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# Paleogenetics of Northern Iberian from Neolithic to Chalcolithic Time

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## Abstract

Dynamics of the Neolithic transition across Europe using ancient DNA datasets have established that Neolithic European populations received a limited amount of admixture from resident hunter-gatherers. However, the genetic diversity of Neolithic and Chalcolithic human populations was shaped predominantly by local processes. In the Iberian Peninsula, the Cantabrian fringe showed different proportions of local hunter-gatherers' ancestry through time. The objective of this chapter is to analyze the mitochondrial variation of populations from the northern Iberian Peninsula from Neolithic to Chalcolithic time using new data from El Aramo mine (Asturias), in the context of the debate about the origin and dispersion of the Beaker culture in Europe.

**Keywords:** paleogenetic, northern Iberia, El Aramo mine, Bell Beaker culture, Chalcolithic

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## 1. Introduction

### 1.1. State of the art

Ancient mitochondrial DNA (mtDNA) provides important insights into the movement and spread of human populations. In particular, European populations exhibit some remarkable changes after the end of the last glacial maximum (after 20,000 YBP). The changes in the early postglacial period are thought to be the result of the arrival of new human population groups to Europe [1]. These new populations brought to Europe new mitochondrial DNAs that caused a change in the frequency of the indigenous mtDNA lineages. By reconstructing

the variability of the mtDNA of past populations, it is possible to infer population movements that shaped the current genetic variability of our species.

One of the most studied population movements is Neolithization, the transition from a nomadic hunter-gatherer to an agro-pastoralist lifestyle. The debate on the mechanisms of the Neolithic transition has been framed within a dichotomy based on either a demic (DD) or a cultural diffusion (CD). According to the DD model, the migrating people bringing new knowledge experienced some gene flow with the local hunter-gatherer groups. On the other hand, the CD model postulates that the Neolithic transition was mediated mainly through the transmission of the agro-pastoralist system without substantial movement of people [2].

However, several DNA studies on different ancient European populations indicated a more complex pattern for the Neolithic transition. Unlike the initial proposal based on classical genetic markers that suggested a major migration wave [3], further studies have shown that the Neolithization process varied in different regions, occurring along several different routes into and across Europe, and having a different genetic impact on the various regions and at various times [4–17].

The mtDNA frequency distribution observed in hunter-gatherers and farmers from Europe provides support for a random dispersion model for Neolithic farmers, with different impacts on the various geographic regions (Central Europe, Mediterranean Europe, and the Cantabrian fringe) [9].

The transition from the Neolithic to the Chalcolithic period in Europe has been debated. Previous mitochondrial DNA analyses on ancient Europeans have suggested that the current distribution of haplogroup H was modeled by the expansion of the Bell Beaker culture (BBC) out of Iberia during the Chalcolithic period. In addition, it has been suggested that these groups with Bell Beaker (BB) culture in Central Europe represented a population movement from the Iberian Peninsula [16]. However, according to the mtDNA variability in Chalcolithic groups from the Cantabrian fringe of Iberia, no genetic relationships have been detected between these Iberian and Central European groups [17]. This suggestion has been confirmed by the recent study [18] about the Beaker phenomenon and genomic data of Europe.

## 1.2. Paleogenetics and paleogenomics

*Paleogenetics* consists of the recovery and analysis of DNA obtained from the remains of individuals from the past, through polymerase chain reaction (PCR) and Sanger sequencing (ancient DNA—*aDNA*). These techniques are mainly applied in the analysis of mtDNA and fragments of nuclear DNA [9, 10, 19–22]. Since 2005, with the development of next-generation sequencing (NGS) technologies, it has been possible to retrieve also genomic data (*Paleogenomics*) from prehistoric European humans [23, 24]. This technology has allowed overcoming the apparently insurmountable difficulties associated with the deficient preservation of genetic material and the contamination of ancient DNA samples by modern DNA. NGS allows sequencing all those molecules that are present in DNA extracts (intact, contaminant, and damaged molecules, DNA from other organisms, etc.). The subsequent bioinformatic analysis allows discriminating endogenous sequences from exogenous sequences.

Massive sequencing has allowed important achievements in the field of human evolution, such as the “Neanderthal Genome” project, the discovery of new species (e.g., denisovans), and the recovery of the genome of very ancient humans (remains of La Sima de los Huesos—Atapuerca, Spain—dated to more than 400,000 years BP) [25–31].

The first paleogenomic studies about modern humans was the 7x coverage genome of the exceptionally well-preserved Tyrolean Ice man, Ötzi, dated to about 5300 years BP [32]. Currently, there are complete genomes from over 90 humans that inhabited Eurasia between 50,000 and 5000 years BP (hunter-gatherers and Neolithic farmers), shedding light on the migratory movements that shaped the genetic variability of modern humans and validating hypotheses proposed from the inference of modern genomes or partial sequences of these individuals [11, 13–15, 23, 24, 33–39]. These paleogenomic studies will enlarge the possibilities of selective and demographic analyses of the European prehistoric populations. The genomic data from European hunter-gatherers and farmers show that there is no evidence that the first modern humans in Europe (~45,000–37,000 years ago) contributed to the genetic makeup of current Europeans; these data rather suggest that individuals between ~37,000 and ~14,000 years descended from a single-founder population that is part of the ancestry of today’s Europeans. During the period of greatest warming after ~14,000 years ago, a genetic component related to the inhabitants of the Middle East region became widespread in Europe. These results document how population rotation and migration have been recurring themes of European prehistory [23].

Recently, 400 European individuals ranging from the Neolithic period to the Bronze Age were analyzed using paleogenomic techniques, including 226 individuals associated to Beaker complex artifacts [18]. Limited genetic affinity between BBC-associated individuals from the Iberian Peninsula and Central Europe was observed, and thus the authors excluded migration as an important mechanism of spread between these regions [18]. This result rejects the hypothesis of the migratory movement of humans from the Iberian Peninsula to Central Europe in the Chalcolithic period accompanied by the BB culture [16].

In the debate about the biological influence of the dispersion of the Beaker culture in Europe, we have analyzed the mtDNA of remains recovered in El Aramo Mine in Asturias (Cantabrian fringe) from the Late Chalcolithic period that were not accompanied by BB cultural artifacts [41]. This human group is contemporaneous to other Iberian Chalcolithic populations both without Beaker complex artifacts associated and with Beaker culture associated. Sites without BBC associated are those of Longar and San Jaun Ante Portam Latinam (SJAPL) in the Basque Country [9]. Contemporaneous sites with Beaker complex culture associated are the central and southern Iberian and central European groups published by [18, 40]. The aim of this study is to contribute new mtDNA data variability of the Chalcolithic site from El Aramo Mine (Asturias) and to determine whether there is either a common genetic signal or a heterogeneous genetic landscape among Chalcolithic European groups (with and without BBC culture).

## 2. Material and methods

In this chapter, we have analyzed the human remains from El Aramo Mine discovered in 1888, a mine located in the Asturias region in the Cantabrian fringe of the Iberian Peninsula [41].

The direct  $^{14}\text{C}$  analysis of the human remains from this mine indicated a dating between the Late Chalcolithic period and the Early Bronze Age. The anthropological remains from El Aramo Mine consists of 9 skulls and 12 skeletal remains. We have isolated DNA mainly from dental pieces (since it is the material that offers the greatest guarantees when recovering DNA). However, in some cases, we had to pulverize bone remains in order to perform DNA extraction, since it was the only anthropological material available.

In the case of teeth, we have selected those without caries or deep fissures that might extend into the dental pulp. The surface of the teeth was thoroughly cleaned with acids and ultraviolet (UV) irradiation to eliminate any possible DNA contaminants [42]. In the case of bones, the surface was thoroughly cleaned by abrasion and pulverized using a Freezer miller. Then we extracted DNA from bone and dental tissue by means of the phenol/chloroform method with some modifications [20–22, 43].

The sequencing of a 399 bp (nps 16,000–16,399) segment of HVS-I and 394 bp (nps1–394) of HVS-II of the mtDNA as per [44] was conducted by amplifying 6 overlapping fragments of 93–133 bp in length. The protocol followed and the primers used are described in [9, 45]. Likewise, in order to verify the obtained mtDNA haplogroups, the nucleotide position of the coding region of mtDNA was determined by means of PCR-restriction fragment length polymorphisms (RFLPs) [43, 46].

The extraction of DNA and the preparation of samples for PCR were performed in a sterile chamber with positive pressure, free of modern DNA, in which no post-PCR process had ever been carried out. Ancient DNA results were validated through the application of the following criteria [47, 48]: (1) suitable clothing was used (disposable cap, gloves, mask and laboratory coat), (2) controls were applied to detect contamination during the extraction process and in each one of the amplifications, (3) Real-time PCR quantification of amplifiable DNA to quantify one mtDNA fragment of 113 bp was conducted [9, 49], (4) a duplicate analysis was performed on the greatest possible number of individuals, and (5) Cloning of PCR products was performed with subsequent sequencing of the clones. The cloning was carried out using TOPO TA Cloning® Kits (Invitrogen), following the supplier's instructions.

The mitochondrial variability resulted from El Aramo Mine was compared with other ancient and present-day populations. With respect to hunter-gatherers, three groups were considered: one from Scandinavia, one from Central Europe [13, 14, 50, 51], and one from the Cantabrian fringe of the Iberian Peninsula [9, 17, 33, 52]. Regarding the Neolithic DNA, 14 populations were selected: 3 from the Near East [15], 4 from Central and Eastern Europe [16, 45], 5 from the Mediterranean area of Europe (Hungary, Romania, Catalonia and France) [6, 7, 10, 12], and 2 from northern Iberia [9, 11]. With regard to the Chalcolithic groups, we considered one from Central Europe with BB artifacts associated [16, 18], one from the Cantabrian fringe of Iberia without BC culture (Longar and SJAPL sites) [9] and another two from Iberia, one with BB culture, and another one without BB culture [BBC: Arroyal (Burgos), Camino de las Yeseras (Madrid), Humanajes (Madrid), La Magdalena (Madrid), and Paris Street (Barcelona). Without BBC: Camino del Molino (Murcia), Bolares (Extremadura), el Sotillo, chabola de la Hechicera (Alava), el Mirador (Burgos), La Mina, Trocs (Huesca), and El Portalón (Burgos)] [18]. The Bronze Age period is represented by three groups from Siberia, Kazakhstan, and

Romania [10, 53, 54]. The present-day populations database corresponds to that described in [10], to which the present-day population of Asturias, where El Aramo Mine is located, has been added [55, 56].

The  $F_{ST}$  distance matrix between present and ancient populations was calculated from the mtDNA haplogroup frequencies using Arlequin 3.11 [57]. Relationships between populations were studied through Multidimensional Scaling analysis (MDS), based on the  $F_{ST}$  distance matrix, using SPSS 20 Software. Median Joining Network (MJN) for certain haplogroups was generated to infer phylogenetic relationships between the mitochondrial lineages from the Paleolithic period to the present day using Network software v4.5.0.0 (available at <http://www.fluxus-engineering.com>). Different mutation weights were applied in accordance with previous papers [58–60], and the point insertions and deletions were excluded from the analysis.

### 3. Results and discussion

Mitochondrial DNA variability was analyzed in 21 skeletal remains recovered from El Aramo Mine, in the Asturias region (Cantabrian fringe of the Iberian Peninsula). The quantification of the template mtDNA number of each of the samples showed values above 1000 molecules/ $\mu$ l in all the DNA extracts from teeth and values below 1000 molecules/ $\mu$ l in all the DNA extracts from bones (**Table 1**). These results indicate the greater efficiency of the DNA extraction from teeth when compared to that from bones. Furthermore, in order to authenticate the results, 5.26% of the samples analyzed were duplicated, and these results were consistent in all the samples (**Table 1**). Moreover, 24 PCR products were cloned, estimating an average of 7.46 mutations per cloned fragment (~100 bp). These mutations have been interpreted as artifacts produced by the postmortem damage of aDNA.

The mitochondrial variability obtained from the 21 human remains from El Aramo Mine showed 15 different haplotypes (genetic diversity:  $0.9608 \pm 0.0394$ ). The nine skulls studied presented nine different mitochondrial haplotypes, which allow us to rule out the existence of maternal kinship among these individuals. The 12 postcranial remains analyzed showed 7 different mitochondrial haplotypes, which, compared with the haplotypes of the skulls, lead to reject possible coincidences, since the postcranial remains were not associated with the skulls. Finally, the minimum estimated number of individuals was 15, with 15 different mitochondrial haplotypes described (**Table 1**). The high genetic diversity obtained in El Aramo site allows us to indicate that it is a representative sample of the original population, with no evidence of kinship among these individuals.

The 15 mitochondrial haplotypes obtained from El Aramo Mine were classified into 5 different mitochondrial haplogroups (H, T, J, U5b, and I3), with a genetic diversity of  $0.6381 \pm 0.1288$  and a heterogeneous distribution of their frequency values (60, 13, 13, 7, and 7%, respectively). Haplogroup H is the most frequent one in the population of El Aramo (60%), whose value is close to that shown by the current population of the Asturias region (56%), where El Aramo Mine is located, and much higher than the average value found in European (45%) and Near Eastern (16%) populations [54, 55, 61] (**Figure 1**). In El Aramo, haplogroup H is represented

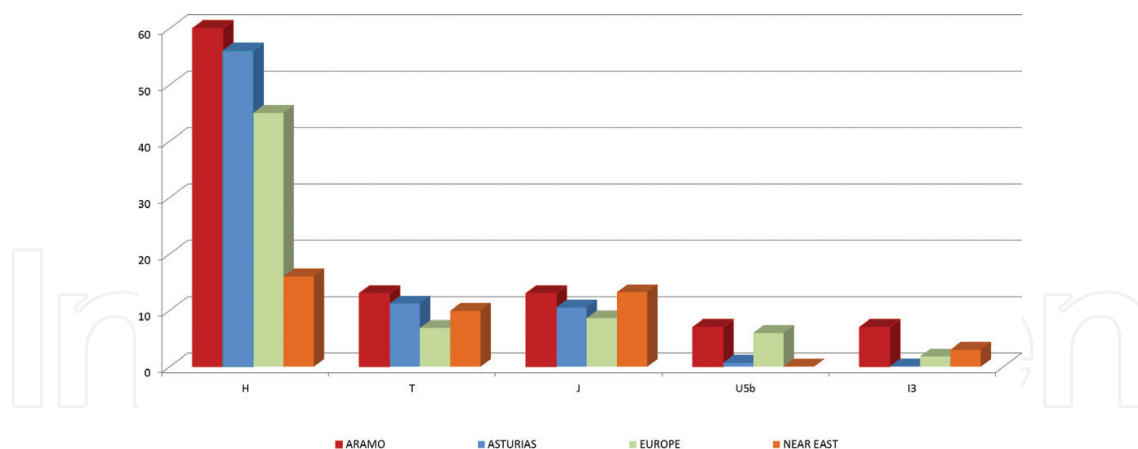
SAMPLE	SAMPLE ID	Molec/ $\mu$ l	HVS-I*	HVS-II	RFLPs	HG
Skull 1 (bone)	AR13	854.6	rCRS	73A	<i>Alu7025-</i>	H
Skull 2 (bone)	AR15	551.4	rCRS	73A-263G-315.1C	<i>Alu7025-</i>	H
Skull 3 (tooth) <sup>d</sup>	AR22	89190.2	183C-189C-270T	73G	<i>DdeI10394-;</i> <i>HaeII9052+;</i> <i>HinfI12308+</i>	U5b1b
Skull 4 (tooth)	AR21	33668.7	189C	73A	<i>Alu7025-</i>	H
Skull 5 (tooth)	AR31	17194.3	rCRS	73A-150T-263-309.1C-315.1C-320C	<i>Alu7025-</i>	H
Skull 6 (tooth)	AR32	18706.4	51G	73A	<i>Alu7025-</i>	H1
Skull 7 (tooth) <sup>d</sup>	AR42	2078.3	rCRS	73A-153T-263G-315.1C	<i>Alu7025-</i>	H
Skull 8 (tooth) <sup>d</sup>	AR41	5847.2	126C-292T-294T-296T	73G	<i>DdeI10394-;</i> <i>NlaIII4216+</i>	T2c
Skull 9 (tooth)	AR5	178331.7	rCRS	73A-263G-315.1C	<i>Alu7025-</i>	H
Mandible (tooth)	AR11	37374.7	126C-355T-362C	73A	<i>Alu7025-</i>	H
Hemimandible (tooth)	AR12	133777.7	69T-126C	73G	<i>DdeI10394-;</i> <i>NlaIII4216+</i>	J1c
Right Hemimandible (tooth)	AR7-9	153133.6	86C-129A-223T	73G	<i>DdeI1715-;</i> <i>Alu10032+</i>	I3
Left Hemimandible (tooth)	AR6	141554.8	69T-126C-320T-360T	73G	<i>DdeI10394-;</i> <i>NlaIII4216+</i>	J1c
Right femur (bone)	ARH10	372.5	126C-163G-187T-189T-294T	73G	<i>DdeI10394-;</i> <i>NlaIII4216+</i>	T1
Fibule (bone)	ARH13	312.6	086C-129A	—	<i>Alu7025-</i>	H

The number of molecules of endogenous mtDNA (molecules/ $\mu$ l), the polymorphisms of HVS-I, HVS-II, those of the coding region (RFLPs), and the mitochondrial haplogroup to which each of the samples belong are indicated.<sup>d</sup>Indicates that this sample has been duplicated.

\*The positions of the polymorphisms of HVS-I must have 16,000 added since it is HVS-I (16,000–16,400 pb).

**Table 1.** Results of the analysis of mtDNA in 15 individuals of El Aramo mine.

by nine different mitochondrial haplotypes, five of which correspond to haplogroup H-rCRS (33%), which is the most frequent one in the present-day European population (15–30%) and in the current population of Asturias (~23.5%). The rest of the haplogroups detected in El Aramo mine have low frequencies in the extant populations of Europe and the Near East [54, 55, 61]. Haplogroup T has a frequency of 13% in El Aramo, which is close to that of the present-day population of Asturias (11.2%) and higher than that of the populations of Europe (6.8%)

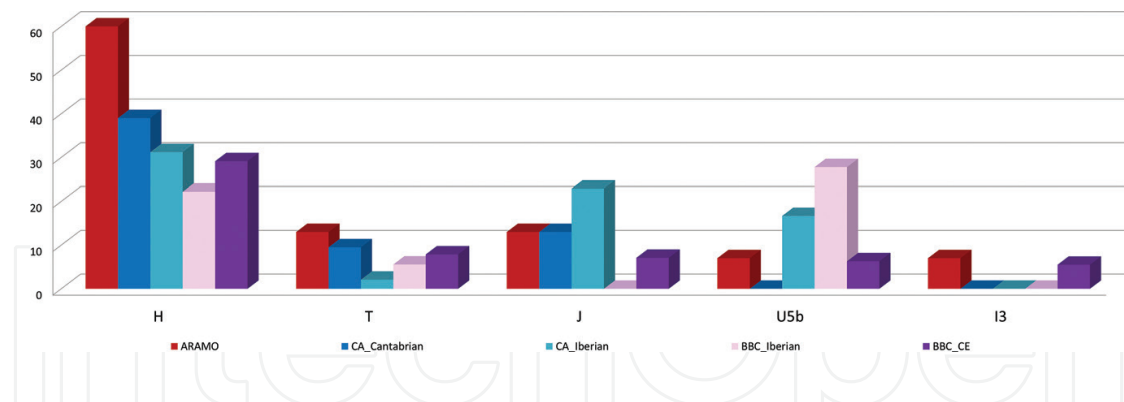


**Figure 1.** Frequency distribution of mitochondrial haplogroups H, T, J, U5b, and I3 in the sample of El Aramo Mine (Asturias, present study) and the modern populations of Asturias, Europe, and the Near East.

and the Near East (9.91%) (**Figure 1**). Haplogroup J shows similar frequencies in El Aramo and in the extant population of the Near East (13%), and lower frequencies in the present-day populations of Asturias and Europe (10.5 and 8.6%, respectively). Haplogroup U5b in El Aramo Mine has a frequency of 7%, which is close to that observed in the extant population of Europe (6%) and considerably higher than that detected in the current populations of Asturias and the Near East (0.7 and 0.05%, respectively) (**Figure 1**). Lastly, haplogroup I3 shows in El Aramo a value (7%) that is considerably higher than that observed in the current populations, among which the population of the Near East (3%) shows the highest frequency value for this haplogroup, which is absent in the current population of Asturias and very infrequent in Europe (1.8%) (**Figure 1**). To sum up, the main differences in the frequencies of the mitochondrial lineages of El Aramo Mine and the present-day populations lie in haplogroups H, U5b, and I3.

The frequency distribution of the mitochondrial haplogroups of El Aramo Mine was compared with that of other Chalcolithic groups of the Iberian Peninsula and Central Europe [9, 18]. For this analysis, five population groups were defined: (1) El Aramo Mine (Late Chalcolithic-Early Bronze Age) without BBC associated (ARAMO), (2) Chalcolithic from the Cantabrian fringe of the Iberian Peninsula without BBC (CA\_Cantabrian), (3) Chalcolithic from Iberia without BBC (CA\_Iberian), (4) Chalcolithic from Iberia associated with BBC (BBC\_Iberian), and (5) Chalcolithic from Central Europe associated with BBC (BBC\_CE) (**Figure 2**). All the populations included in this analysis share the presence of haplogroups H and T, although the distribution of their frequencies is different. The group of El Aramo shows the highest frequency value for haplogroup H (60%), followed by the two Chalcolithic groups of the Iberian Peninsula without BBC, CA\_Cantabrian and CA\_Iberian (39 and 31.2%, respectively); the lowest frequencies of haplogroup H were found in the BBC-associated groups of the Iberian Peninsula (BBC\_Iberian, with 22%) and Central Europe (BBC\_CE, with 29%). This frequency distribution of haplogroup H does not support the hypothesis of Brandt et al. [16], who suggest that the BBC was spread from the Iberian Peninsula toward Central Europe by haplogroup H carriers, since the highest frequency values were not found in population associated with the BB culture; moreover, the group of the Iberian Peninsula (BBC\_Iberian) has the lowest value (22%) (**Figure 2**).

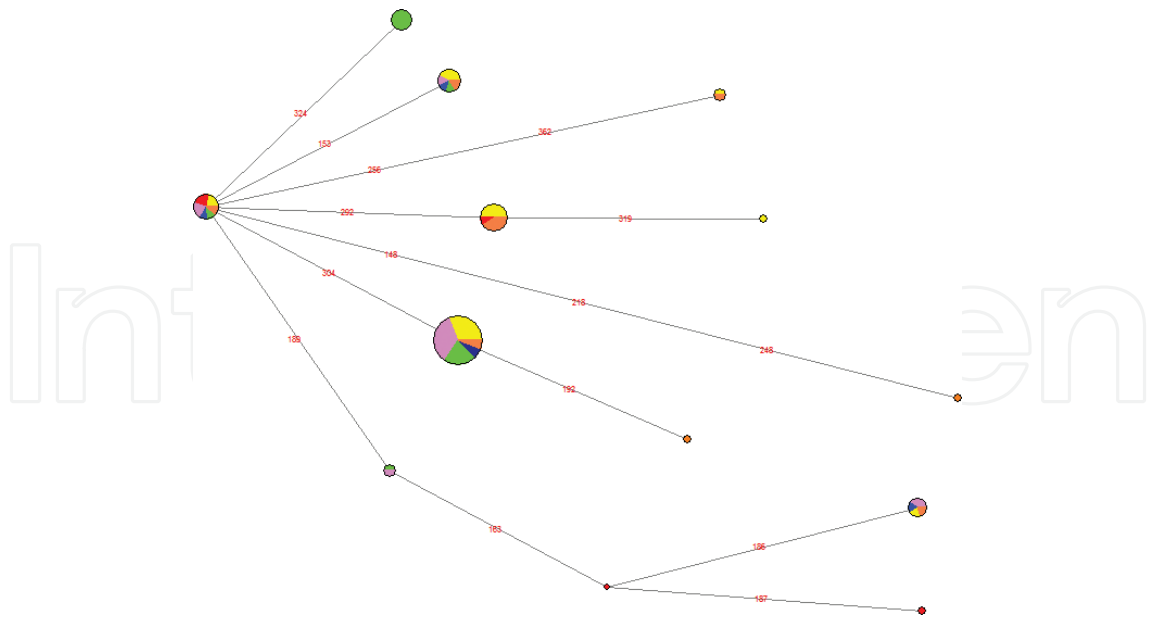




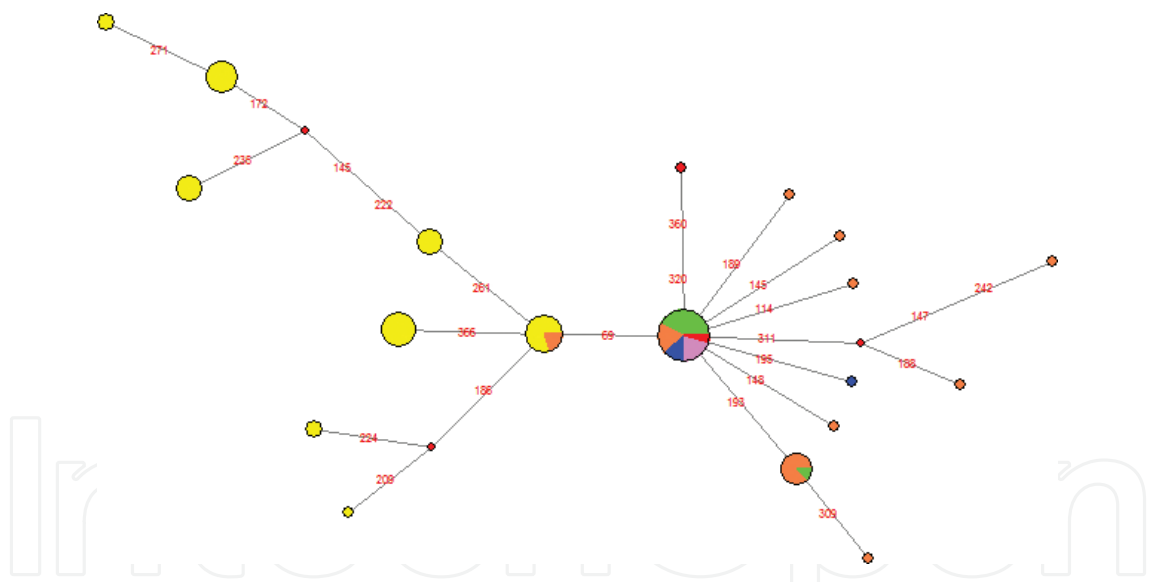
**Figure 2.** Frequency distribution of mitochondrial haplogroups H, T, J, U5b, and I3 in the sample of El Aramo Mine (Asturias, present study), and in other Chalcolithic groups, both without BB culture (CA\_Cantabrian and CA\_Iberian) and with BBC associated (BBC\_Iberian and BBC\_Central Europe).

With respect to haplogroup T, there is also a heterogeneous distribution among the five population groups analyzed. The group of El Aramo shows the highest value for this haplogroup (13%); the Chalcolithic groups associated with BBC (BBC\_Iberian and BBC\_CE) have frequencies of 5.5 and 7.8%, respectively, which are lower than that of CA\_Cantabrian (9.5%) and higher than that of CA\_Iberian (2.08%). Given the fact that haplogroup T has been proposed as a marker of the diffusion of the Neolithic culture, the heterogeneity of the frequencies of this haplogroup in the Chalcolithic populations supports the model of random Neolithic cultural diffusion (Hervella et al. [9]). This behavior has also been observed in the MJN (**Figure 3**), where T haplotypes of prehistoric and present-day populations were shown. In MJN main nodes are shared by all the populations compared, both prehistoric and modern. The polymorphisms that define the two T haplotypes of El Aramo Mine are shared with Neolithic and Chalcolithic groups, indicating their relation. Furthermore, the polymorphisms of one of the lineages found in El Aramo Mine are shared with current samples of Europe and the Near East, showing its prevalence to the present time (**Figure 3**).

Haplogroup J shows high-frequency values in the groups that are not associated with BBC (Aramo (13%), CA\_Cantabrian (13%), and CA\_Iberian (23%)), with the sample of CA\_Iberian showing the highest value. The BBC-associated groups have lower frequencies, with 7.8% in the sample of BBC\_CE and 0% in BBC\_Iberian, showing once again the difference between the Chalcolithic groups with BBC and without BBC. Haplogroup J has also been proposed as a marker of the Neolithic diffusion in Europe. The distribution of frequencies of this haplogroup in the populations analyzed (**Figure 2**) shows a greater Neolithic influence in the non-BBC Chalcolithic groups. This influence of Neolithic diffusion is also observed in the MJN analysis of haplogroup J (**Figure 4**), which shows that the haplotypes of the Chalcolithic samples non- BBC are included in the mitochondrial variability of the Near East. With regard to the BBC-associated populations, the J lineages correspond solely to the BBC\_CE population, and not to the BBC\_Iberian population (**Figures 2 and 4**), highlighting the differentiation between the two groups with BB culture (BBC\_CE and BBC\_Iberian). The frequency of haplogroup U5b in El Aramo (7%) indicates the persistence of Paleolithic lineages even after the Neolithic period. The prevalence of this lineage seems to be higher in the Chalcolithic groups of the Iberian Peninsula (16.6% in CA\_Iberian and 27.8% in BBC\_Iberian) when compared to



**Figure 3.** Median Joining Network of mitochondrial haplotypes of haplogroup T. El Aramo (red), Neolithic populations (green), Chalcolithic (blue), BBC (pink), modern Europeans (yellow), and modern Near Easterns (orange).



**Figure 4.** Median Joining Network of mitochondrial haplotypes of haplogroup J. El Aramo (red), Neolithic populations (green), Chalcolithic (blue), BBC (pink), modern Europeans (yellow), and modern Near Easterns (orange).

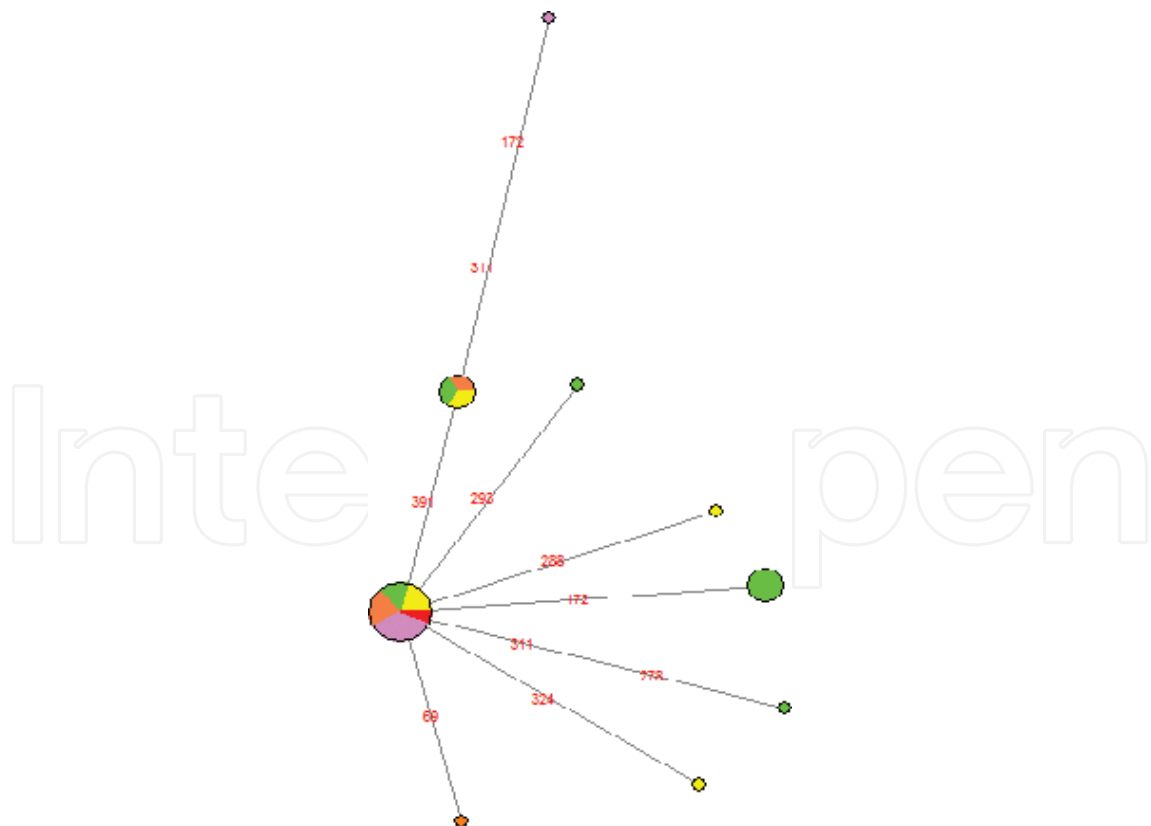
those of Central Europe (6.3%) (**Figure 2**). The absence of haplogroup U5b in the Chalcolithic sample of the Cantabrian region (CA\_Cantabrian) is due to the lack of differentiation in the U lineages published in this sample [43].

Lastly, haplogroup I3 appears only in the sample of El Aramo (7%) and in the BBC population of Central Europe (5.5%). The origin of this mitochondrial lineage has been thoroughly debated, and the consensus reached is that it began in the recent Neolithic period [1], with

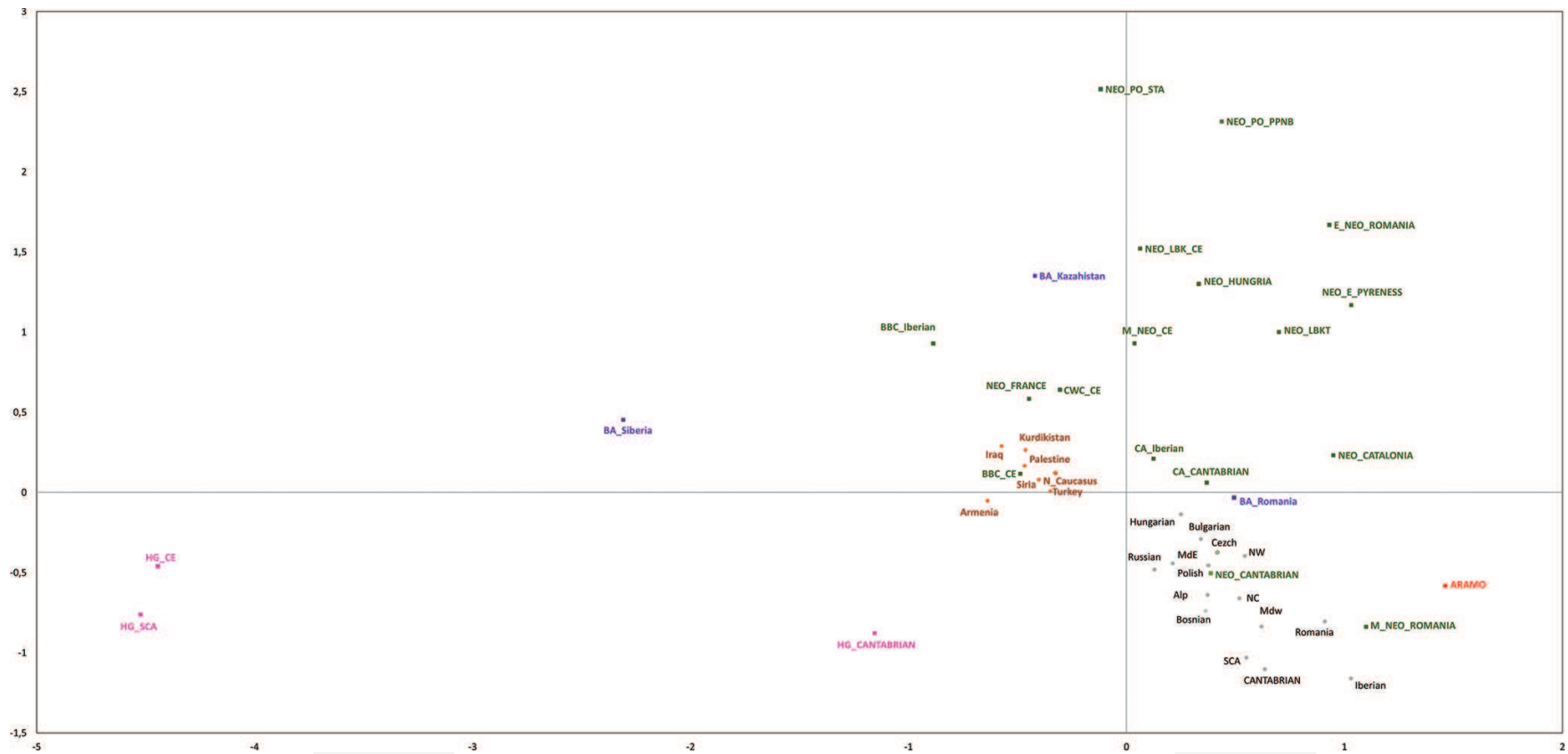
very low-frequency values found in the modern populations. In the MJN conducted for haplogroup I3, it was observed that El Aramo shares polymorphisms with Neolithic and Chalcolithic groups and with the current populations of Europe and the Near East, showing a low diversity for this lineage (**Figure 5**).

After the comparative analysis of the frequency distribution of the mitochondrial haplogroups of El Aramo and other prehistoric populations, it is possible to distinguish some general tendencies. On the one hand, there is a clear genetic differentiation between the Chalcolithic groups with BB culture (BBC\_CE and BBC\_Iberian) and those without BB culture (CA\_Iberian, CA\_Cantabrian and El Aramo); on the other hand, there is a differentiation between the BBC groups of Central Europe and those of the Iberian Peninsula (**Figure 2**). The population of El Aramo Mine shows a distribution of mitochondrial haplotypes that is closer to that of Chalcolithic populations without BBC than to that of chalcolithic populations with BBC, highlighting the specificity of El Aramo, the high frequency of haplogroup H (60%) and the presence of haplogroup I3, whose frequency is, in addition, one of the highest ones described to the present day.

The mitochondrial variability of the population of El Aramo was analyzed in the context of other prehistoric groups and the modern populations of Europe and the Near East through a Multidimensional Scaling analysis (MDS) (**Figure 6**). The MDS was done through a matrix of  $F_{ST}$  distances, calculated by the frequencies of the mitochondrial haplogroups of hunter-gatherer,



**Figure 5.** Median Joining Network of mitochondrial haplotypes of haplogroup I3. El Aramo (red), Neolithic populations (green), Chalcolithic (blue), BBC (pink), modern Europeans (yellow), and modern Near Easterns (orange).



**Figure 6.** Multidimensional Scaling Analysis performed for haplogroup frequencies of the ancient and present-day European and Near Eastern populations. Hunter-Gatherer groups (pink), Neolithic and Chalcolithic populations (green), Bronze Age groups (purple), El Aramo Mine (red), present-day European populations (grey), and present-day Near Eastern populations (orange). Stress: 0.142 and RSQ: 0.94548. Abbreviations: Hunter-Gatherers (HG), Neolithic (NEO), Bronze Age (BA), present-day populations in Europe: Eastern Mediterranean (MdE), Central Mediterranean (MdC), Western Mediterranean (MdW), Northeast Europe (NE), North-Central Europe (NC), Northwest Europe (NW), Southeast Europe (SE), and Alps (ALP).

farmer, Chalcolithic, Bronze Age, and present-day populations from the Cantabrian fringe, Europe, and the Near East. This analysis shows the differentiation between the two hunter-gatherer populations from Central Europe and Scandinavia (**Figure 6**). It has been proposed that the mtDNA variation of these groups indicates a genetic discontinuity between the hunter-gatherers and later populations in these two geographic regions [13, 14, 50, 51]. However, this suggested discontinuity is not so obvious in the case of the hunter-gatherers from the Cantabrian fringe who separated from those of Central Europe and Scandinavia in this analysis [9, 17] (**Figure 6**).

With regard to the European Neolithic populations, the heterogeneity observed in the mtDNA haplogroup frequency variation is revealed by their position on the two-dimensional plot of the MDS analysis (**Figure 6**). On the one hand, a group of populations (Near East, Central Europe, Hungary, and Eastern Pyrenees) with high-frequency values for haplogroup N is separated from the other Neolithic populations. On the other hand, heterogeneity is also apparent within the Mediterranean area, with a Neolithic population of Southern France being closer to present-day populations in the Near East, due to its high frequency for haplogroups J and U, whereas the Neolithic populations from the Iberian Peninsula (Catalonia and the Cantabrian fringe) show lower frequency for those haplogroups (J and U) (**Figure 6**). The genetic distances observed between the European Neolithic groups suggest a different genetic impact of the Neolithic farmers from the Near East on Central Europe, Mediterranean Europe, and the Cantabrian fringe. These data support a random dispersion model for Neolithic farmers, with different impact on the various geographic regions [9].

With respect to the Chalcolithic prehistoric groups included in the analysis (BBC\_CE, BBC\_Iberian, CA\_Iberian, CA\_Cantabrian and Aramo), those with BB culture (BBC\_CE and BBC\_Iberian) are differentiated from the rest. On the other hand, the distance between the BBC groups of Central Europe and the Iberian Peninsula is due to both the greater persistence of Paleolithic U5 lineages in the BBC\_Iberian group and the higher frequency of Neolithic lineages T and J in the BBC\_CE group. This differentiation suggests that the relation between these two Chalcolithic groups (BBC\_CE and BBC\_Iberian) is only cultural but not genetic, supporting the study [18] about the Beaker phenomenon and genomic data from Europe, who reject the hypothesis that the genetic substrate of the BBC\_CE groups came from the BBC\_Iberian groups [16].

Regarding the Chalcolithic populations without BB culture, CA\_Iberian and CA\_Cantabrian are genetically close to one another, with El Aramo being further from them due to the high frequencies of haplogroups H and I3. These Chalcolithic groups are not distant from their Neolithic ancestor populations, although they are distant from the present-day populations of these regions (**Figure 6**), which could be attributed to a post-Neolithic population restructuring [20].

In view of the results obtained, it can be inferred that the influence of the Neolithic period on the local groups was complex, and its result could generate the existence of Chalcolithic groups in the Iberian Peninsula with genetic and cultural differences, with the latter being mainly related to the Beaker phenomenon.

The human group of El Aramo Mine, without artifacts associated with BBC, shows some peculiarities. Its chronology expands from the Chalcolithic period to the Early Bronze Age,

during which a change in the landscape and the subsistence conditions have been detected in this region [62]. El Aramo consists of males who had a strong relationship with mining, and the burials reflect a ritual related to the exploitation of the mine [63]. Therefore, it is a group with differentiated cultural characteristics within the Chalcolithic groups of the Iberian Peninsula. This cultural differentiation seems to be accompanied by a genetic differentiation, since in the MDS, El Aramo is distant from the rest of the Chalcolithic groups (**Figure 6**). These results indicate the existence of local processes in the Chalcolithic period that could be related to subsistence strategies linked to the characteristics of the environment. In the case of El Aramo (Asturias), these strategies are linked to mining activity, since the region of Asturias is one of the traditional mining areas since the Neolithic period [63].

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## Conflict of interest

The authors have declared that no competing interests exist.

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