GenBank, and corresponding accession numbers are provided in Table 1.

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Supplementary Materials

{~?~IM: insert Supplementary Table here.}

Supplementary table 1. Consensus autosomal profiles of aDNA samples and DNA profiles of lab authors