Insights Into the "Isolation" of the Basques: mtDNA Lineages from the Historical Site of Aldaieta (6th–7th Centuries AD)

Ainhoa Alzualde,[1] Neskuts Izagirre,[1] Santos Alonso,[1] Antonio Alonso,[2] Cristina Albarrán,[2] Agustin Azkarate,[3] and Concepción de la Rúa[1]*

[1]*Genetika, Antropologia Fisikoa, eta Animali Fisiologia Saila, Zientzia eta Teknologia Fakultatea, Euskal Herriko Unibertsitatea, 48080 Bilbo, Spain*
[2]*Servicio de Biología, Instituto Nacional de Toxicología y Ciencias Forenses, 28002 Madrid, Spain*
[3]*Filologia eta Geografia-Historia Fakultatea, Euskal Herriko Unibertsitatea, 01006 Vitoria-Gasteiz, Spain*

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ABSTRACT We analyzed the hypervariable region I (HVR-I) sequence variability of the mitochondrial DNA (mtDNA) of individuals buried at Aldaieta (6th–7th centuries AD) in order to find out more about the biosocial implications of this cemetery. The results, fully authenticated by means of diverse criteria (analysis of duplicates, replication in an independent laboratory, quantification of target DNA, and sequencing and cloning of polymerase chain reaction products), suggest that Aldaieta largely consists of autochthonous individuals who shared common funereal customs with the late Ancient North Pyrenean cemeteries of Western Europe (the *Reihengräberfelder*), a cultural influence possibly accompanied by a certain genetic flow. Furthermore, the distribution of mtDNA lineages in the cemetery highlighted the existence of a significant number of family relationships, supporting the belief that it was a stable settlement and not a group that had haphazardly settled in the area. Finally, this paper stresses the importance of ancient DNA data for reconstructing the biological history of human populations, rendering it possible to verify certain hypotheses based solely on current population data. The presence at Aldaieta of an mtDNA lineage originating in Northwest Africa testifies to the existence of contact between the Iberian Peninsula and Northwest Africa prior to the Moorish occupation. Both this latter discovery and the high frequency of haplogroup J at the Aldaieta cemetery raise questions about the generally accepted belief that, since ancient times, the influence of other human groups has been very scarce in the Basque Country. Am J Phys Anthropol 000:000–000, 2006.

Technological advances in molecular biology allow for the recovery and analysis of DNA of ancient human remains, whereby we can adopt an objective approach to the genetic composition of past populations. In this paper, we undertook a genetic analysis of the ancient population of Aldaieta, as it is of considerable interest both for its late ancient chronology (6th–7th centuries AD), one of the more obscure periods in Basque history, and for its anthropological and cultural characteristics. The cemetery was uncovered in 1987, offering up well over 100 burials in which the corpse was accompanied by grave goods of significant quality in certain cases. These burials were arranged in groups that were clearly separated from each other by extensive open areas (Azkarate, 1999). Aldaieta features two sectors with major differences: the southeast (SE) sector of the cemetery is occupied by superimposed burial groups with an initial foundational level and, on top it, up to three different burial levels, accompanied by numerous personal possessions and funereal items. The wealth of these groups (with an abundance of weapons) is in contrast to the northwest (NW) sector of the cemetery, in which burials are arranged in nonsuperimposed rows with personal possessions of lesser importance. Certain striking features of this cemetery are, on the one hand, the high percentage of weaponry present in the burials, and on the other, the fact that certain mortuary objects associate Aldaieta with the Frankish cemeteries of Western Europe (Azkarate, 2004).

Various interpretations of Aldaieta have been forthcoming. On the one hand, Böhme (2002) suggested that Aldaieta must be the burial site of members of a foreign army, probably a Frankish military unit, judging by the type of weapons recovered. Böhme (2002) tended to favor the expedition that the Merovingian kings undertook in AD 541 and which suffered a severe defeat at the hands of the Visigoths. However, Azkarate (2004) believed that Aldaieta does not correspond to a specific episode in history, but rather represents a stable population. The origin of the archeological evidence of a Northern Pyrenean

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*Correspondence to: Dr. Concepción de la Rúa, Genetika, Antropologia Fisikoa, eta Animali Fisiologia Saila, Zientzia eta Teknologia Fakultatea, Euskal Herriko Unibertsitatea, Posta Kutxa 644, 48080 Bilbo, Spain. E-mail: conchi.delarua@ehu.es

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Fig. 1. Location of the cemetery of Aldaieta in Basque Country. Also shown are the boundaries in the late ancient period that, according to historians, were not exceeded by the Franks (Pyrenees) and the Visigoths (Ebro River).

(Frankish) nature recovered in this cemetery may be attributed to the spread of funereal customs, trading links, or possibly an indication of temporary control of Basque territory by the Franks (Azkarate, 2004).

In a prior analysis of ancient populations in the Basque Country based on mitochondrial DNA (mtDNA) haplogroup frequencies (determined by means of restriction fragment length polymorphisms (RFLPs); Alzualde et al., 2005), we found that between 1,500–5,000 YBP, there was a restructuring of the distribution of mtDNA frequencies in the population of the Basque Country and possibly in more regions of Western Europe, probably as a result of gene flow and/or genetic drift. The aforementioned analysis pointed to the existence of a recent relationship between the historical population of Aldaieta and those constituting the Atlantic Fringe. In the present paper, based on hypervariable region I (HVR-I) sequences in the population of Aldaieta, we analyzed the relationship between this ancient population in the Basque Country and present-day populations in Europe, with a view to providing a response to the historical problem posed regarding the origins of the inhabitants of Aldaieta and their relationship to human groups within a Peninsular and North-Pyrenean setting.

MATERIALS AND METHODS

Materials

The human remains analyzed came from the late ancient (second half of 6th through 7th century AD) cemetery of Aldaieta (Nanclares de Gamboa, Alava, Basque Country) (Fig. 1). An analysis was made of the mtDNA HVR-I of 65 individuals out of the 105 that constituted the minimum number of individuals (de la Rúa, 1999), as some were only represented by a few bone remains, and others did not preserve dental pieces in good condition.

The comparative population analysis was performed by exploring the haplotypes described in Aldaieta in worldwide populations, including sequences from regions in Europe, Asia, and Africa (Table 1). Overall, the database used for the analysis of shared haplotypes consisted of over 10,000 Euro-Asiatic and African sequences.

Methods

In order to minimize the risk of contamination, the ancient samples were handled with care during each step undertaken, and authentication criteria were upheld (Pääbo et al., 2004). During the excavation process, samples were handled as little as possible, being packed up as quickly and carefully as possible without being washed, and in many cases they arrived at our laboratories still embedded in sediment, whereupon they were cleaned in dry conditions. The extraction of DNA and the preparation of samples for polymerase chain reaction (PCR) were performed in a positive-pressure sterile chamber, free of modern DNA, in which no post-PCR process had ever been carried out. The worktops were regularly cleaned with diluted bleach and irradiated with ultraviolet (UV) light. Furthermore, suitable clothing was used: disposable cap, gloves, mask, and laboratory coat. Controls were applied for detecting contamination during the extraction process and in each one of the amplifications. Whenever possible, the analysis of samples was duplicated in our laboratory, and in certain cases it was replicated in an independent laboratory (Instituto Nacional de Toxicología y Ciencias Forenses (INTyCF), Madrid). The HVR-I sequences of the researchers who handled the samples were analyzed.

Selection and extraction of DNA from dental samples. A selection was made of 123 teeth (without caries or fissures) belonging to 65 individuals, of which 45 underwent duplicate analysis (69.2%), and 10 underwent triplicate (15.4%) analysis, in our laboratory. Certain individuals could not be duplicated due to a shortage of anthropological material. Acids and UV irradiation were used to give the surface of the teeth a thorough cleaning and remove any possible contaminants (Ginther et al., 1992). DNA was then extracted from dental tissue by means of the phenol/chloroform method, with certain modifications (Izagirre and de la Rúa, 1999). In addition, a further nine dental pieces were sent to the INTyCF to undergo replication, where the entire process was undertaken by different researchers.

Sequencing mtDNA HVR-I. The sequencing of a 403-bp segment of the mtDNA HVR-I (nps 15,998–16,400, as per Andrews et al., 1999) was undertaken by amplifying six overlapping fragments between 93–133 bp in length. The protocol and primers are described in Alonso et al. (2003), although instead of using two multiplex-PCRs, each fragment was amplified in a separate PCR to obtain greater amplification efficiency. Likewise, in order to verify a specific haplotype (haplogroup M), determination was made of nucleotide position 10,400 by the use of primers 5′-TCTTATTAATCATCATCCTAGC-3′ and 5′-TTTAATGAGTCGAAATCATTCG-3′ (annealing temperature, 53°C), which gave rise to a fragment 115 bp in length. This fragment was subsequently analyzed by both sequencing and digestion by means of the *Alu*I enzyme, following the supplier's instructions (New England Biolabs).

Authentication criteria. Besides the standard precautions mentioned above, the following authentication criteria, as described in Alzualde et al. (2005), were also used:

- Quantification of amplifiable DNA: Nine individuals were used to quantify the amplifiable DNA of two fragments, one of 113 bp, and the other of 287 bp (Alonso et al., 2004).

TABLE 1. Populations constituting database of HVR-I sequences of mtDNA compiled for population analysis[1]

Population	Description	n[2]	Reference
Aldaieta	Ancient population from northern Iberian Peninsula (6th–7th centuries AD)	48	This study
Basques	Northern Iberian Peninsula	156	Bertranpetit et al., 1995; Corte Real et al., 1996; Richards et al., 2000
Cantabria	Northern Iberian Peninsula	88	Maca-Meyer et al., 2003a
Liebana	Isolate from Cantabria	72	Maca-Meyer et al., 2003a
Pas	Isolate from Cantabria	82	Maca-Meyer et al., 2003a
Galicia	Northern Iberian Peninsula	135	Salas et al., 1998; Gonzalez et al., 2003
Andalusia	Southern Iberian Peninsula	114	Corte-Real et al., 1996; Larruga et al., 2001; Plaza et al., 2003
Castile	Central Iberian Peninsula	38	Larruga et al., 2001
León	Central Iberian Peninsula	61	Larruga et al., 2001
Maragatos	Isolate from León	49	Larruga et al., 2001
Catalonia	Eastern Iberian Peninsula	61	Corte-Real et al., 1996; Plaza et al., 2003
France		312	Monson et al., 2002; Dubut et al., 2004
Portugal		353	Corte-Real et al., 1996; Gonzalez et al., 2003
Germany		500	Lutz et al., 1998; Poetsch et al., 2003
Norway		316	Helgason et al., 2001
Great Britain		97	Piercy et al., 1993
Scotland		1,190	Helgason et al., 2001
Italy		462	Di Rienzo and Wilson 1991; Stenico et al., 1996; Francalacci et al., 1996; Cali et al., 2001; Bini et al., 2003
Etruria	Ancient population from Italy (7th–3rd centuries B.C.)	28	Vernesi et al., 2004
Near East		1,234	Richards et al., 2000
North Caucasus		208	Richards et al., 2000
Europe		2,804	Richards et al., 2000
Poland		436	Malyarchuk et al., 2002
Russia		201	Malyarchuk et al., 2002
Iraq		216	Al-Zahery et al., 2003
East and Central Asia		910	Quintana-Murci et al., 2004
India		550	Kivisild et al., 1999
Bantu speakers		307	Salas et al., 2002
Mozambique		109	Pereira et al., 2001
Morocco		50	Brakez et al., 2001
North Africa		268	Rando et al., 1998
Northwest Africa		172	Plaza et al., 2003
Ethiopia		270	Kivisild et al., 2004
Yemen		115	Kivisild et al., 2004
Tunisia (Berbers)		155	Fadhlaoui-Zid et al., 2004

[1] Upper half contains populations of Western Europe, and lower half (Near East and below) features Euro-Asiatic and African populations classified into more extensive regions.
[2] Number of individuals in each population.

- Duplication: A duplicate analysis was performed on the greatest possible number of individuals (out of 65 individuals selected, 45 were analyzed in duplicate, and 10 in triplicate).
- Replication: A subsample of nine individuals was used to replicate the mtDNA HVR-I sequence at INTyCF, as per the protocol described in Alonso et al. (2003).
- Cloning of PCR products: This was performed with subsequent sequencing of a fragment in a subsample of 10 individuals. Cloning was undertaken using TOPO TA Cloning® Kits (Invitrogen), following the supplier's instructions.

Statistical analysis

Calculation was made of the nucleotide diversity (Nei, 1987) in the ancient population of Aldaieta and in other populations in Western Europe (Table 1) by means of the Arlequin software package (Schneider et al., 2000). Likewise, a neutrality test was carried out through calculation of Tajima's D value (Tajima, 1989, 1996), using the Arlequin software package (Schneider et al., 2000), and its statistical significance was assessed by means of coalescence simulations undertaken with the software SIMCOAL version 1.0 (http://cmpg.unibe.ch/software/simcoal). Different simulations were performed (each one of 10,000 iterations): on the one hand, considering that mutation rates are uniform in the different sites in the control region (assuming a gamma distribution with $\alpha = 0.26$; Meyer et al., 1999) and that the population size is constant; and on the other, considering both the heterogeneity of mutation rates and population increase.

The search for the haplotypes described in Aldaieta was carried out considering a total of over 10,000 Euro-Asiatic and African sequences (see Materials). Regarding the Aldaieta haplotypes not found among these sequences, an additional search was made in Genbank. In order to compare the haplotypes described in Aldaieta with the greatest possible number of sequences published, the size of the fragment analyzed was restricted to that between nucleotide positions 16,092–16,362 (Andrews et al., 1999).

TABLE 2. Haplotypes and haplogroups of mtDNA described in late ancient cemetery of Aldaieta (6th–7th centuries AD)

Haplotype	n[1]	n_p[2]	HVR-I haptotype[3]	nt73 HVR-II	RFLP motifs[4]	Haplogroup[5]
ht1	11	6	CRS	G	−14766*Mse*I; −7025*Alu*I	H
ht2	12	9	CRS	A	−14766*Mse*I; −7025*Alu*I	H
ht3	11	3	362	A	−14766*Mse*I; −7025*Alu*I	H
ht4	1	1	298	A	−14766*Mse*I; +7025*Alu*I; +4577*Nla*III	V
ht5	1	1	126, 266, 274, 294, 304	G	−10394*Dde*I	T2
ht6	1	1	126, 294, 296, 304	G	−10394*Dde*I; + 4216*Nla*III	T2
ht7	1	1	126, 189, 294	G	+4216*Nla*III	T
ht8	1	1	192, 270	G	+12308*Hinf*I	U5
ht9	1	1	176, 270	G	+12308*Hinf*I	U5
ht10	1	1	172, 189, 192, 270, 311	G	+12308*Hinf*I	U5
ht11	1	1	051, 092, 129C, 192, 362	G	+12308*Hinf*I	U2
ht12	3	1	051, 129C, 183, 189, 362	G	+12308*Hinf*I	U2
ht13	1	1	224, 311	G	Not determined	K
ht14	3	2	069, 126, 390	G	+10394*Dde*I; +4216*Nta*III	J
ht15	3	1	069, 126	G	+10394*Dde*I; +4216*Nta*III	J
ht16	2	2	069, 126, 278, 366	G	+10394*Dde*I; +4216*Nta*III	J
ht17	2	1	129, 185, 189, 223, 249, 311	G	Not determined	M1

[1] Number of individuals carrying the haplotype.
[2] Number of individuals corrected as per kinship criteria.
[3] Nucleotide positions (−16,000) ranging 15,998–16,400 that are different from CRS (Cambridge reference sequence, Anderson et al., 1981).
[4] RFLP motifs for mtDNA haplogroup determination (Macaulay et al., 1999) analyzed in Alzualde et al. (2005).
[5] Subhaplogroups determined by HVR-I sequences (Macaulay et al., 1999).

RESULTS

Sequencing of mtDNA HVR-I

An analysis was made of the variability of HVR-I in 65 individuals from the late ancient cemetery of Aldaieta. In 56 individuals, sequencing was possible of a fragment of 403 bp of HVR-I, with a description of 17 different haplotypes taking into account both the HVR-I sequence and position 73 of HVR-II (Table 2). The sequences were duplicated in 55% of the individuals. The supplementary material contains detailed information on each individual analyzed and the researchers' HVR-I sequences.

A non-Caucasian haplotype belonging to haplogroup M1 (ht17) was observed in two individuals in Aldaieta (Table 2). These two individuals, who on the basis of RFLP analysis could not be classified into any of the more frequent haplogroups in Caucasians (Alzualde et al., 2005), are likewise difficult to classify by the polymorphisms of the HVR-I sequence (16,129-16,185-16,189-16,223-16,249-16,311), as they could correspond to the M1 or U1a lineages (Richards et al., 2000). However, as no shared sequences belonging to U1 were found in the database of over 10,000 Euro-Asiatic and African sequences, although shared sequences were indeed found belonging to haplogroup M1, whose HVR-I motifs are 16,129-16,189-16,223-16,249-16,311 (Quintana-Murci et al., 1999), we verified the membership of these two individuals in haplogroup M, which presents a transition of C to T at nucleotide position 10,400 (Macaulay et al., 1999).

Kinship

The Aldaieta cemetery was used for approximately 150 years, from the middle of the 6th century AD through the 7th century AD. Therefore, among the samples analyzed, there may be related individuals across the generations. The high frequency at Aldaieta of the 00073G mutation of the mtDNA among the lineages of haplogroup H, and its differential distribution in certain burials, denote the existence of kinship on the maternal side, which is also supported by archaeological data (Izagirre et al., 2005). Given that kinship may alter a population's true frequencies, it is advisable to detect the possible family relationships present at the site and remove the bias they cause. The present mtDNA analysis reveals that certain lineages present a differential distribution in the cemetery, whereby some are concentrated in specific burials. For example, the two sole individuals at Aldaieta belonging to ht17 were buried side by side on their own (B3 and B4, Fig. 2); the three individuals belonging to ht14 were buried in the same group burial (Fig. 2); the three individuals belonging to ht12 were buried beside one another (Fig. 2); identification was made of 11 individuals of ht3, of whom 8 belonged to the same burial group (Fig. 2). This analysis therefore enables us to identify which mitochondrial lineages are associated with kinship. In order to avoid the distortion of frequencies due to kinship, a correction factor was applied whereby if a haplotype is represented by two or more individuals interred in the same burial, we only consider one of them. This correction criterion is similar to that applied by Vernesi et al. (2004) to the ancient Etruscan population, the only ancient DNA population study hitherto published. The haplotypes whose frequency was corrected are shown in Figure 2. As a result of this correction, 22 of the 56 individuals sequenced were ruled out, meaning that the sample for statistical analyses consists of 34 individuals. Haplogroup and haplotype frequencies are shown in Table 3.

The application of this criterion entails certain risks and considerably reduces the sample size, and increases the value of the standard deviation. However, the strength of the present paper is based mainly on a qualitative analysis that takes into account the presence/absence of specific haplotypes.

Authentication of results

The result of the quantification of amplifiable DNA gives a sufficient number of DNA copies for molecular analysis, and likewise indicates an inverse relationship

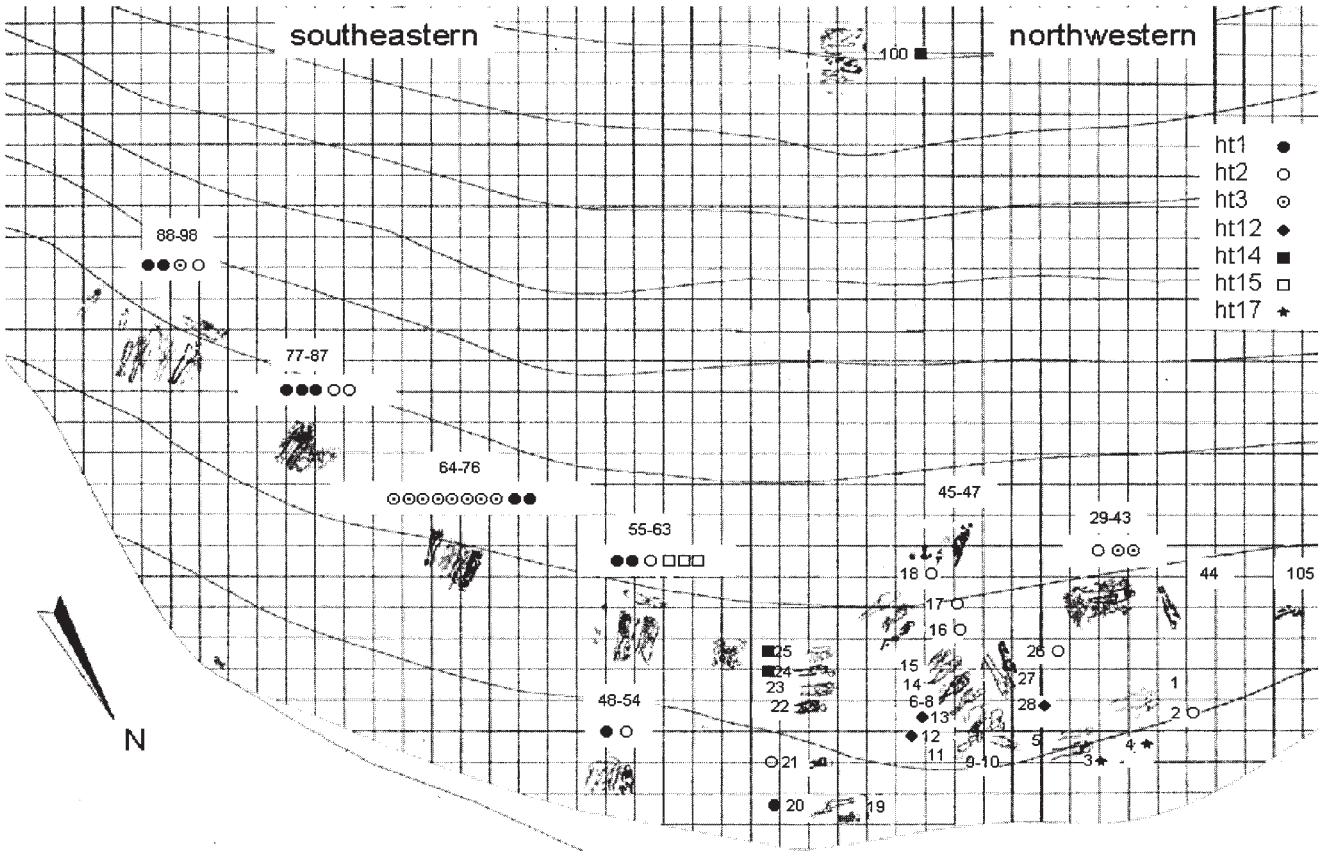

Fig. 2. Map of the cemetery of Aldaieta that includes distribution of the mtDNA haplotypes whose frequency was corrected as per kinship criteria.

TABLE 3. Frequencies of haplotypes and haplogroups in Aldaieta cemetery[1]

Haplotype	Corrected frequencies (%) ± SD[2]	Haplogroup	Corrected frequencies (%) ± SD[2]
ht1–2	44.12 ± 8.64	H	52.94 ± 8.7
ht3	8.82 ± 4.94		
ht4	2.94 ± 2.94	V	2.94 ± 2.94
ht5	2.94 ± 2.94	T	8.82 ± 4.9
ht6	2.94 ± 2.94		
ht7	2.94 ± 2.94		
ht8	2.94 ± 2.94	U5	8.82 ± 4.9
ht9	2.94 ± 2.94		
ht10	2.94 ± 2.94		
ht11	2.94 ± 2.94	U2	5.88 ± 4.1
ht12	2.94 ± 2.94		
ht13	2.94 ± 2.94	K	2.94 ± 2.94
ht14–15	8.82 ± 4.94	J	14.71 ± 6.2
ht16	5.88 ± 4.10		
ht17	2.94 ± 2.94	M1	2.94 ± 2.94

[1] Frequencies were corrected as per kinship criteria.
[2] Values are means ± SD (standard deviation).

between the size of the amplified fragment and the efficiency of the PCR (see Appendix). Moreover, we describe a wide variability of HVR-I sequences, which would not have occurred in the event of widespread contamination. Nine individuals were replicated in an independent laboratory (INTyCF), with coinciding results in the case of eight individuals, as the ninth was not replicated satisfactorily due to inhibition problems (see Appendix). Likewise, the cloning of an HVR-I fragment was undertaken in a subsample of 10 individuals, and in all cases the consensus sequence of the clones confirmed the result obtained by means of direct sequencing involving the PCR products (Alzualde et al., 2005).

Statistical analysis

Neutrality test. The value of Tajima's D for the individuals considered in Aldaieta is D = −1.509. Different simulations were carried out (each one of 10,000 iterations) to assess the statistical significance of this value: on the one hand, considering the uniformity of the mutation rates and constant population size, it was noted that the test is not significant. However, considering both the heterogeneity in mutation rates, and a population increase with different levels of growth (0.001 and 0.0001), the test is indeed statistically significant. These results suggest that the absence of neutrality in the mtDNA control region observed in the first simulation might be due to a population increase, although we cannot rule out the action of natural selection.

Nucleotide diversity. Calculation was made of nucleotide diversity in the ancient population of Aldaieta and likewise in the populations of Western Europe considered for comparison (Table 4). Although Aldaieta is the population with the least diversity value (0.0145

TABLE 4. Nucleotide diversity[1] in late ancient cemetery of Aldaieta[2] (6th–7th centuries AD) and in other populations from Western Europe

Population	Nucleotide diversity ± SD
Aldaieta	0.0145 ± 0.0087
Basques	0.0158 ± 0.0091
Cantabria	0.0185 ± 0.0105
Liabana	0.0205 ± 0.0115
Pas	0.0219 ± 0.0121
Galicia	0.0204 ± 0.0113
Andalusia	0.0270 ± 0.0145
Castile	0.0216 ± 0.0122
León	0.0203 ± 0.0114
Maragatos	0.0186 ± 0.0106
Catalonia	0.0216 ± 0.0120
France	0.0224 ± 0.0126
Portugal	0.0234 ± 0.0127
Germany	0.0216 ± 0.0119
Norway	0.0212 ± 0.0117
Great Britain	0.0226 ± 0.0124
Scotland	0.0233 ± 0.0127
Italy	0.0266 ± 0.0142
Etruria	0.0228 ± 0.0129

[1] Nei (1987).
[2] Considering haplotype frequencies corrected as per kinship criteria.

± 0.0087 SD), the present-day population of the Basque Country also presents a low diversity (0.0158 ± 0.0091), similar to that found in Aldaieta. Two further populations that present low values of diversity (below 0.02) are the neighboring population of Cantabria and the isolated population of Maragatos (in the Spanish province of León).

Shared haplotypes. The comparison of sequences had to be limited to nps 16092–16362 of HVR-I, and accordingly, the number of haplotypes from Aldaieta used for comparison was reduced from 17 to 15 (it is not possible to differentiate between ht14 and ht15, or between ht1 and ht2; see Table 2). Of these 15 haplotypes described in Aldaieta, three are unique (ht5, ht9, and ht11; not found in Genbank or among the more than 10,000 sequences used for the comparison), and a further six from Aldaieta (ht7, ht8, ht10, ht12, ht16, and ht17) are uncommon, i.e., they present a frequency in Western Europe of below 1% (Table 5). The six remaining haplotypes are widely distributed in present-day populations of Western Europe (ht1–2, ht3, ht4, ht6, ht13, and ht14–15) (Table 5). Of the 15 haplotypes described in Aldaieta, nine are haplotypes that are uncommon or unique in Europe. In order to verify whether this proportion of uncommon or unique haplotypes is to be expected in any sample of European populations, an analysis was made of the distribution of common and rare haplotypes in the populations of the Cantabrian fringe. Resampling of 34 individuals (the number of individuals from Aldaieta considered in the statistical analyses) was carried out, with 100 iterations. We observed that the frequency of the resampled haplotypes in the Cantabrian fringe is 47.9% ± 16.2% (for haplotypes that are uncommon in Europe) and 27.3% ± 15.5% (for haplotypes not described so far in other European populations). The frequencies of uncommon and unique haplotypes in Aldaieta are 40% and 20%, respectively, which fall within the range of values obtained in the resampling of populations on the Cantabrian fringe, whereby we may conclude that they do not stray from the values expected in the present-day populations of the same region.

Within the three haplotypes unique to Aldaieta (ht5, ht9, and ht11) (Table 5), ht5 was determined by the separate analysis of two dental samples in our laboratory, and in addition, a third sample was replicated at INTyCF, with the results coinciding in all cases. Furthermore, the fragment containing mutations 16,294 and 16,304 was cloned, which enables the result to be fully authenticated. The second haplotype unique to Aldaieta is ht9. The individual presenting this haplotype underwent duplicate analysis, with replication also being undertaken at INTyCF, and the result was authenticated by cloning of the fragment containing mutation 16,270. The third haplotype unique to Aldaieta is ht11, as described in an individual from Aldaieta who underwent duplicate analysis in our laboratory.

Among the haplotypes described in Aldaieta that are widely distributed throughout Western Europe (Table 5), there are the two haplotypes of haplogroup H (ht1–2, ht3), which account for 53% of the population of Aldaieta. Haplogroup H is also the most common one in all present-day populations of Western Europe. Regarding haplogroup V, a unique haplotype was found in Aldaieta (ht4, 2.94%) that is also common in all populations of Western Europe. Haplogroup V presents a frequency of 2.94% in Aldaieta, whereas the present-day populations of Western Europe with higher frequencies of haplogroup V are the present-day population of the Basque Country (11%) and those of the neighboring region of Cantabria (Pas, 16%; Cantabria, 15%; and Liebana, 11%). Ht6 (2.94%), ht13 (2.94%), and ht14–15 (the most common within haplogroup J, and which presents a frequency of 8.82% in Aldaieta) are also widely distributed throughout Western Europe.

The haplotypes of Aldaieta that are infrequent in Western Europe are ht7, ht8, ht10, ht12, ht16, and ht17 (Table 5). The haplotypes of the ancient population of Aldaieta that were found in the populations on the Cantabrian fringe are ht8, ht10, and ht16. Ht8 (2.94%) was found in an individual in the present-day population of the Basque Country, and in another in León (center-north of the Iberian Peninsula); a further five individuals with ht8 were found in the rest of Western Europe: two in Germany, two in France, and one in England (Table 5). Ht10 (2.94%) was only detected in three present-day individuals belonging to the neighboring population of Cantabria (one in Valle de Liebana, and two in Valle de Pas). Ht16 (5.88%) was found in 10 individuals among the 4,184 sequences in Western Europe (0.24%): half of them in neighboring populations of the Basque Country and Cantabria, and the other half spread throughout Western Europe (Table 5). Moreover, the haplotypes from Aldaieta that are related to non-peninsular populations are ht7, ht12, and ht17. Ht7 (2.94%) was only found in eight present-day individuals, located as follows: four in Eastern Europe, and the remainder in Italy, Syria, the African population of Nubia (Richards et al., 2000), and Azerbaijan (Quintana-Murci et al., 2004). Ht12 was found only in one individual among present-day populations, in the sample from England. It is present at Aldaieta in three individuals from the cemetery, two of them buried alongside each other, and the third perpendicular to them beside their heads; only one of them was considered for the statistical analysis (2.94%).

TABLE 5. Distribution in European, Asian, and African populations of haplotypes found in Aldaieta cemetery

Haplotypes	Haplogroup	Distribution
ht1–2	H	Widely distributed throughout Western Europe
ht3	H	Widely distributed throughout Western Europe
ht4	V	Widely distributed throughout Western Europe
ht6	T	Widely distributed throughout Western Europe
ht13	K	Widely distributed throughout Western Europe
ht14–15	J	Widely distributed throughout Western Europe
ht5	T	Unique to Aldaieta site
ht9	U5	Unique to Aldaieta site
ht11	U2	Unique to Aldaieta site
ht7	T	Eastern Europe (1%), Italy (<0.1%), Syria (1.4%), Nubia (1.2%), and Azerbaijan (2.5%)
ht8	U5	Basque Country (0.6%), León (1.6%), Germany (0.4%), France (0.6%), and Great Britain (1%)
ht10	U5	Liebana (1.4%) and Pas (2.4%)
ht12	U2	Great Britain (1%)
ht16	J	Basque Country (1.9%), Cantabria (1.1%), Liebana (1.4%), Portugal (0.2%), Castile (2.6%), Andalusia (0.9%), Germany (0.2%), and Scotland (<0.1%)
ht17	M1	Northwest African origin[1]

[1] See text for explanation of distribution of this haplotype.

The only non-Caucasian haplotype described in Aldaieta, ht17, belongs to haplogroup M1, whose origin was attributed to Eastern Africa (Quintana-Murci et al., 1999). The M1 lineage has a very scarce presence in Europe; only 13 of 4,184 sequences in Western Europe belong to this lineage (0.31%): eight in the Iberian Peninsula, four in Italy, and one in Scotland, and considering the whole of the European continent, its frequency is 0.11% (Richards et al., 2000). Apart from the motifs defining haplogroup M1, ht17 from Aldaieta contains a transition in position 16,185, characteristic of one of the three clusters described within M1, i.e., cluster M1c. This M1c lineage is the most common within M1 in populations of Northwestern Africa and the Canary Islands (Kivisild et al., 2004). Sequences coincident with ht17 from Aldaieta were found in five individuals belonging to the same lineage, M1c, from Northwestern Africa (two Berber speakers from Ghardaia (an isolated population in the north of Algeria), Corte-Real et al., 1996; one from Algeria, Plaza et al., 2003; one from the western Sahara, Rando et al., 1998; and one from the Canary Islands, Rando et al., 1998), and in one individual from the Eastern Mediterranean (Richards et al., 2000), which revealed a relationship between Aldaieta and Northwestern Africa to be addressed later.

DISCUSSION

Population affinities

Considering the fragment of mtDNA HVR-I between nucleotide positions 16,092–16,362, 15 haplotypes were described in Aldaieta, of which nine are not common to Western Europe or are unique to Aldaieta. Focusing on the six haplotypes described in Aldaieta that are infrequent in Europe, three (ht8, ht10, and ht16) are to be found in populations of the Basque Country or the adjacent population of Cantabria, with two of them (ht10 and ht16) having greater frequency in the region of the Cantabrian fringe, which reinforces the greater affinity between Aldaieta and the populations of those regions than with others in Europe. A similar interpretation was made of the fact that the highest frequencies of subhaplogroup U2 were found in Western Europe, in the populations of León (4.9%), Cantabria (2.27%), and Maragatos (2.04%), and in the ancient population of Aldaieta, with an even higher frequency (5.88%). Furthermore, Aldaieta has a characteristic that is shared with the present-day population of the Basque Country, i.e., the presence of basal lineages of subhaplogroup U5 and the absence of those derived (U5a1 and U5a1a) (Larruga et al., 2001), which suggests an in situ evolution of these lineages.

As regards haplogroup J, emphasis should be placed on the differences found between present-day Basques and ancient populations of the Basque Country. In the present-day population of the Basque Country, the frequency of haplogroup J is very low (Richards et al., 2000; Maca-Meyer et al., 2003a), whereas in Aldaieta, it is 14.7% (represented by three different J haplotypes), and in the prehistoric post-Neolithic Basque populations studied so far, its frequency oscillates around 16.7%, except in the population of Longar, where it is absent (Izagirre and de la Rúa, 1999). The haplotype that presents transitions 16,069–16,126 (ht14–15) is the most common haplotype within haplogroup J in Europe and also in Aldaieta. Nevertheless, it is absent in the present-day Basque population. This absence might be due to the extinction of the lineage due to genetic drift, as the probability that a lineage with a frequency similar to that observed in Aldaieta (8.82%) should disappear in a population with a stable size of 500 individuals over 75 generations is approximately 25% (Hartl and Clark, 1997). Accepting that haplogroup J is a lineage of Neolithic origin, the high frequency of this haplogroup in ancient populations of the Basque Country suggests a Neolithic influence in the Basque Country similar to that experienced by all other European populations, in contrast to the hypothesis suggesting that this impact was minimal in the Basque Country (Richards et al., 1996, 2000). We are aware that the sample size is limited; however, it is highly significant that three different lineages of haplogroup J are present at Aldaieta, and that the most common of these does not appear in the present-day population of the Basque Country. The case of haplogroup J, as with others addressed in Alzualde

et al. (2005), highlights the fact that the inferences made on the basis of present-day genetic data should take into account historic and prehistoric variations that may have taken place: data that may only be verified by means of the analysis of DNA recovered from skeletal remains.

Northwest African influence in the Iberian Peninsula

It was estimated that 10% of the mtDNA haplotypes and chromosome Y existing in the Iberian Peninsula is of African origin (Corte-Real et al., 1996; Larruga et al., 2001; Flores et al., 2000; Pereira et al., 2000; Bosch et al., 2001; Scozzari et al., 2001; Gonzalez et al., 2003; Alonso et al., 2005), specifically due to an influence of Northwest African origin. There are two hypotheses for explaining the Northwest African influence in the Iberian Peninsula. Certain authors attribute this influence to the Moorish occupation that began in AD 711 and ended with their expulsion in 1492 (Bosch et al., 2001). On the other hand, some authors, while accepting this hypothesis, furthermore propound a prehistoric relationship between the Iberian Peninsula and Northwest Africa (Pereira et al., 2000; Larruga et al., 2001; Maca-Meyer et al., 2003a; Brion et al., 2003; Flores et al., 2004). Maca-Meyer et al. (2003b), on the basis of the phylogeography of haplogroup U6, affirmed that this prehistoric relationship must have existed 10,000 years ago at most, and may be linked to the spread of Capsian culture, coming from the Near East, which at the beginning of the eighth millennium BC displaced the Iberomaurisians (populations of the Mahgreb) toward the Atlantic coast, whereby they reached the Canary Islands, Malinese Sahara (Ferembach, 1985), and in all probability the Iberian Peninsula (Maca-Meyer et al., 2003b). The presence was detected in Aldaieta of a lineage whose origin lies in Northwest Africa (M1c), and which was probably introduced into Europe from the south of the Iberian Peninsula, as the presence of haplogroup M1 in the rest of Europe is very scarce, with the greater part of Europe's M1 being concentrated in the Iberian Peninsula and Italy. The presence of this haplogroup in Aldaieta cannot be attributed to historical contact with the Moorish population, as the chronotypology of the assemblage found at Aldaieta cannot be attributed to the 8th century AD, the time when the Islamic occupation began. Consequently, the discovery of a lineage coming from NW Africa in the population of Aldaieta supports the existence of contacts between the Iberian Peninsula and Northwest Africa across the Straits of Gibraltar prior to the 8th century Moorish occupation.

Ancestry of Aldaieta

The discovery of the Aldaieta cemetery has cast doubts on certain historical interpretations of the isolation of the Basques, as the tendency is to look upon the Pyrenees as a barrier that once clearly separated the Iberian Peninsula from continental Europe. The Aldaieta cemetery questions this approach, because it reveals a funereal culture closely linked to the *Regnum Francorum* (Kingdom of the Franks). Following the definition of the Aldaieta cemetery as a stable settlement (Azkarate, 2004), the next question to resolve is whether the Northern Pyrenean cultural influence at Aldaieta has its corresponding biological influence. Accordingly, the analysis of mtDNA lineages performed in the present study indicates that the population of Aldaieta presents greater genetic proximity to the present-day population of the Cantabrian fringe than to Central European populations, such as Germany or France.

There is, however, one datum that might be significant. As was noted in the introduction, Aldaieta features two areas with major differences: the SE sector where there are group burials, with an abundance of grave goods, and on the other hand, the NW sector where there is a prevalence of burials in rows with personal possessions of lesser importance. These differences in terms of material culture lead one to consider factors of a social nature regarding the burial of individuals (Izagirre et al., 2005). It is highly significant, therefore, that the mitochondrial lineages in the SE sector are those most commonly found in present-day Europe, whereas the NW sector contains the haplotypes that associate Aldaieta with populations on the Cantabrian fringe. Nevertheless, the genetic differences between the two sectors are not statistically significant (exact test of population differentiation, $P = 0.58$; Raymond and Rousset, 1995). Furthermore, two individuals in a group burial in the SE revealed the existence of a mutation in the Y chromosome (haplogroup R1b3b; unpublished data), considered to originate in the Basque Country, where it now presents the highest frequency in Europe (7.1%, as opposed to 0.9% in the Iberian Peninsula; Alonso et al., 2005). Consequently, although we cannot altogether rule out the influence of some Trans-Pyrenean population at Aldaieta, it appears to have been minimal.

CONCLUSIONS

The genetic data obtained in this study, together with archaeological and anthropological data, suggest that the Aldaieta cemetery largely consists of individuals originating in the Basque Country who shared the funereal customs of the *Regnum Francorum*, although we cannot rule out that this was accompanied by a certain genetic component. Moreover, this paper highlights the importance of the genetic data recovered from past skeletal remains in terms of verifying hypotheses formulated solely on the basis of data involving present-day populations. On the one hand, the frequency of haplogroup J in ancient populations of the Basque Country suggests a Neolithic influence in this territory similar to that experienced by other European populations. On the other hand, the discovery in Aldaieta of a lineage of mtDNA coming from Northwest Africa supports the existence of contact between the Iberian Peninsula and Northwest Africa across the Straits of Gibraltar prior to the Moorish occupation in historical times. Finally, these results suggest that the ancient Basque population had biological contact with foreign populations. This evidence leads us to play down the importance of genetic isolation as the main factor contributing to the genetic peculiarities described in the present-day Basque population.

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APPENDIX. Additional information on each individual from historical site of Aldaieta[1]

Sample reference	No. of samples	Replicated	Quantified[2] 113 bp	Quantified[2] 287 bp	Cloned	Sequenced[3]	Subhaplogroup
B1	2						
B3	2					129-185-189-223-249-311	M1
B4	1				Yes	129-185-189-223-249-311	M1
B5	2						X
B12	1					051-129C-183-189-362	U2
B13	2					051-129C-183-189-362	U2
B14	2					069-126-278-366	J
B16	2					CRS	H
B18	2					CRS	H
B19	1				Yes	126-294-296-304	T2
B20	1					CRS	H
B24	1					069-126-390	J
B25	1					069-126-390	J
B28	1					051-129C-183-189-362	U2
B29-42 (individual 1)	2					069-126-278-366	J
B29-42 (individual 2)	1						J
B29-42 (individual 3)	2						J
B29-42 (individual 4)	1					362	H
B29-42 (individual 5)	1					172-189-192-270-311	U5
B43	2					CRS	H
B45	2				Yes	051-092-129C-192-362	U2
B46	1						J
B48	2					CRS	H
B48-53 (individual 1)	3					CRS	H
B48-53 (individual 2)	2					CRS	H
B55	2					069-126	J
B56	2					069-126	J
B58	2						
B59	1				Yes	CRS	H
B60	2					069-126	J
B61	3					224-311	K
B62	2					CRS	H
B63	2					CRS	H
B64	2				Yes	362	H
B65	2					362	H
B66	2					362	H
B67	2		50–500		Yes	362	H
B68	3	Yes	50–500		Yes	362	H
B69	2					362	H
B70	2						H
B71	2					362	H
B73	3					362	H
B75	3	Yes	50–500			CRS	H
B76	2					CRS	H
B77	3	Yes	50–500		Yes	126-266-274-294-304	T2
B78	3	Yes	50–500			CRS	H
B79	4	Yes	50–500			CRS	H
B85	2					CRS	H
B86	3	Yes	50–500		Yes	176-270	U5
B87	3	Yes	50–500			CRS	H
B89	1					CRS	H
B90	2					CRS	H
B92	2					126-189-294	T
B93	3	Yes	>3,000	<50	Yes	298	V
B100	2					069-126-390	J
B104	1					192-270	U5
Researcher 1						192-270-319	U
Researcher 2						CRS	H
Researcher 3						189	H
Researcher 4						111-223-290-319-362	Other
Researcher 5						126-271-294-296-304	T
Researcher 6						316	
Researcher 7						189	

[1] Lower part of table shows mtDNA HVR-I sequences of researchers who handled samples. "–" indicates that no positive results were forthcoming.
[2] In event of positive result, number of copies of DNA per microlitre is shown.
[3] In event of transition, indication is only made of nucleotide position that bears mutation numbered according to Anderson et al. (1981), subtracting 16,000; and in case of transversion, in addition to mutated nucleotide position, resulting nucleotide is indicated. CRS, Cambridge reference sequence.

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