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# How Mitochondrial DNA Can Write Pre-History: Kinship and Culture in Duero Basin (Spain) during Chalcolithic and Bronze Age

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**Abstract:** The chronological period from the beginning of the Chalcolithic Age to the end of the Bronze Age on the Iberian northern sub-plateau of the Iberic Peninsula involves interesting social and cultural phenomena, such as the appearance of the Bell Beaker and, later, the Cogotas I cultures. This work constructs a genetic characterisation of the maternal lineages of the human population that lived on the northern sub-plateau between 5000 and 3000 years ago through an analysis of mitochondrial DNA (mtDNA), a kind of genetic marker that is inherited through maternal lineages, unaltered from generation to generation. Population and cultural questions are investigated through mtDNA analyses. This study intends to shed light on the following questions. Were individuals who were buried together in multiple or collective burials biologically related through their maternal lineages? Were there distinct maternal human lineages in the same or different geographical areas if different material cultures (Bell Beaker and Cogotas I) were associated with the arrival of new human populations who established close biological relationships with the endogenous populations? Or could this be the result of the transmission of knowledge without human populations mixing? Another important question is whether the material cultures were related to the female populations. We analysed 91 individuals from 28 different archaeological sites of the Iberian northern sub-plateau from four different chrono-cultural periods (Pre-Bell Beaker, Bell Beaker, Proto-Cogotas I, and Cogotas I), from the end of the Chalcolithic Age up to the Bronze Age. There were two historical moments of new populations arriving: the first during the Pre-Bell Beaker period, associated with the K mtDNA haplogroup, and the second during the Proto-Cogotas I culture, with new lineages of the H, HVO, and T haplogroups. Neither of these new population flows were directly associated with the maximum development of the two main material cultures Bell Beaker and Cogotas I, so they must have occurred immediately beforehand, during the Pre-Bell Beaker and Proto-Cogotas I periods, respectively. However, we cannot discard an association between the populations and material cultures. Curiously, it has also been observed that there was also a tendency towards multiple burials, in which the individuals who were buried together belonged to the same maternal lineage, during these two periods of population change. This study has shed some light on the populational changes that occurred through these different periods in this specific geographical area of the northern sub-plateau of the Iberian Peninsula.

**Keywords:** ancient DNA; kinship analysis; mitochondrial DNA; lineage markers; archaeogenetic; Cogotas I culture; Bell Beaker period; Iberian Peninsula



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## 1. Introduction

The first studies on ancient DNA were published towards the middle of the 1980s (Higuchi et al. 1984; Pääbo 1985, 1989). Since then, the discipline has continued to evolve, standardising protocols and implementing increasingly efficient techniques, such as PCR testing (Mullis and Faloona 1987), a great milestone in the field of molecular biology, which made the amplification and analysis of ancient DNA possible (Pääbo and Wilson 1988; Hagelberg et al. 1989). PCR technology, when applied to the study of ancient DNA, can isolate DNA fragments from human or, in general, biological remains and obtain millions of copies of a sequence of interest or objective (Fernández et al. 2014; Olalde et al. 2015; Villalba-Mouco et al. 2019; Palomo-Díez et al. 2018; Esparza-Arroyo et al. 2017).

Since these early works, the field of archaeogenetics has evolved remarkably, moving from the analysis of small DNA fragments to the amplification of complete mitochondrial genomes, thanks to new massive sequencing techniques (Morris et al. 2014; Meyer et al. 2014; Olalde et al. 2015; Alves-Cardoso et al. 2022).

Nevertheless, obtaining DNA from ancient archaeological samples depends on the degree of preservation of the sample. A poor state of DNA preservation will manifest itself in different ways in DNA sequences or STR markers (Palomo-Díez 2015).

In this paper, we focus our research on the human populations that lived during the 2nd and 3rd millennia B.C. in the Duero Basin (Central Spain), especially in its most central areas, where a certain number of individuals have been able to gather sufficiently well-preserved, culturally characterised, and radiocarbon-dated samples, enough to form the corpus of paleogenetic research.

The periods studied in our geographical area of interest were characterised by different material traits (ceramics, metals, etc.) and different funeral traditions. Regarding ceramics and other materials, two great cultures have been detected along this lapse of time: the Bell Beaker culture and the Cogotas I culture, each of them preceded by a previous stage, called the Pre-Bell Beaker period and the Proto-Cogotas I period, respectively. As previously mentioned, different funeral traditions were implemented over time, alternating between collective and individual burials. Both cultural changes and funeral patterns can be studied from an archaeogenetic perspective. On the one hand, genetics can provide information about whether these cultures were transmitted by learning without any biological change in human populations or if these cultural changes were accompanied by the arrival of new people. On the other hand, the analysis of possible lineage kinship among individuals buried together can provide relevant information about social relationships as well as the concept of family in different cultures and times.

The chronological framework of this work incorporated the Copper (Chalcolithic) and Bronze Ages, using traditional terminology. Herein, we will take a brief tour through the chronological periods analysed in the Duero Basin or northern sub-plateau of the Iberian Peninsula and its main characteristics from the point of view of its material culture and funeral traditions.

The Chalcolithic period involved the incorporation of copper into material culture (Rovira Lloréns 2005). In the Iberian Peninsula, metal smelting had become widespread by the end of the 4th millennia B.C., when several cultural areas were documented to have had copper metallurgy, for example, Los Millares in the southeast, Vila Nova de São Pedro in the Portuguese Estremadura, and the area surrounding the Duero river.

The Chalcolithic Age took place over more than 1000 years, and the forms of burial that we can observe during this time were very diverse, probably due to different reasons (Aliaga 2008).

As for the geographical scope of this study, which was limited to the Duero Basin (Spain), the Chalcolithic Age was characterised by the subdivisions of the Initial Chalcolithic or Pre-Bell Beaker period and Chalcolithic Bell Beaker period. This distinction was marked by the absence/presence of a particular type of ceramic, which was the most visible sign of a profound cultural change (Delibes de Castro 1977).

The Pre-Bell Beaker (3rd millennia B.C.) period was characterised by "pit fields", sets of exhausted silos, structures filled with what, at first glance, appeared to be domestic rubbish. In these sites, copper objects were discovered (flat axes, simple awls, and daggers), crucibles, and slag that testified that such metallic artefacts were not imported from other peninsular areas but, rather, manufactured here, with minerals from the mountainous edges of the region. Some of such pits ended up as individual burial graves (sometimes double), with the deceased placed in a retracted position and accompanied by grave goods consisting of ceramics, sometimes stone or bone ornaments, and even metallic objects (Delibes de Castro 1987; Garrido-Pena 1999, 2005).

Regarding the funeral costumes, the old megalithic-dolmens and corridor tombs that emerged during the Neolithic continued to be in use along the Chalcolithic in the areas of the large central area of the Duero Basin, but the most frequent burial is the individual grave, although, sometimes, as in Aldeagordillo (Ávila), one of the archaeological sites that we studied, there were still graves with collective burials. This change in the funerary rite was surely related to a change in the forms of social organisation, having pointed out that the Initial Chalcolithic period was not only the time in which the new copper metallurgy arose but also the time when social differences begin to emerge, evident in some tombs where some metallic instruments are buried as exceptional grave goods, after the segmental societies of the Neolithic period (Delibes de Castro 1987; Garrido-Pena 1999). In addition, vestiges have been found that testified to the importance of ritual activities, such as the deposits of remains of sacrificed animals, and even a singular case of human sacrifices, such as those of Los Cercados (Mucientes Valladolid) (García Barrios 2007; Palomo-Díez et al. 2017).

The funeral ritual is the material expression of an active ideological discourse, which intervenes in social and economic relations. The key is to find out the reasons that prompted those prehistoric groups to gather their dead in common graves where their remains would eventually mix. On the other hand, an individual burial segregates the deceased from the rest of the group, highlighting and singling them out, as opposed to collective rituals that tend to erase the individuality of the deceased, diluting it in the group. Another intermediate point would be constituted by the pantheons where there are several individualised tombs. Regarding this question, the paleogenetic study can shed light on the knowledge of the interrelationships between these individuals, knowing the existence or absence of possible close kinship ties, as well as their linkage through the maternal lineage (through the study of mitochondrial DNA (mtDNA)) or paternal (through the study of Y chromosome markers) (Palomo-Díez and López-Parra 2022).

The second relevant archaeological culture covered by our study was the Bell Beaker culture, which was defined by the pottery that bore the same name, yet, simultaneously, it was also accompanied by a change in the funeral ritual. It was a restricted chronological period approximately between 2700/2600 and 2000 B.C. (Aliaga 2008; Garrido-Pena 2005). Bell Beaker was a complex cultural phenomenon with an enormous geographical scope, which completely went beyond traditional cultural areas. It would, essentially, be a set of cultural manifestations of great symbolic contents that the emerging elites adopted in areas of Europe, ranging from the Iberian Peninsula to the Netherlands and from the British Isles or the Western Mediterranean to Central Europe.

In addition, the transition from a collective ritual in the Dolmen period to an individual ritual was consolidated. The characteristic grave goods were the so-called Bell Beaker-shaped ceramics (bowl, glass, and casserole), together with some weapons (tongue daggers, javelin heads, bows and arrows, and archer's bracelets) (Rojo-Guerra et al. 2006). This period witnessed the progressive implantation of individual funerary structures, as with the reuse of previous collective tombs, especially the megalithic ones (Rojo-Guerra et al. 2006). At first, this presence of the Beaker cultures in the megaliths was assumed as a testimony of the arrival of new people that implanted their funerary costumes and desecrated the tombs of the local groups. But, for some years now, the progressive multiplication of Bell Beaker finds modified this view since it was shown that the reuse of megaliths was not

occasional but was the important, and even the preferred, funerary formula. Nonetheless, it is critical to recall that megaliths were no longer built but only repurposed in the Bell Beaker period. Therefore, according to archaeological data, the disappearance of these large collective structures could also imply the disappearance of the social order that gave them meaning (Rojo-Guerra et al. 2006). Now, was this change in the social order simply cultural, or was it about different populations from other regions? This question can be addressed by paleogenetic evidence, and we will attempt to achieve that.

Another question that we will attempt to answer is where lies the origin of this new culture.

Taking a chronological step forward, we find ourselves with the Bronze Age, the period of Prehistory in which the metallurgy of this metal was developed, which was the result of copper-tin alloys.

The progress of archaeological research in the Duero Basin region has made it possible to recognise the existence of the Early Bronze Age, which is still not well-defined due to the scarcity of funerary findings. Furthermore, a certain overlapping of the radiocarbon dating of these sites with the Bell Beaker's made it harder to define; although, the Early Bronze Age could be placed especially in the 20th and 19th B.C. centuries (Blasco-Bosqued 1997; Rodríguez Marcos 2007). On the geographical area studied along the Bronze Age, we could distinguish a great northern sub-plateau culture, Cogotas I.

Cogotas I was developed throughout the Plateau for about eight centuries, i.e., almost the entire 2nd millennia B.C: firstly, the Formative moment, also called Proto-Cogotas I (Middle Bronze Age, 1850–1450 B.C.); later, the Fullness phase, called more strictly Cogotas I (Late Bronze Age).

In the settlement type, there is some continuity to the Early Bronze Age since some deposits have very striking high-altitude locations, but the vast majority are deposited in the open field, on the plain. These deposit features, commonly called "pit sites", are very numerous, and are sometimes quite close together, giving the impression that the habitat shifted, perhaps related to the depletion of soil fertility.

Regarding the materials, the change when compared with the Early Bronze Age is most noticeable in the decorated ceramics. Although there are still smooth containers or ones decorated in relief with fingerings, difficult to distinguish from the previous ones, there is now a multitude of open vessels, with a fairing profile and well-ordered geometric decorations based on spikes, zigzags, etc., which make the vessel very recognisable as belonging to the Proto-Cogotas I style. Subsequently, the pure Cogotas I style is much more complicated, with a mixture of techniques (incision, Boquique impression, excision) that occupy more and more surface area in the vessels; these were also being made with new shapes, such as the truncated cone vessels (Abarquero Moras 2005).

Moving on to the funerary world, one could also continue with the comparison to Early Bronze Age, which shows some continuity but also novelties. Certainly, the most characteristic funerary type continues to be burial in a pit, generally in flexed lateral decubitus. Some use of other formulas persisted, for example, collective burial in caves, such as the one mentioned in La Revilla (Atapuerca, Burgos, Spain), in which some ceramics belong to the Proto-Cogotas I phase. Some of the skeletons from this site have been dated by radiocarbons to that phase, surely illustrating a population continuity that is interesting to analyse from a paleogenetic point of view (Abarquero Moras 2005). But, in the face of these continuous aspects, the pit burials found inside the habitation deposits present a striking absence of grave goods, which clearly distinguishes them from those mentioned in the Chalcolithic, Bell Beaker, and Early Bronze ages. The total number of burials found was very low, concerning the high number of known sites. The aforementioned lack of grave goods and other arguments such as the surprising proportion of triple burials or the demographic structure of the segment of those buried recently led to the suggestion that this segment is exceptional. Furthermore, based on the observation of two skeletons from the Tordillos site and some other cases, it has been proposed that the usual burial rite of the Proto-Cogotas I and Cogotas I communities was the exposure of corpses (Esparza Arroyo et al. 2012).

While only a very specific fraction of the population was buried in a pit, probably those people who were considered to have had a "bad death" (for example, due to having died early in accidents, from childbirth, etc.), and even socially rejected individuals who would be buried in holes but without the careful placement that the former had, were waived. Both subtypes of burials made up the set of human remains that have been able to be analysed in this study, with the majority belonging to Proto-Cogotas I and the fewest being those of Cogotas I Plenary since one of the characteristics of this phase is the decrease in the number of burials concerning the Formative phase, as already stated.

It had been assumed that the Cogotas I communities were subject to constant mobility, with transitory occupations, motivated by economic practices of an itinerant condition, including cattle transhumance (Abarquero Moras 2005). Recently, the hypothesis of Cogotas I's livestock transhumance was questioned (Blanco González and Arroyo 2019). After Cogotas I, a new archaeological culture emerged, the Soto Formativo (Final Broze Age, circa 1100-800 B.C) in which the manipulation of human remains seems to persist (Delibes de Castro and Manzano 2000; Delibes de Castro and Carnicero 2011; Esparza Arroyo et al. 2016).

Considering all the different cultural characteristics, it is intended to establish whether the different collective burial rituals throughout the different cultures studied are related to biological family ties or if the decision to bury different individuals together is conditioned by another type of social relationship. On the other hand, the mtDNA compositions of the populations will be studied at the same time to compare them with different cultures. This will try to establish whether there is an association between cultural changes and the biological compositions of the populations.

## 2. Materials and Methods

### *2.1. Materials: Selected Samples from Each Chronological Period Studied*

The distribution of our sampling extended over the entire northern sub-plateau of the Iberian Peninsula, specifically within the modern Autonomous Community of Castilla y León (modern Spain) and in a single case (Terrazas del Manzanares Site, in Rivas Vaciamadrid) in the modern Community of Madrid (modern Spain). In Figure 1, one can observe the geographical localisation of the archaeological sites analysed.

Twenty-eight archaeological sites from 4 different periods (Pre-Beaker, Bell-shaped, Proto-Cogotas I culture, and Cogotas I culture) were sampled. In addition, two samples corresponding to a single individual from the *Soto formativo* (Final Bronze Age) were selected. In total, 91 individuals from 28 archaeological sites were analysed for mitochondrial DNA polymorphisms. We endeavoured to select at least two different samples for each individual whenever possible.

Table 1 collects information about the chronological period of each archaeological site: the dating information, the location, the number of individuals analysed, and the name assigned in the genetic laboratory.

**Table 1.** Relevant information about each archaeological site studied.

| Archaeological Site | Lab. Name | Estimated Antiquity | Location in Spain | Individuals | Period |
|---|---|---|---|---|---|
| El Tomillar | ATOM | 4000 BP | Bercial de Zapardiel, Ávila | 8 | Pre-Bell Beaker |
| El Tomillar | ATOM | 3780 ± 100–3830 ± 95 BP | Bercial de Zapardiel, Ávila | 11 | Pre-Bell Beaker |
| Los Areneros | ARE | 4240 ± 35–4125 ± 25 BP. | La Lastrilla, Segovia | 8 | Pre-Bell Beaker |
| Los Cercados | CER | 3970 ± 60 BP. | Mucientes, Valladolid | 3 | Pre-Bell Beaker |
| Camino de Trascabañas | TC | 4180 ± 35 BP. | Simancas y Ciguñuela | 1 | Pre-Bell Beaker |
| Aldeagordillo | ALG | 3690 ± 50 BP | Ávila | 4 | Bell Beaker |

**Table 1.** *Cont.*

| Archaeological Site | Lab. Name | Estimated Antiquity | Location in Spain | Individuals | Period |
|---|---|---|---|---|---|
| El Mirador | MIR | 3650 ± 35 BP | La Lastrilla, Segovia | 1 | Bell Beaker |
| Perro Alto | PA | 3730 ± 65 BP | Fuente Olmedo, Valladolid | 1 | Bell Beaker |
| Santa Cruz de Cabezón II | CAB | Not available | Cabezón de Pisuerga, Valladolid | 1 | Bell Beaker |
| Pago de Valhondo | PAD | 3970 ± 50 BP | Pajares de Adaja, Ávila | 1 | Bell Beaker |
| Terrazas del Manzanares | RIV | 3050 ± 100 BP | Rivas Vaciamadrid, Madrid | 1 | Bell Beaker |
| Tablada del Rudrón | TR | Not available | Tubilla del Agua, Burgos | 4 | Bell Beaker |
| Santioste | SAN | 3780 ± 50 BP | Otero de Sariegos, Zamora | 1 | Bronze Age |
| Cueva de la Revilla | REV | 3550 ± 40–3325 ± 35 BP | Atapuerca, Burgos | 8 | Bronze Age |
| Carrelasvegas | CV | 3230 ± 80 BP | Santillana de Campos, Palencia | 1 | Proto-Cogotas I |
| El Cementerio | QUO | 480 ± 35 BP | Quintanilla de Onésimo, Valladolid | 1 | Proto-Cogotas I |
| El Cerro de la Horra | HOR | 3180 ± 50–3225 ± 30 BP | La Horra, Burgos | 3 | Proto-Cogotas I |
| El Juncal | JUN | 3.335 ± 35 BP | Villaralbo, Zamora | 1 | Proto-Cogotas I |
| Fuente de la Mora | FM | Not available | Valladolid | 3 | Proto-Cogotas I |
| La Huelga | HU | 3290 ± 35 BP | Dueñas, Palencia | 1 | Proto-Cogotas I |
| Los Rompizales | RPZ | 3165 ± 30–3250 ± 35 BP | Quintanadueñas, Burgos | 4 | Proto-Cogotas I |
| Las Cañamonas | CÑ | 3205 BP | San Cristobal de Entreviñas, Zamora | 1 | Proto-Cogotas I |
| Los Tolmos | TOL | 3600–3.200 BP | Caracena, Soria | 4 | Proto-Cogotas I |
| Tordillos | TOR | 3205 ± 35 BP | Aldeasca de la Forentera, Salamanca | 12 | Proto-Cogotas I—Cogotas I |
| La Requejada | LR | 3020 ± 35–3120 ± 30 BP | San Román de Hornija, Valladolid | 3 | Cogotas I |
| Cerro de la Cabeza | CC | 2160 ± 50 BP | Ávila | 2 | Cogotas I |
| Canto Blanco | CB | 3123 ± 30 BP | Sahagún, León | 1 | Cogotas I |
| El Cerro de San Pelayo | SP | 2715 ± 30 BP | Salamanca | 1 | *Soto formativo* |
| Total Number of Individuals | | | | 91 | |

### *2.2. Methodology*

#### 2.2.1. Experimental Process and Authenticity Criteria

We have selected two samples for each individual to carry out the experimental process in duplicate. At the end of the process, only those results that were identical in the two samples processed for each individual were taken as valid, according to ancient DNA authenticity criteria (Pääbo et al. 2004). Moreover, not only has a duplicate analysis of each individual been carried out in the same laboratory and by the same person, but also a large number of the second selected samples (specifically from 31 of the analysed individuals) were processed in a different laboratory (which will be named as laboratory 2). Also, in both laboratories, different ancient DNA authenticity criteria were taken into account (Pääbo et al. 2004; Hummel 2003; Fulton 2012; Palomo-Díez 2015; Palomo-Díez et al. 2016).

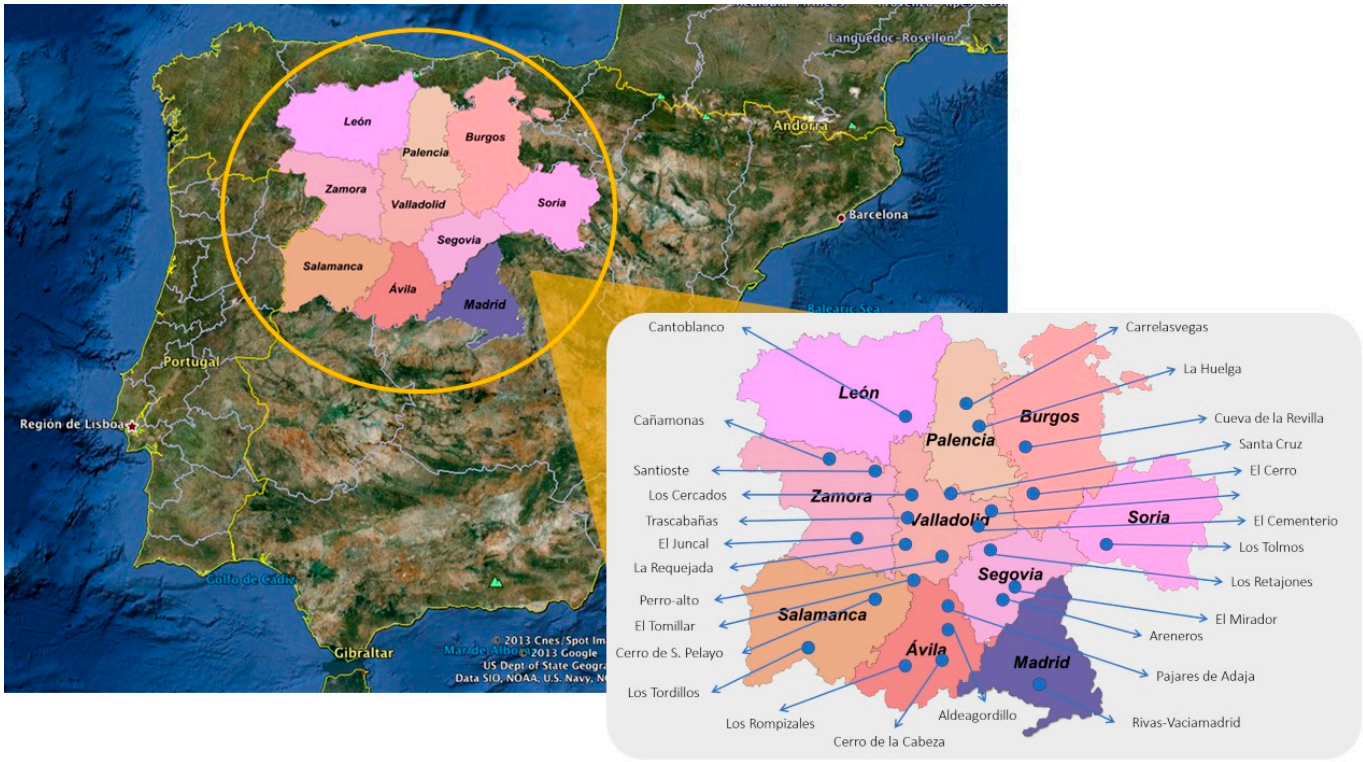


**Figure 1.** Map of the archaeological site's localisation.

- Pretreatment of samples and ancient DNA extraction in Laboratory 1:

Firstly, possible contaminating molecules attached to the surfaces of the samples were removed by abrasion with aluminium oxide by a sandblaster (Dentalfarm Base 1 Plus). Then, the samples were irradiated with ultraviolet light in a Crosslinker using ultraviolet radiation. Subsequently, most of the samples were pulverised using a liquid-nitrogen-cooled mill (SPEX Model 6700). Once the powder was obtained, it was stored in its corresponding sterile falcon tube at −20 °C to preserve the DNA (Fulton 2012). This grinding process was not performed on all the samples since some of them were analysed by a non-destructive DNA extraction methodology (Gomes et al. 2015; Palomo-Díez 2015). DNA from all the ground samples was extracted by the Rohland and Hofreiter modified method (Rohland and Hofreiter 2007a, 2007b; Rohland et al. 2009; Palomo-Díez 2015). The DNA pretreatment and extraction process in this laboratory was carried out in isolated rooms with restricted access to a limited number of researchers who always wore specific clothing (overalls, gloves, glasses, shoe covers, etc.). The rooms were also equipped with UV radiation and were cleaned before and after each use with diluted bleach and ethanol.

- Samples pretreatment, ancient DNA extraction, and DNA quantification in Laboratory 2:

Regarding the samples processed in Laboratory 2, the pretreatment used in this case was a superficial cleaning of the sample using a sterilised scalpel, previously cleaned with 70% diluted ethanol and bleach and irradiated with ultraviolet light. Cleaning consisted of scraping the surface of the sample to remove adhering dirt. This first removal of the superficial layer was completed by irradiating the sample with ultraviolet light for 24 h.

In this case, two different grinding methodologies were employed: an agate mortar or a dental drill.

In this case, after the DNA lysis by a mix of proteinase K and EDTA, DNA extraction was carried out using the commercial PCR product purification kit QIAquick PCR Purification Kit® (Qiagen, Germantown, MD, USA), following the manufacturer's recommendations. Also, in laboratory 2, the DNA quantifications of DNA extracts were

performed by Real-Time PCR (RT-PCR). Concretely, the RT-PCR consisted of amplifying two mitochondrial DNA from the HVI and HVII regions (Scorrano et al. 2014).

In this case, the laboratory was composed of different isolated rooms with restricted access to a limited number of researchers who always wore specific work clothing within each room. The rooms are also equipped with UV radiation and were cleaned before and after each use with diluted bleach and ethanol.

- DNA amplification by PCR, results analysis, and cloning.

MtDNA amplification was carried out with the same primers and PCR protocol in both laboratories (1 and 2), using overlapping short fragments. MtDNA amplification was carried out in two phases: the first for the amplification of overlapping fragments of the Hypervariable Region I (HVI) of the mitochondrial D-loop, and the second for the HVII.

The sequences of the primers used for HVI region amplification are those created and used by Fernández E. (Fernández 2005), and the primers used for the amplification of the HVII region were designed by (Martínez-Labarga and Rickards 1999), following their PCR conditions. Before sequencing, the success of the PCR was verified by agarose gel electrophoresis (Hummel 2003).

For the acceptance of a valid consensus profile, all mtDNA fragments were amplified, at least in duplicate, from two samples from each individual, i.e., at least 8 amplifications per individual (4 of each fragment) were obtained, considering valid only those individuals that provided reproducible profiles. For the mtDNA amplification, the Qiagen® multiplex Kit was used, regarding the manufacturer's recommendations.

To isolate the amplification product, two methods were used: a commercial PCR product purification kit (laboratory 1) with columns and an ExoSAP™ PCR Product Cleanup Reagent (Applied Biosystems™) enzyme (laboratory 2).

Sequencing of mtDNA PCR products was performed in an Applied Biosystems 3730 DNA Analyser sequencer.

All the PCR products that showed DNA mixes or certain doubts to be interpreted were cloned. To achieve this, the TOPO® TA Cloning® kit from Life Technologies was following the manufacturer's indications. Finally, 12 clones of each amplification product were analysed and sequenced to determine the endogenous sequence of the sample (Palomo-Díez 2015). All cloning products are summarised in the Supplementary material (Supplementary Materials 1_clones).

All the electropherogram analyses were performed using Chromas version 2.0 software and Mutation Surveyor V4.0.9 software. Comparing all the sequences with the revised Anderson reference sequence (rCRS) (Anderson et al. 1981; Andrews et al. 1999) to determine each sample's haplotype. Subsequently, to determine the mitochondrial haplogroup from the obtained haplotypes, the EMPOP database (Parson et al. 2014; Parson and Dür 2007; Zimmermann et al. 2011; Huber et al. 2018) was used. All the haplogroups assigned were checked directly in the Phylotree (van Oven and Kayser 2008). Also, the frequencies of the obtained haplogroups in ancient populations by the AmDB database were searched (https://amtdb.org/ (accessed on 2 February 2023)).

#### 2.2.2. Analytical Procedure

Once the haplotypes and haplogroups of each of the individuals analysed were known, we proceeded to analyse the variations in the genetic compositions of the populations throughout the periods studied. We also analysed variations in the different geographical regions that occupied our study population.

- Data organisation

In the first place, a diachronic study was carried out to find out the existence of genetic variations in the populations analysed throughout the different 6 periods studied (Periods: Pre-Bell Beaker, Bell Beaker, Early Bronze Age, Proto-Cogotas I, Cogotas I, and Soto Formation of the northern sub-plateau of the Iberian Peninsula), except for the

*Soto Formativo* period, due to the low number of individuals from this period (only one individual studied).

Moreover, the interest in comparing the analysed individuals with other periods' populations from the Iberian Peninsula was taken into account. To address this issue, a set of individuals from different geographical areas and chronological periods were considered (Prieto et al. 2011; Hervella et al. 2012), organised into three more groups: Paleolithic–Mesolithic, Neolithic, and XXI Century.

Using this structure in 8 groups (Paleolithic–Mesolithic, Neolithic, Pre-Bell Beaker, Bell Beaker, Bronze Age, Proto-Cogotas I, Cogotas I, and XXI Century), an analysis of molecular variance (AMOVA) was performed to know the variance between the different groups as well as within them (between sites belonging to the same period) and between the individuals of each of the sites.

To carry out this approach to diachronic variations, the Arlequin version 3.5 software was used (Excoffier and Lischer 2010). To analyse the data with the Arlequín software in a diachronic way, the following structure, organised into different chrono-cultural groups, was established (Table 2).

**Table 2.** Chronological Groups were established for the diachronic study. The table shows the 5 chronological groups and the archaeological sites belonging to each one of them, and the total number of individuals (N) from each chronological group.

| **Group 1: Paleolithic-Mesolithic N = 4 (Hervella et al. 2012)** | **Group 2: Final Neolithic N = 42 (Hervella et al. 2012)** | **Group 3 Pre-Bell-Beaker N = 17** | **Group 4 Bell-Beaker N = 9** | **Group 5 Ancient Bronze N = 11** | **Group 6 Proto-Cogotas I N = 25** | **Group 7 Cogotas I N = 3** | **Group 8 XXI Century N = 335 (Prieto et al. 2011)** |
|---|---|---|---|---|---|---|---|
| Erraya | Cascajos | Los Areneros | El Mirador | La Revilla Cave | Tordillos | Cantoblanco | XXI Century Northern Iberian Subplateau |
| La Pasiega | Fuente Hoz | Los Cercados | Rivas-Vaciamadrid | Santioste | Las Cañamonas | La Requejada | XXI Century Cantabric Coast |
| La Chora | Paternabidea | Trascabañas | Santa Cruz de Cabezón | Urtiaga (Hervella et al. 2012) | El Cerro de la Horra | | XXI Century Mediterranean Coast |
| Aizpea | Cueva de Marizulo | El Tomillar | Tablada de Rudrón | | Fuente de la Mora | | XXI Century Basque Country |
| | | | Pajares de Adaja | | El Cementerio | | |
| | | | Perro Alto | | La Huelga | | |
| | | | | | Los Rompizales | | |
| | | | | | Carrelasvegas | | |

Secondly, a geographic–synchronous analysis of the data was performed. The geographical divisions that were used were included in the following map (Figure 2).

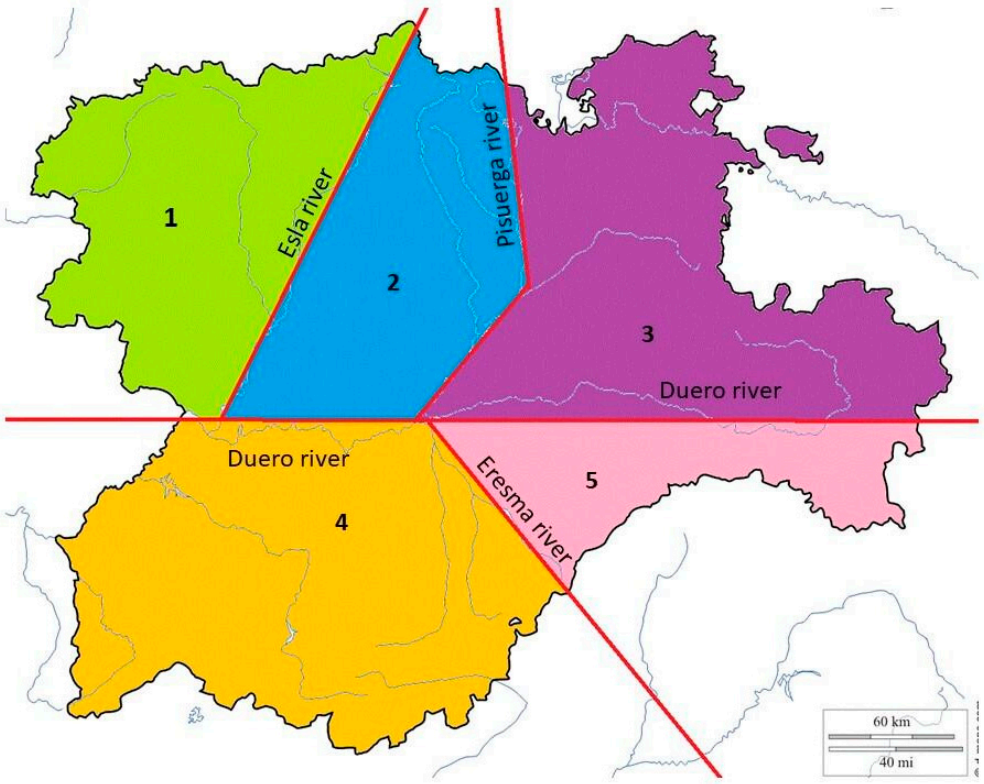


**Figure 2.** Geographical divisions were established to perform the geographical analysis.

In this case, the structure in the groups that were used to carry out the analysis of molecular variance (AMOVA) with the Arlequin v.35 software was summarised in Table 3.

**Table 3.** Chronological Groups were established for the geographical analysis.

| Group 1: Region 1 N = 2 | Group 2: Region 2 N = 12 | Group 3: Region 3 N = 19 | Group 4: Region 4 N = 16 | Group 5: Region 5 N = 14 |
|---|---|---|---|---|
| Las Cañamonas | Santa Cruz de Cabezón | El Cerro de la Horra | El Tomillar | Los Areneros |
| Santioste | Fuente de la Mora | Tablada de Rudrón | Pajares de Adaja | El Mirador |
| | Los Cercados | Cueva de la Revilla | Perro Alto | El Cementerio |
| | Trascabañas | Los Rompizales | Tordillos | Los Tolmos |
| | La Huelga | | Cerro de San Pelayo | |
| | Cantoblanco | | | |
| | La Requejada | | | |

The Terrazas del Manzanares site (Rivas-Vaciamadrid) was omitted from this second set of groupings because it was geographically located outside the northern sub-plateau. However, as can be seen in the Region 4 groups, on this occasion, the San Pelayo site was included, which was omitted in the diachronic study because it constituted a group of a single individuals from *Soto Formativo*. Nevertheless, in this case, it was part of geographic group 4 (Table 3).

- Analysis of Molecular Variance (AMOVA) and $F_{ST}$ distances

AMOVA, or Analysis of Molecular Variance, is a statistical model that measures the molecular variation in a species (Excoffier et al. 1992). Together with the study of the Genetic Distances $F_{ST}$, it was computed with Arlequin 3.5 software (Excoffier and Lischer 2010), and, specifically, Slatkin's genetic distances were used (Slatkin 1995).

- Multidimensional Scaling Analysis (MDS)

MDS was performed to obtain a visual representation of the data distribution by comparing the different chronological groups. It was performed by SPSS Statistics 24 software.

- Correspondence Analysis: Performed by SPSS Statistics 24 software.
- Creation of Maps of Genetic Distances and Haplogroup Frequencies

Another method used to represent the genetic distances between different chrono-cultural groups was through the use of maps. It was considered interesting to know not only when a greater genetic distancing between populations took place but also in the specific geographical regions where this change took place. For this reason, the genetic distances were represented on maps of the studied regions, and, for this, the Surfer V. 11 software was used. The exact geographic coordinates were previously determined using Google Earth®.

Human populations from successive chrono-cultural periods were compared with each other, geographically locating each of the sites. In this way, one could observe in which geographical region the change in the genetic composition of the population took place. The full information about $F_{ST}$ distances and geographical coordinates employed are collected in Supplementary Materials (Supplementary Material 2: $F_{ST}$ and Coordinates).

## 3. Results

### *3.1. mtDNA Haplotypes and Haplogroups Determined*

Supplementary Material 3 of the Supplementary Materials shows the mitochondrial haplotypes and haplogroups determined for each of the individuals studied. In Supplementary Material 3, only the individuals who provided valid (replicable) results were included, obtained by the replication of the same mtDNA sequences from the two different samples in all cases.

Considering the 91 analysed individuals, 68 provided valid results, which conferred nearly 75% of success. Table 4 summarises the individuals who could have been linked through the maternal lineage, taking into account each individual who shared the same burial or archaeological site and the same mtDNA haplotype simultaneously.

### *3.2. Analysis of Variations among the Chronological Groups: AMOVA and Genetic Distances $F_{ST}$*

The results of the general AMOVA analysis between the different established chronological groups (Pre-Bell Beaker period, Bell Beaker period, Ancient Bronze Age, Proto-Cogotas I culture, and Cogotas I culture) are collected in Table 5. Here, it is possible to observe that the greatest variation occurs between individuals and the least between the different groups. To obtain a more detailed view, AMOVA was performed comparing pairwise groups, the results of which are shown in Table 6.

The AMOVA showed that there were no significant genetic differences among the different chronological periods studied (Table 5). Nevertheless, when comparing groups two to two (Table 6), we could observe that most of the differences appeared among the Pre-Bell Beaker Period and Proto-Cogotas I and among Proto-Cogotas I and Cogotas I.

On the other hand, the genetic distances between chrono-cultural groups were studied, creating a matrix of Slatkin's $F_{ST}$ genetic distances, which were represented in the form of an MDS graph (Figure 3). The $F_{ST}$ distances and correspondent *p*-values are available in Supplementary Materials (Supplementary Material 4: Chronological MDS).

In this case, the individuals were also grouped according to which chronological group they belonged to, and it was observed that the only group that presented significantly elevated $F_{ST}$ distances concerning the rest of the periods was the Pre-Bell Beaker period, located further away from the rest.

The AMOVA and $F_{ST}$ distances analyses among the studied sites and the other previous (Hervella et al. 2012) and posterior Iberian populations (Pre-Bell Beaker period different archaeological sites studied, with the immediately previous Final Neolithic populations (Hervella et al. 2012), has provided the results collected on Tables 7 and 8.

**Table 4.** Relation of individuals who share the same mtDNA haplotype from each archaeological site. Possible private mutations are marked in bold letters.

| Period | Individual | Range | mtDNA Haplotype |
|---|---|---|---|
| Pre-Bell Beaker | 7BTOM | (16,105–16,399) (29–309) | 16,183C 16,189C 16,234A 16,299G 263G 291.1A |
| | 9BTOM | (16,105–16,399) (29–309) | 16,183C 16,189C 16,234A 16,299G 263G 291.1A |
| Pre-Bell Beaker | 1ARE | (16,106–16,399) (29–309) | 16,224C 16,311C 73G 263G |
| | 2ARE | (16,105–16,399) (29–389) | 16,224C 16,311C 16,319A 73G 152C 263G **277T** 315.1C |
| | 5ARE | (16,105–16,399) (29–389) | 16,224C 16,311C 16,319A 73G 152C 263G 309.1C 315.1C |
| | 6ARE | (16,105–16,399) (29–389) | 16,224C 16,311C 73G 150T 239C 263G 309.1C 315.1C |
| | 20ARE | (16,105–16,399) (29–80) (240–390) | 16,224C 16311C 73G 263G 309.1C 315.1C |
| Proto-Cogotas I | 1ELC | (16,105–16,399) (50–190) | 16,298C **64T** 72C |
| | 2ELC | (16,105–16,399) (50–190) | 16,298C **64T** 72C |
| | 3ELC | (16,105–16,399) (50–190) | 16,298C **64T** 72C |
| | LTB3 | (16,105–16,399) (29–120) | 16,224C 16,311C 16,362C 73G |
| | LTB2 | (16,105–16,399) | 16,224C 16,311C |
| | LTB1 | (16,105–16,399) (29–120) | 16,224C 16,311C 16,362C 73G |
| | 2TOR | (16,105–16,399) (29–310) | 16,362C 73G 150T 263G 291.1A |
| | 6TOR | (16,105–16,399) (29–390) | 263G 315.1C |
| | 7TOR | (16,105–16,399) (29–310) | 16,362C 73G 150T 263G 291.1A |
| | 8TOR | (16,105–16,399) (29–390) | 263G 315.1C |
| | 1RPZ | (16,105–16,399) (29–128) | 16,192T 16,270T 16,304C 73G |
| | 3RPZ | (16,105–16,399) | 16,192T 16,270T |

**Table 5.** General AMOVA results among the chronological groups. The Index value $F_{ST}$ obtained for this analysis was 0.09884, and the *p*-value was 0.06452 (they were not significantly different).

| Source of Variation | d.f. | Sum of Squares | Components Variation | % of Variation |
|---|---|---|---|---|
| Among groups | 4 | 8.035 | 0.024 Va | 1.74 |
| Among populations | 18 | 27.414 | 0.113 Vb | 8.14 |
| Among individuals | 40 | 50.392 | 1.259 Vc | 90.12 |

**Table 6.** AMOVA comparing groups two to two the chronological groups. The index value $F_{ST}$ and *p*-values obtained for each one of the pairwise analyses are also collected in the table. The $F_{ST}$ distances with significative *p*-values are marked in bold letters.

| % Variation among Groups | Pre-Bell Beaker | Bell Beaker | Ancient Bronze Age | Proto-Cogotas I |
|---|---|---|---|---|
| Pre-Bell Beaker (n = 20) | | | | |
| Bell Beaker (n = 9) | 5.37 ($F_{ST}$ fixation Index = 0.05745; $p$ = 0.33920) | | | |
| Ancient Bronze Age (n = 9) | 2.91 ($F_{ST}$ fixation index = 0.13076; $p$ = 0.06061) | 16.06 ($F_{ST}$ fixation index = −0.25644; $p$ = 0.94917) | | |
| Proto-Cogotas I (n = 26) | **5.99 ($F_{ST}$ fixation index = 0.25409; $p$ = 0)** | 4.78 ($F_{ST}$ fixation index = 0.11769; $p$ = 0.09873) | 9.39 ($F_{ST}$ fixation index = 0.08724; $p$ = 0.06549) | |

**Table 6.** *Cont.*

| % Variation among Groups | Pre-Bell Beaker | Bell Beaker | Ancient Bronze Age | Proto-Cogotas I |
|---|---|---|---|---|
| Cogotas I (n = 4) | 5.28 ($F_{ST}$ fixation index = 0.15807; $p$ = 0.13490) | 0.77 ($F_{ST}$ fixation index = −0.42670; $p$ = 0.87292) | 16.87 ($F_{ST}$ fixation index = 0.29316; $p$ = 0.89345) | **12.47 ($F_{ST}$ fixation index = 0.13386; $p$ = 0.02639)** |

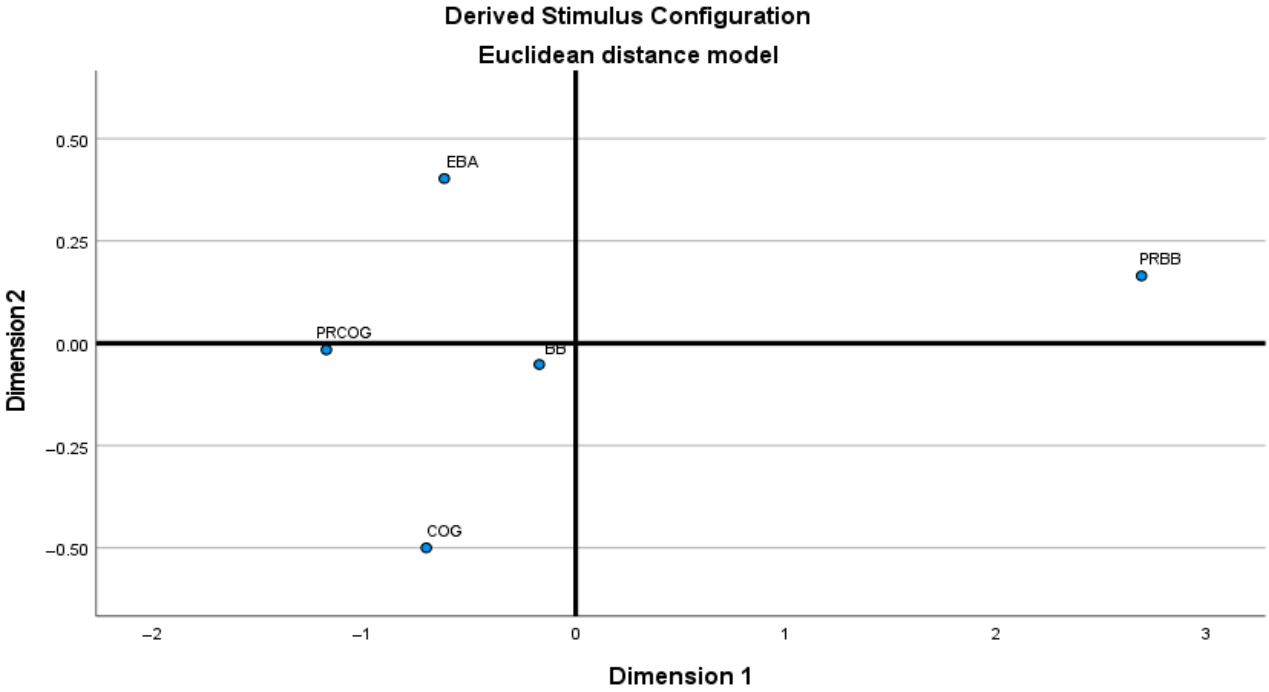


**Figure 3.** MDS of $F_{ST}$ distances among chronological groups. Performed by Alscal analysis, Euclidean distance model. Stress = 0.04763; RSQ = 0.99337. Legend: PRBB: Pre-Bell Beaker period; BB: Bell Beaker Period; EBA: Early Bronze Age; PRCOG: Proto-Cogotas I culture; COG: Cogotas I culture.

**Table 7.** General AMOVA results among the chronological groups. The Index value $F_{ST}$ obtained for this analysis was: 0.01286, and the $p$-value: was 0.32258 (they are not significantly different).

| Source of Variation | d.f. | Sum of Squares | Variance Components | % of Variation |
|---|---|---|---|---|
| Among groups | 8 | 17.337 | 0.02850 Va | 1.75 |
| Among populations | 24 | 36.951 | −0.00756 Vb | −0.46 |
| Among individuals | 276 | 443.557 | 1.60709 Vc | 98.71 |

According to Table 8 data, the most significant $F_{ST}$ distances are observed among Proto-Cogotas I culture and Paleolithic–Mesolithic, Neolithic, Pre-Bell Beaker period, and Cogotas I culture. Also, it is interesting that we observed a significative genetic distance between the Pre-Bell Beaker period and the immediately previous Neolithic period.

The $F_{ST}$ distance analysis of consecutive periods was used to elaborate MDS graphical representations, which we can observe in Figure 4. In this image (Figure 4), only the distances observed among the Final Neolithic and Pre-Bell Beaker periods and among the Proto-Cogotas I and Cogotas I cultures were significant, according to AMOVA analysis (Table 8).

**Table 8.** AMOVA comparing groups two to two the chronological groups. The index value $F_{ST}$ and *p*-values obtained for each one of the pairwise analyses are also collected in the table. The $F_{ST}$ distances with significant *p*-values are marked in bold letters.

| % Variation among Groups | Paleolithic–Mesolithic | Final Neolithic | Pre-Bell Beaker | Bell Beaker | Ancient Bronze Age | Proto-Cogotas I | Cogotas I |
|---|---|---|---|---|---|---|---|
| Paleolithic–Mesolithic | | | | | | | |
| Final Neolithic | 10.28 ($F_{ST}$ fixation index = −0.13857; $p$ = 0.23656) | | | | | | |
| Pre-Bell Beaker (n = 20) | 11.28 ($F_{ST}$ fixation index = −0.22096; $p$ = 0.07722) | **11.36 ($F_{ST}$ fixation index = −0.23925; $p$ = 0)** | | | | | |
| Bell Beaker (n = 9) | 8.63 ($F_{ST}$ fixation index = −0.28990; $p$ = 0.68817) | 0.06 ($F_{ST}$ fixation index = −0.02227; $p$ = 0.56891) | 3.94 ($F_{ST}$ fixation index = −0.09343; $p$ = 0.20919) | | | | |
| Ancient Bronze Age (n = 9) | 11.39 ($F_{ST}$ fixation index = −0.57862; $p$ = 0.88172) | 2.25 ($F_{ST}$ fixation index = −0.01562; $p$ = 0.637349 | 5.21 ($F_{ST}$ fixation index = −0.13987; $p$ = 0.07527) | 15.50 ($F_{ST}$ fixation index = −0.32931; $p$ = 0.97458) | | | |
| Proto Cogotas I (n = 26) | **7.78 ($F_{ST}$ fixation index = −0.13671; $p$ = 0.01466)** | **0.15 ($F_{ST}$ fixation index = −0.14797; $p$ = 0)** | **7.79 ($F_{ST}$ fixation index = −0.27062; $p$ = 0)** | 4.45 ($F_{ST}$ fixation index = 0.08344; $p$ = 0.10655) | 6.03 $F_{ST}$ fixation index = 0.06619; $p$ = 0.07136) | | |
| Cogotas I (n = 4) | 7.84 ($F_{ST}$ fixation index = −0.01961; $p$ = 0.42620) | 2.65 ($F_{ST}$ fixation index = −0.004181; $p$ = 0.55621) | 3.28 ($F_{ST}$ fixation index = −0.18059; $p$ = 0.07527) | 0.77 ($F_{ST}$ fixation index = −0.42670; $p$ = 0.87977) | 16.44 ($F_{ST}$ fixation index = −0.39237; $p$ = 0.95112) | **11.80 ($F_{ST}$ fixation index = −0.09426; $p$ = 0.01564)** | |
| XXI Century | 3.31 ($F_{ST}$ fixation index = −0.03832; $p$ = 0.71750) | 0.88 ($F_{ST}$ fixation index = −0.00487; $p$ = 0.48387 | **8.58 ($F_{ST}$ fixation index = −0.10048; $p$ = 0)** | 1.60 ($F_{ST}$ fixation index = −0.03276; $p$ = 0.95601) | 2.64 ($F_{ST}$ fixation index = −0.03194; $p$ = 0.85826) | 0.23 ($F_{ST}$ fixation index = −0.01411; $p$ = 0.06843) | −8.95 ($F_{ST}$ fixation index = −0.09194; $p$ = 0.76442) |

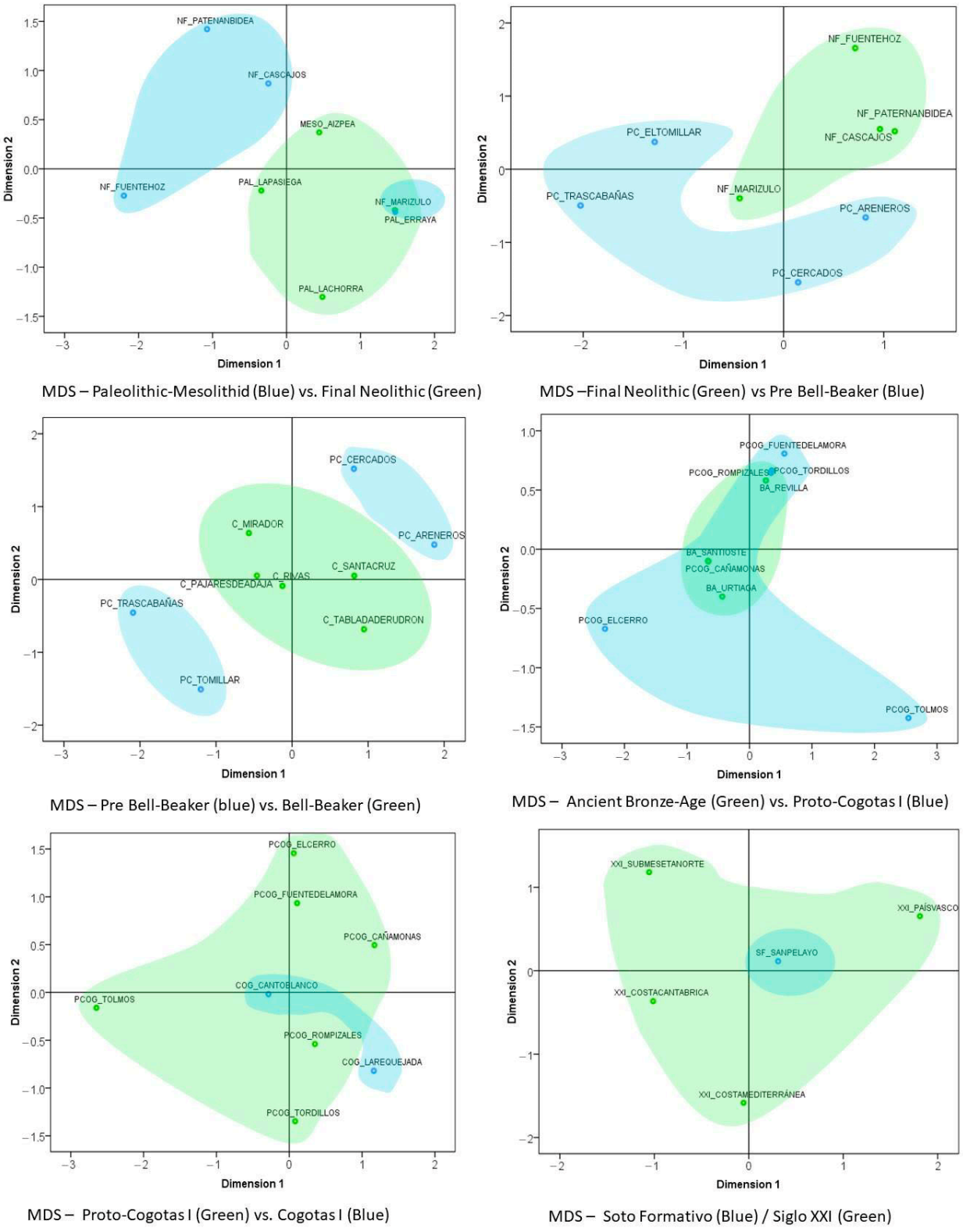


**Figure 4.** MDS of $F_{ST}$ distances among chronological groups. Performed by Alscal analysis, Euclidean distance model. Paleolithic vs. Final Neolithic: Stress = 0.15824; RSQ = 0.85399. Final Neolithic vs. Pre-Bell Beaker period: Stress= 0.10574; RSQ = 0.92337. Pre-Bell Beaker period vs. Bell Beaker period: Stress = 0.18026; RSQ = 0.80932. Ancient Bronze Age vs. Proto-Cogotas I: Stress = 0.24644; RSQ = 0.83248. Proto-Cogotas I vs. Cogotas I: Stress = 0.09300; RSQ = 0.95261. *Soto Formativo* vs. XXI Century: Stress = 0.0496; RSQ = 0.98115.

To visualise the haplogroup distribution over time, a linear graphic was created (Figure 5).

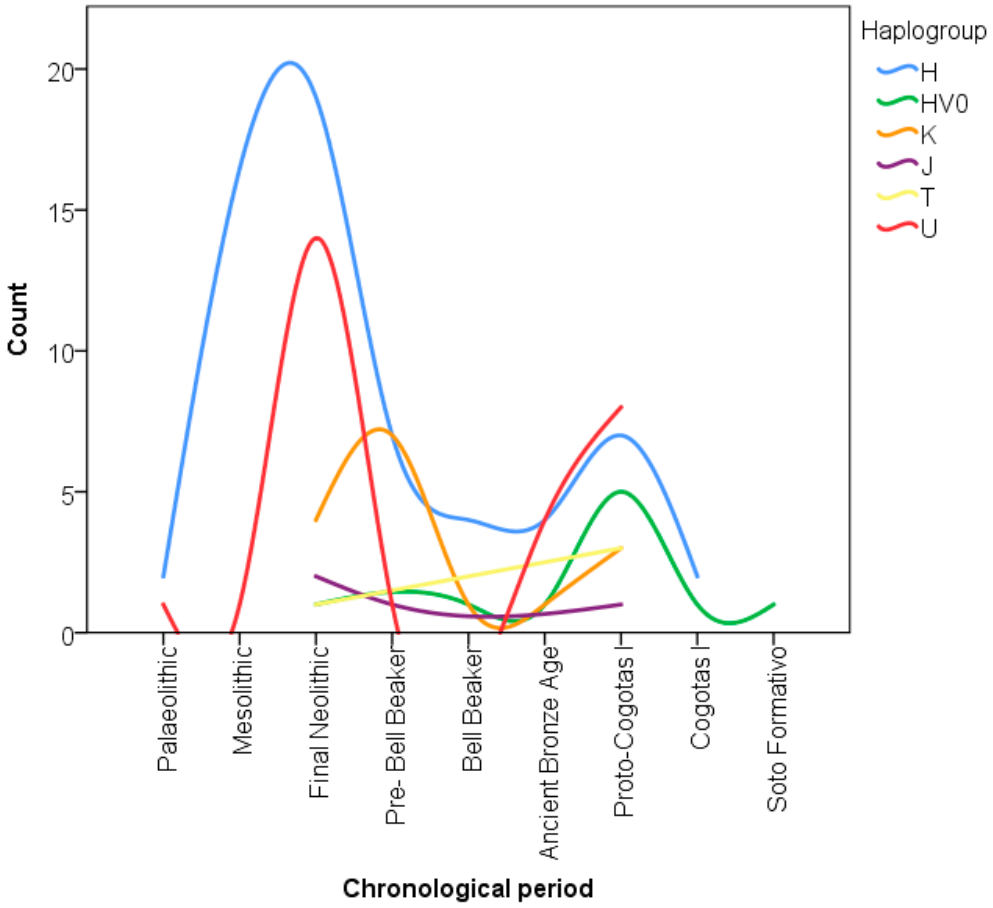


**Figure 5.** Haplogroups frequency count (Y-axis) along the different chronological periods (X-axis) studied linear representation.

### *3.3. Analysis of Variations among the Geographical Groups: AMOVA and Genetic Distances $F_{ST}$*

Mirroring the prior analysis, the geographical groups' AMOVA analysis (Table 9) and the pairwise analysis among the different geographical groups (Table 10) were carried out. The MDS graphic of genetic distances $F_{ST}$ is available in Figure 6. The $F_{ST}$ distances and correspondent *p*-values are available in Supplementary Materials (Supplementary Material 5: Geographical MDS).

**Table 9.** General AMOVA results among the geographical groups. $F_{ST}$ Fixation Index: 0.11303; $p = 0.04790$ c. There are significant genetic distances between the geographical groups.

| Source of Variation | d.f. | Sum of Squares | Components Variation | % of Variation |
|---|---|---|---|---|
| Among groups | 4 | 10.308 | 0.087 Va | 6.11 |
| Among populations | 18 | 25.840 | 0.074 Vb | 5.19 |
| Among individuals | 40 | 50.392 | 1.260 Vc | 88.70 |

**Table 10.** AMOVA comparing groups to geographical groups. The values that show significative differences are marked in bold letters.

| | % Variation | Region 1 | Region 2 | Region 3 | Region 4 |
|---|---|---|---|---|---|
| Region 1 | Among groups | | | | |
| Region 2 | Among groups | 3.65 ($F_{ST}$ fixation index: −0.29677; $p$ = 0.849469) | | | |
| Region 3 | Among groups | 9.81 ($F_{ST}$ fixation index: 0.05415; $p$ = 0.11144) | 1.04 ($F_{ST}$ fixation index: −0.03169; $p$ = 0.63441) | | |
| Region 4 | Among groups | 9.68 ($F_{ST}$ fixation index: 0.00790; $p$ = 0.31085) | 4.87 ($F_{ST}$ fixation index: 0.01899; $p$ = 0.50733) | 1.81 ($F_{ST}$ fixation index: −1.81; $p$ = 0.12805) | |
| Region 5 | Among groups | **13.60 ($F_{ST}$ fixation index: 0.34047; $p$ = 0.02737)** | 3.92 ($F_{ST}$ fixation index: 0.11036; $p$ = 0.19648) | **11.30 ($F_{ST}$ fixation index: 0.22580; $p$ = 0.00196)** | **15.63 ($F_{ST}$ fixation index: 0.30023; $p$ = 0.00196)** |

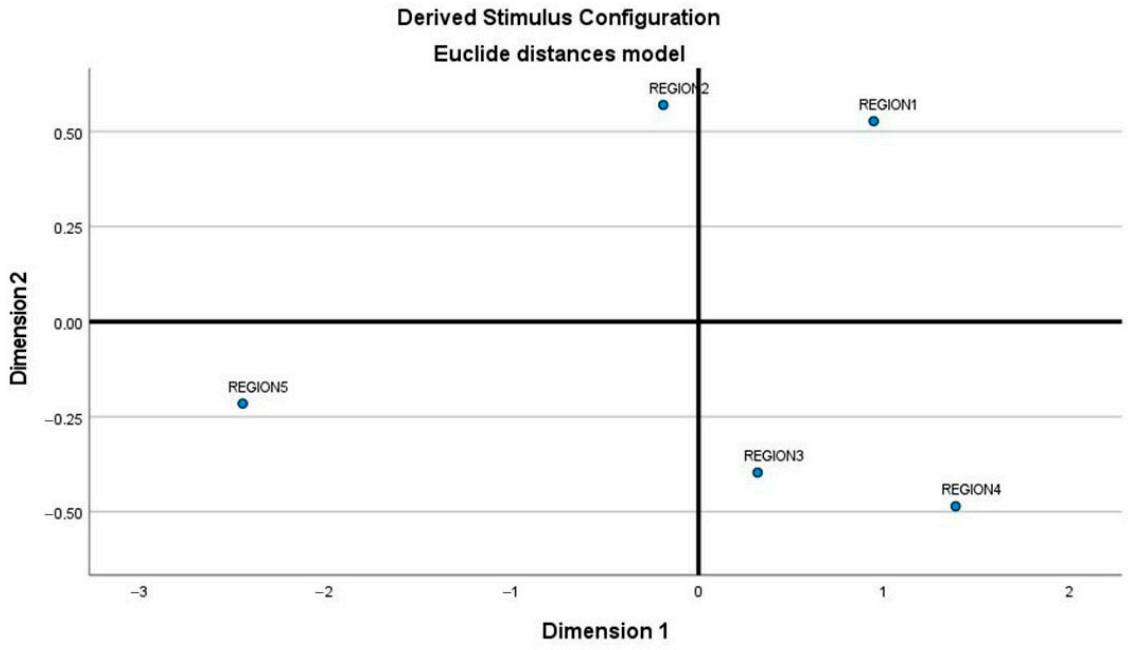


**Figure 6.** MDS of $F_{ST}$ distances among chronological groups. Performed by Alscal analysis, Euclidean distance model. Stress = 0.00684; RSQ = 0.99977.

Finally, a combined chronological–geographical analysis was performed to compare the genetic distances among consecutive chronological periods on the geographical plain. Figure 7 shows the results of its analysis. The $F_{ST}$ distances and geographical coordinates to perform this analysis are available in the Supplementary Materials (Supplementary Materials 2: $F_{ST}$ and coordinates; Supplementary Material 6: $F_{ST}$ periods).

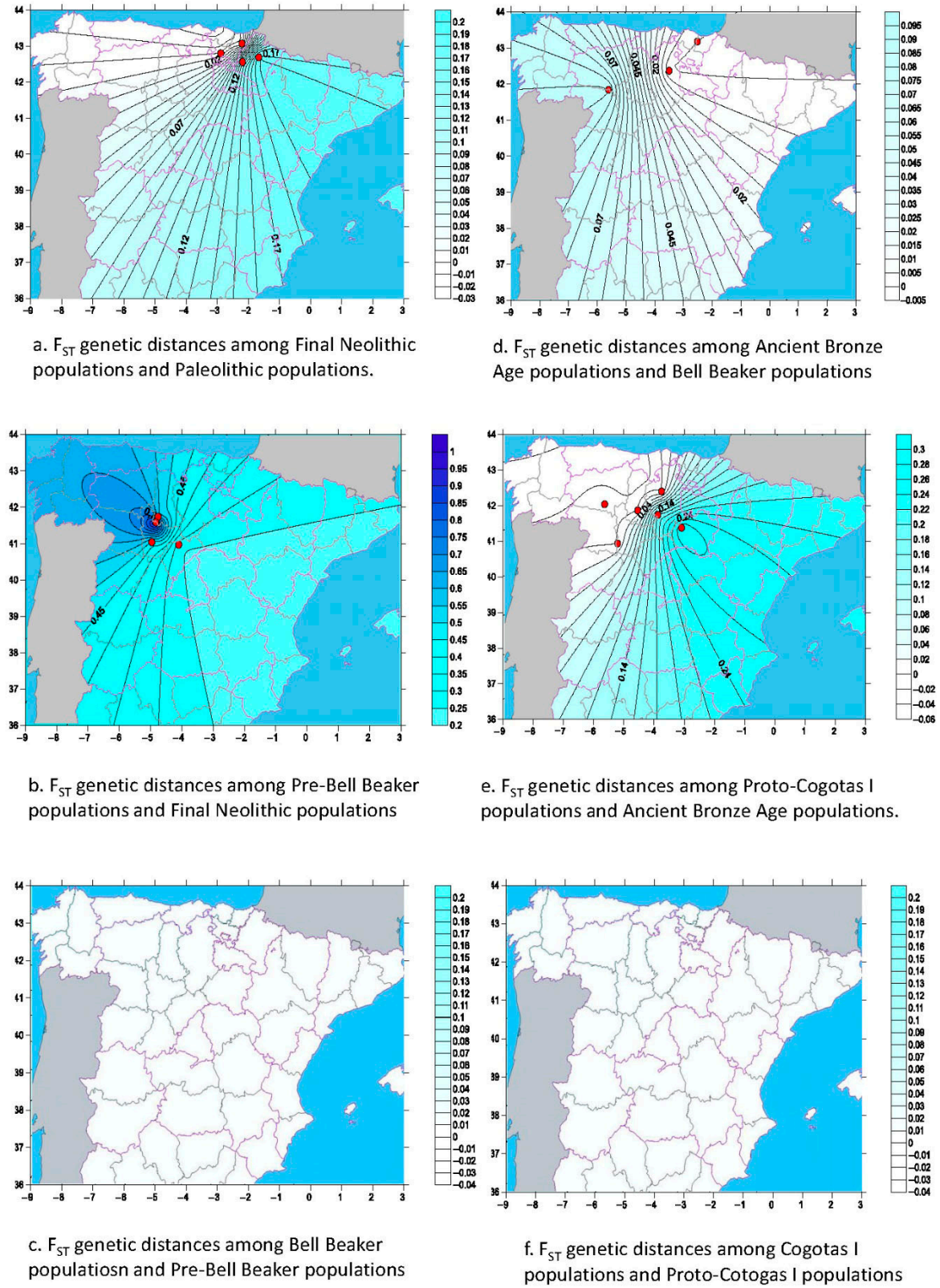


**Figure 7.** $F_{ST}$ genetic distances among the different consecutive periods studied (**a**–**f**). Clear colour means less $F_{ST}$ genetic distance, and the gradually intense blue colour indicates more $F_{ST}$ genetic distance. The last period *Soto Formativo* has not been considered in this analysis because of its low number of i.ndividuals.

## 4. Discussion

Regarding the possible family ties among individuals inhumed together, possible maternal lineage kinship was detected among individuals from the Proto-Cogotas I culture and the Pre-Bell Beaker period (Table 4). Looking at the Pre-Bell Beaker period, two of the three collective burials studied from this chronological period also showed maternal lineage relations among the individuals. The only Pre-Bell Beaker site that did not show any maternal lineage linkage was Los Cercados; nevertheless, it was a special case because of its characteristic ritual (García Barrios 2007). Therefore, in general, we can affirm that the tendency during the Pre-Bell Beaker was to bury together those individuals who shared a certain common lineage (maternal in this case).

As we can observe in Supplementary Material 3 and Table 4, all the collective burials from the Proto-Cogotas I culture studied showcased biological kinship through the maternal lineage because some of their individuals shared the same mtDNA haplotype. Moreover, in the case of El Cerro de la Horra (ELC), as we could observe in Supplementary Material 3 and Table 4, the three individuals inhumed together also shared a private mutation (marked in red bold letters) (64T); it was a sign that enforced the hypothesis of maternal lineage kinship among the individuals.

Nevertheless, there was no detected lineage kinship through the maternal lineage among individuals inhumed together in the collective burials from the Bell Beaker period, Early Bronze Age, or Cogotas I culture. However, it must be taken into account that we found a higher number of collective burials in Proto-Cogotas I culture than in the other periods, but it certainly looks like that it was more frequent to inhumate biologically related individuals together during this Proto-Cogotas I culture period; meanwhile, in other cases, it appears that the criterion to bury different individuals together could be another kind of criteria, like group associations. It must be considered that the concept of "family", which nowadays is reflected in the way of inhumation, was perhaps not the same in past times and societies (Gomes et al. 2021).

Regarding the diachronic analysis, the percentage of variation between chronological groups was not significant (Tables 5 and 7), which indicated that there was no sudden population change but rather a gradual contribution. Regarding the AMOVA among pairwise groups (Tables 6 and 8), we could appreciate that two main chronological periods showed significant genetic $F_{ST}$ distances concerning the previous periods: the Pre-Bell Beaker and Proto-Cogotas I periods.

Below, we will discuss the characteristics of these two key moments in the arrival of a new population flow.

A change in the genetic composition of the population was observed during the Pre-Bell Beaker period, concerning the previous populations. In addition, attending to Figure 7 (Figure 7b), it was observed that the greatest genetic distances were found among the Final Neolithic and Pre-Bell Beaker period in the north-western region of the peninsula. This could indicate that the arrival of a new population flow associated with the Pre-Bell Beaker culture, that could have arrived through that peninsular region. In addition, according to the haplogroup frequencies (Figure 5), this new population arrival during the Pre-Beaker seemed to be linked to haplogroup K, which increased notably during this period, being previously absent, so this haplogroup appeared at the end of the Neolithic and reached its maximum plenitude during the Pre-Beaker period. According to the AmtDB database search (Supplementary Material 3), this K haplogroup mainly extended through the Iberian Peninsula, Germany, the Netherlands, and the Czech Republic. Therefore, we could interpret that this haplogroup K, which arrived in the peninsula during this period, may have come from these regions.

Subsequently, the population stabilised, and there did not seem to be a new gene flow during the Bell Beaker period (Tables 6 and 8), which would indicate that the material culture could have arrived earlier (during the Pre-Bell Beaker period) and been transmitted without being accompanied by a new population flow. Nevertheless, it was possible to observe a slight $F_{ST}$ distance increasing around the Iberian northwest (Figure 7c), but it was

not statistically significant (Tables 6 and 8). Regarding the compositions of haplogroups during the Bell Beaker period, the haplogroup K, characteristic of the previous period (Pre-Bell Beaker), was reduced, resembling the levels of other haplogroups already existing during the Mesolithic and Neolithic periods (H, HV0, J, and T) (Figure 6). For this reason, during the Bell Beaker period, we observed a balance between the previously existing mitochondrial haplogroups (Figures 5 and 6) without the new arrival of a population and significant genetic distances from the previous periods. This marked a stage of stability, during which the Beaker culture would develop, probably transmitted by the populations that arrived in the previous stage, but without a new population flow.

Regarding the haplogroups' compositions of the populations during the Bell Beaker period, it should be noted that the presence of haplogroup U has been detected (Figure 5), which was considerably reduced during the Pre-Bell Beaker period until it disappeared at this stage.

The population stability observed during the Bell Beaker period would also be maintained during the Bronze Age, during which neither significant genetic distances were perceived concerning previous periods (Tables 6 and 8), nor the arrival of new notable population flows. Nevertheless, it was possible to observe a slight $F_{ST}$ distance increasing around the Iberian northwest (Figure 7c), but it was not statistically significant (Tables 6 and 8).

With the arrival of the Proto-Cogotas I culture, the second important new genetic flow appeared. Significant genetic $F_{ST}$ distances among the Proto-Cogotas I culture and the previous periods were observed: the Pre-Bell Beaker, Neolithic, and Mesolithic periods, also concerning the posterior Cogotas I culture (Tables 6 and 8) (Figure 7e).

As we previously commented, also during the Cogotas I period, the culture of burying individuals together linked by their lineage (maternal in this case) was recovered (Table 4). In addition, we observed that according to the genetic distances observed (Tables 6 and 8), as well as their distributions, it seemed that a new population flow arrived during this period. In particular, it seemed that this would be the place of entry for this new population through the south-eastern region of the Duero Basin (Figure 7e). This new population flow did not seem to be associated with the appearance of new mitochondrial haplogroups but rather with the expansion of other already existing haplogroups, specifically the haplogroups: U, H, and HV0 (Figure 5). Thus, this new populational flow was associated with the arrival of new haplotypes but not with a new haplogroup input.

This was the last one immediately before the arrival of the Cogotas I material culture. This would indicate that, during the Proto-Cogotas I period, there was a change in the genetic compositions of the populations immediately before the development of the Cogotas I material culture. However, this change would have been gradual since there were no significant genetic distances between Proto-Cogotas I and Bell Beakers or ancient Bronze, so it should be a gradual change, produced by a gradual arrival of a new foreign population (not like what occurred during Pre-Bell Beaker period, which supposed a great change to the period immediately before: the Late Neolithic). Attending to the haplogroups determined (Supplementary Material 3), we could observe that the main haplogroups observed during this Proto-Cogotas I period were, in general, more frequently observed in other populations of Germany, Great Britain, and Spain; so, Germany or Great Britain could have been the possible origins of them (Supplementary Material 3).

At this point in the discussion, it is worth noting that all the data analysed here was related to the study of mtDNA, which is why they described changes in matrilineal lineages since women are carriers of mtDNA (all populations, both men and women, possess it, but only women pass it on to descendants). Despite this type of DNA being present in all individuals, both men and women, the changes in the composition depend on the population movements of women because they are the ones who transmit it. Therefore, it is a very appropriate marker to find out about women's movements (Palomo-Díez and López-Parra 2022), and, in this case, it is especially interesting to discover if there is a certain link between the movement of a female component of the population, accompanied by novelties in material culture, such as those that characterise the

Beaker population or the Cogotas I culture. This is important because the archaeological data suggest that the production of ceramics in these cultures was the work of the women of the studied communities (Whallon 1968; Garrido-Pena 1999; García Barrios 2007). Therefore, the study of mtDNA could allow us to verify or deny this hypothesis. However, from a genetic point of view, we can say that it could have been a new female population arrival during the Pre-Bell Beaker Period parallel to the arrival of the new material culture (the posterior Bell Beaker pottery). Still, it is not enough to ensure that this kind of ceramic was made exclusively by women. In other words, the genetic results point to the possible arrival of a new female population, but it could be possible that the ceramics were made both by women and men; we cannot know who exactly carried out the manufacturing, although it does seem that these new ceramics arrived at the same time as the new flow of the female population. But we can say that there may be a connection between the arrival of this new population and the starting of the appearance of the culture.

To carry out a geographical analysis of the data, five groups were established that had not been based on the political divisions of the current provinces (Figure 2). Instead, a division based on the hydrographic divisions of the northern sub-plateau was used. This criterion was chosen since the orographic divisions within the Sub-plateau did not seem to pose great geographical barriers for the traffic of individuals from one region to another, so the river networks were taken as possible geographical divisions. In this way, we intended to observe if these hydrographic "frontiers" were real barriers that emerged in reaction to the genetic compositions of the populations or on the contrary, if the communities who settled in positions spaced by kilometres but in the basins of the same rivers shared similar and different genetic characteristics to the rest. The latter would be indicative of the possible use of rivers as means of transportation and communication between residents.

Regarding the differences in the genetic composition of the populations of the different geographical areas (Tables 9 and 10; and Figure 6), marked by the riverbeds, in this case, it is striking that the genetic variance between populations (5.19) is lower than the variance between the groups formed by the different geographical regions (6.11) (Table 4). The variability between groups is rarely greater than between populations within the same group, and this indicates a possible differentiation between groups that live in different geographic regions. However, to refine this analysis, it is necessary to take into account whether these differences are due to a geographical or temporal issue, making it also necessary, in this case, to analyse the variances between specific groups two by two (Table 10), assessing, in each case, if the molecular variance is due to geographical or chronological issues. Broadly speaking, we can say that no significant differences were observed in the genetic compositions of the populations, except in geographic area number 5 (located south of the Duero River and east of the Eresma River), which did seem to present a genetic composition significantly different from that of the other regions (except for Region 2). This would indicate that there must have been a certain geographical barrier that kept Region 5 isolated to a certain degree from the rest of the regions, but the same would not happen among the rest of the geographical regions that seemed to maintain similar genetic compositions among their populations. It should be noted that several authors already proposed the existence of communication and transportation routes in the western region of the northern sub-plateau (Abarquero Moras 2005), and this fact may also be supported by these genetic data, which demonstrate greater interactions between the populations located to the north and south of the western zone and between the northern and southern parts of the eastern half, which remained more isolated (Region 5).

## 5. Conclusions

To summarise as the main conclusion of this research, it can be said that we have observed two historical moments of new population arrival: the first during the Pre-Bell Beaker period, associated with the K mitochondrial haplogroup, and the second during Proto-Cogotas I culture, with new lineages of the H, HVO, and T haplogroups. Neither of the new population flows were directly associated with the full development of the

two main material cultures Bell Beaker and Cogotas I, which occurred immediately before; so, we cannot discard an association. This study has provided us with an approximation of the populational changes that occurred during these different periods in this specific geographical area of the north sub-plateau of the Iberian Peninsula.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genealogy7030051/s1, Sup.Mat.1_cones, Sup.Mat.2_FSTs, and coordinates; Sup.Mat.3; Sup.Mat.4_Chronologica MDS; Sup.Mat.5_Geographical MDS; Sup.Mat.6_FSTs_PERIODS.

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