

An mtDNA Analysis in Ancient Basque Populations: Implications for Haplogroup V as a Marker for a Major Paleolithic Expansion from Southwestern Europe

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Summary

mtDNA sequence variation was studied in 121 dental samples from four Basque prehistoric sites, by high-resolution RFLP analysis. The results of this study are corroborated by (1) parallel analysis of 92 bone samples, (2) the use of controls during extraction and amplification, and (3) typing by both positive and negative restriction of the linked sites that characterize each haplogroup. The absence of haplogroup V in the prehistoric samples analyzed conflicts with the hypothesis proposed by Torroni et al., in which haplogroup V is considered as an mtDNA marker for a major Paleolithic population expansion from southwestern Europe, occurring ~10,000–15,000 years before the present (YBP). Our samples from the Basque Country provide a valuable tool for checking the previous hypothesis, which is based on genetic data from present-day populations. In light of the available data, the most realistic scenario to explain the origin and distribution of haplogroup V suggests that the mutation defining that haplogroup (4577 *NlaIII*) appeared at a time when the effective population size was small enough to allow genetic drift to act—and that such drift is responsible for the heterogeneity observed in Basques, with regard to the frequency of haplogroup V (0%–20%). This is compatible with the attributed date for the origin of that mutation (10,000–15,000 YBP), because during the postglacial period (the Mesolithic, ~11,000 YBP) there was a major demographic change in the Basque Country, which minimized the effect of genetic drift. This interpretation does not rely on migratory movements to explain the distribution of haplogroup V in present-day Indo-European populations.

Received September 10, 1998; accepted for publication April 23, 1999; electronically published June 3, 1999.

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0002-9297/99/6501-0026\$02.00

Introduction

Molecular analyses have shown that most mtDNA mutations have accumulated sequentially along radiating maternal lineages that have diverged as human populations have colonized different geographic regions of the world. Studies based on sequence data from hypervariable segments of mtDNA, in conjunction with RFLP haplotype data from the entire mtDNA, have revealed the existence of European-specific haplogroups (H–K, T, V, and W), whereas other haplogroups (such as U and X) are shared between Europeans and Africans and between Europeans and modern Amerindians, respectively (Torroni et al. 1996, 1998).

In a recent paper, Torroni et al. (1998) analyzed the distribution and frequency of haplogroups V and H in a wide range of modern populations from Eurasia. On the basis of analysis of the frequency and extent of variation accumulated within haplogroup V, these authors suggested that this haplogroup is most likely to have originated 10,000–15,000 years before the present (YBP) in a region covering the northern part of the Iberian Peninsula and the southwestern part of France. Similarly, they proposed that haplogroup V might constitute a marker of a major Paleolithic expansion from southwestern Europe, which took place after the Last Glacial Maximum. Thus, the Paleolithic population of southwestern Europe would have contributed extensively to the mitochondrial gene pool of all central and northern European populations, including the Saami and the Finns.

Interdisciplinary studies involving mtDNA sequences, RFLP data, and archeological findings can provide a better insight into population history (Bertranpetit et al. 1995; Richards et al. 1996). Still, several points remain to be resolved satisfactorily. At the genetic level, one of the main difficulties is the estimation of mutation rates, which vary considerably in different regions of the mitochondrial genome (Howell et al. 1996; Mumm et al. 1997; Parsons et al. 1997). Archeologically, hypotheses based on migrational events (Otte 1990) are losing ground, since it has become difficult to sustain the idea that the presence of similar artifacts in distant popula-

tions can always be interpreted in migrational terms (Clark and Lindly 1991). Thus, despite the great many recent theoretical advances in population genetics (Slatkin and Hudson 1991; Rogers and Harpending 1992; Harpending et al. 1993), the possibility of extracting and characterizing DNA from the remains of prehistoric humans themselves constitutes a unique and highly valuable contribution to the debate on the origin of the genetic diversity of human populations.

In this study we report the frequency of mtDNA haplogroups in four prehistoric populations of the Basque Country that are distributed over different regions of the Basque area (fig. 1). Here, we will focus on the analysis of haplogroup V in prehistoric Basque populations; this will enable us to discuss the recently proposed value of this haplogroup as a marker for a major Paleolithic expansion from southwestern Europe (Torrioni et al. 1998).

Subjects and Methods

The sample considered in our work is large (121 teeth) and covers different regions of the Basque Country (fig. 1). Two of the sites—San Juan Ante Portam Latinam (SJAPL) (Araba) (Etxeberria and Vegas 1988) and Longar (Nafarroa) (Armendariz and Irigarai 1995)—are located in the southern part of the Basque Country, whereas the others—Pico Ramos (Bizkaia) (Zapata 1995) and Urratxa (Bizkaia) (Muñoz and Berganza 1997)—lie in the Cantabrian fringe. Habitation of these sites has been chronologically dated as having occurred between the Neolithic and the Bronze Age. Radiocarbon dates of the occupation periods, as well as the geographic location of the prehistoric sites analyzed in this work, are presented in figure 1.

Before molecular analysis, all the fossil teeth recovered were classified by an expert in dental anthropology, according to type (incisors, canines, premolars, and molars), side, and jaw (upper/lower). This allowed us to choose the single dental piece that maximized the sample size and thus to avoid the possibility of analyzing the same individual twice. Genetic analysis of this prehistoric material was performed only on intact permanent teeth not showing any cracks or caries, so that the dental pulp was preserved from possible contaminants. For that reason, only 121 canine teeth from a total of 1,099 were used for analysis. The most suitable tooth was selected at each site, on the grounds of frequency and conservation; for instance, at site SJAPL (Araba) it was the right maxillary canine, and 63 intact teeth were selected from the total of 232 right-maxillary canines. Additionally, the selected teeth were subjected to a sterilization process based on acids (Ginther et al. 1992), a technique with a high depurinating power capable of inactivating any potential exogenous contaminating DNA present on the surface of the teeth. The roots of the teeth were

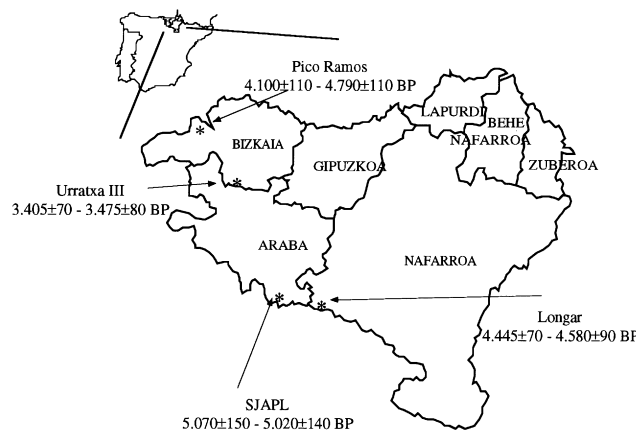


Figure 1 Geographic map of Basque Country, showing locations and radiocarbon dates of periods of occupation of prehistoric sites.

removed by a saw in a sterile environment and then were subjected to a DNA-extraction procedure.

DNA extraction was performed according to the phenol-chloroform method (Hagelberg and Clegg 1991). In brief, the roots of the teeth were incubated overnight at 37°C, with agitation in a lysis buffer (0.5 M EDTA pH 8.0–8.5, 200 µg proteinase K/ml, 0.5% SDS, 50 mM Tris-HCl), followed by one extraction with phenol and one final extraction with chloroform. The DNA extract obtained was concentrated and purified by means of Centricon-30 columns, down to a volume of 300 µl, which was stored at –20°C, as three aliquots of 100 µl each.

In each extraction round, a control sample (a sample with no tissue) was also included and was processed similarly. The DNA extraction and amplification of the ancient DNA (aDNA) samples were performed in a dedicated laboratory in which no work with modern DNA had been performed. All the materials and work surfaces used were routinely washed with bleach and alcohol (Prince and Andrus 1992) and were irradiated with UV light of short wavelength (Fox et al. 1991).

We typed these prehistoric samples in order to identify those haplogroups previously defined by Torrioni et al. (1996, table 3) in modern DNA. We typed those nucleotide positions strictly necessary to correctly identify the nine haplogroups characteristic of Caucasians: 7025 *AluI*, 4577 *NlaIII*, 8249 *AvaII*, 9052 *HaeII*, 8994 *HaeIII*, 12308 *HinfI*, and 13704 *BstNI*. Table 1 shows the primers used to amplify the fragments containing the mutation that defines each haplogroup, as well as the size of both the amplicon and the restriction fragments. We designed all these primers ourselves, except the reverse primer used to detect the mutation in nucleotide 12308 by *HinfI*. PCR amplifications were performed in a final volume of 35 µl, composed of the following: 1 × final

Table 1**Sequence of Primers, Length of Each Amplification Fragment and Corresponding Restriction Fragments, and Annealing Temperature of Each Pair Of Primers**

mtDNA SITE AND PRIMER	PRIMER SEQUENCE ^a (5'→3')	LENGTH (bp)		ANNEALING TEMPERATURE (°C)
		Amplicon	Restriction Fragment	
7025 <i>AluI</i> :				
L6958	5'-CCT GAC TGG CAT TGT ATT-3'			
H7049	5'-tgt aaa acg acg gcc agt TGA TAG GAC ATA GTG GAA GT 3'	109	68 + 41	58
8249 <i>AvaII</i> :				
L8191	5'-AAC CAC AGT TTC ATG CCC AT-3'			
H8312	5'-TAA GTT AGC TTT ACA GTG GGC T -3'	121	58 + 63	59
13704 <i>BstNI</i> :				
L13626	5'-CCT AAC AGG TCA ACC TCG CT-3'			
H13729	5'-tgt aaa acg acg gcc agt CTG CGA ATA GGC TTC CGG CT-3'	122	80 + 42	64
9052 <i>HaeII</i> :				
L9003	5'-CCT AAC CGC TAA CAT TAC-3'			
H9105	5'-tgt aaa acg acg gcc agt GAA GAT GAT AAG TGT AGA GG-3'	120	54 + 66	51
8994 <i>HaeIII</i> :				
L8908	5'-TTC TTA CCA CAA GGC AC A CC-3'			
H9033	5'-AGG TGG CCT GCA GTA ATG T-3'	126	88 + 38	65
12308 <i>HinfI</i> :				
L12216	5'-CAC AAG AAC TGC TAA CTC ATG C-3'			
H12338	5'-ATT ACT TTT ATT TGG AGT TGC ACC AAG ATT-3'	123	93 + 30	55
4577 <i>NlaIII</i> :				
L4519	5'-CAC TCA TCA CAG CGC TAA GC-3'			
H4620	5'-TGG CAG CTT CTG TGG AAC-3'	120	62 + 58	55

^a The lowercase portions of some primers represent a tail added to enlarge the length of the amplified product.

concentration of PCR reaction buffer (Perkin-Elmer), 2 mM MgCl₂, 20 μg BSA, 200 μM each dNTP, 0.4 μM of each primer, 2 units of *Taq* polymerase (Perkin-Elmer), and 10 μl of a 1:15 dilution of the DNA extract. This reaction mixture was cycled as follows: 1 cycle at 94°C for 5 min; 40 cycles of 94°C for 15 s, 51°–65°C (depending on the primer pair; see table 1) for 5 s, and 72°C for 10 s; and 1 final extension cycle at 72°C for 5 min. At this stage we also used another control to check for contamination. It consisted of an amplification reaction mixture with no DNA extract, called the “amplification control.” The amplification of the samples and the absence of contamination in the controls were ascertained by PAGE and silver staining.

Subsequent analysis of the mutations that define each haplogroup was performed by cleavage with the corresponding restriction enzymes. Typing was performed by means of PAGE and silver staining. For each sample, both the digested and undigested PCR products were run in adjacent lanes. Only those samples negative for the extraction and amplification controls were subjected to restriction analysis.

Contamination during the excavation of the skeletal remains is more difficult to detect. The best way to deal with this is by the simultaneous analysis of two independent samples from the same individual. However, in the prehistoric sites analyzed in our study it was not

always possible to obtain duplicate samples (when there were collective burials, we could not always identify two skeletal remains as belonging to the same individual). Only in those cases in which the tooth analyzed was articulated in the maxilla with other teeth was it possible to perform a duplicate analysis with a second independent sample (tooth) from the same individual. It was possible to perform this check on only 12 samples, whose haplotypes were determined (by analysis of seven polymorphic restriction sites). Because the number of samples on which duplicate analysis was possible was so low, we analyzed 92 left femurs from the same sites, to double-check the results obtained in dental samples. For these bone samples (which it was not possible to associate with particular dental remains), only sites 4577 *NlaIII* and 8249 *AvaII* were analyzed. The same digestion pattern was found in the bone samples as was seen in the teeth—that is, the presence of cleavage for 4577 *NlaIII* and the absence of cleavage for 8249 *AvaII*.

Additionally, in the case of the haplogroups defined by the absence of a particular restriction site, we double-checked the typing by both positive and negative restriction of other linked sites that characterize those haplogroups (Torroni et al. 1996, table 3). Thus, in the case of haplogroup H, for instance, which is characterized by the absence of restriction at 7025 *AluI*, typing was confirmed by positive restriction of 4577 *NlaIII*, 8994

*Hae*III, 9052 *Hae*II, and 13704 *Bst*NI and by negative restriction of 8249 *Ava*II and 12308 *Hinf*I.

Results

We studied mtDNA sequence variation in 121 dental samples from four prehistoric Basque sites, using RFLP analysis (Izagirre 1998). The main problem in the analysis of aDNA is the possibility of contamination during either excavation or the manipulation of samples in the laboratory. Such contamination can be revealed by the use of controls at the DNA-extraction and -amplification stages. Thus, samples showing contamination in the extraction control were discarded. If, however, the contamination appeared only in the amplification control, then new aliquots of all the reagents and a thorough cleaning of all the work surfaces sufficed to eventually obtain an uncontaminated amplification control. Therefore, only those samples showing a negative amplification pattern (i.e., without any visible trace of a specific amplified product) in the control reactions were subjected to restriction analysis. Following these criteria, we were able to successfully type 97% of the dental samples. The amplification efficiency in the case of the bone samples analyzed in parallel, to confirm the results, is 76.1%, much lower, as has been described by others (DeGusta and White 1996; Zierdt et al. 1996). Although the amplification efficiency obtained in our study may be considered high, it must be taken into account that only the best-preserved teeth in the sample (11% of the total dental sample) were analyzed. On the other hand, similar rates of success (80%–100%) have been found by other authors using the same method of analysis (restriction enzymes) (Stone and Stoneking 1993; Lalueza et al. 1997; Parr et al. 1996). However, when the analysis performed comprises the sequencing of the amplified fragment, the rate of success decreases to 55%–70% (Hagelberg and Clegg 1993; Oota et al. 1995; Ribeiro-Dos-Santos et al. 1996).

The results obtained for the prehistoric sites analyzed in the present study are shown in table 2. The frequency distribution of the haplogroups is summarized in figure 2 (in which results from the site of Urratxa have been excluded, owing to the small sample size). Haplogroup

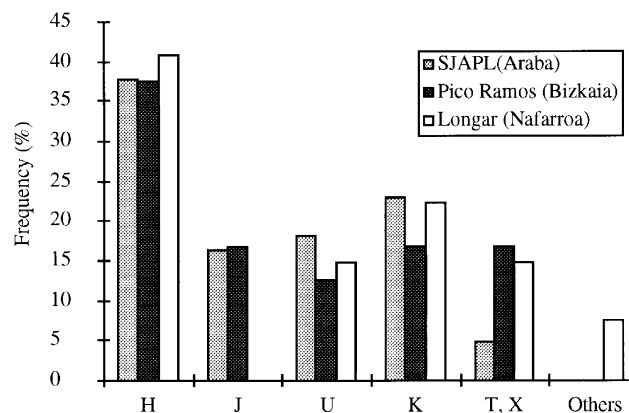


Figure 2 Distribution of frequency (%) of mtDNA haplogroups detected at three prehistoric sites in the Basque Country: SJAPL (Araba), Pico Ramos (Bizkaia), and Longar (Nafarroa).

H showed the highest frequency in all the prehistoric sites whose habitation corresponded to a period between the Neolithic and the Bronze Age (fig. 1). Haplogroup H is the most common haplogroup in all present-day European populations and reaches its highest frequency (40%–60%) in western and northern Europe (Torroni et al. 1998). The absence of haplogroups I, W, and V in all the prehistoric samples is noteworthy. Haplogroup V is defined by the absence of the 4577 *Nla*III site; haplogroup I is defined by the presence of the 8249 *Ava*II site, and, finally, haplogroup W is defined by the absence of the 8994 *Hae*III site. All of our prehistoric dental samples yielded positive restriction in the 4577 *Nla*III and 8994 *Hae*III sites and negative restriction in the 8249 *Ava*II site, which indicates the absence of haplogroups V, W, and I in our samples. To corroborate the results obtained with the dental samples, we analyzed, in parallel, 92 DNA extracts from bones (femurs), for these same sites. These analyses of bone samples confirmed the results for dental samples, since both showed the same typing.

Both modern Basques (A. Torroni, personal communication) and prehistoric Basques show absence of haplogroups I and W. However, the situation for haplogroup V differs; in modern Basques, there is considerable var-

Table 2

Absolute Frequencies of mtDNA Haplogroups Obtained at Four Prehistoric Sites in Basque Country

PREHISTORIC SITE	NO. IN HAPLOGROUP									Not Determined
	H	I	J	K	U	V	W	T+X	Other ^a	
SJAPL (Araba) (<i>n</i> = 63)	23	...	10	14	11	3	...	2
Pico Ramos (Bizkaia) (<i>n</i> = 24)	9	...	4	4	3	4
Urratxa (Bizkaia) (<i>n</i> = 5)	2	...	1	...	2
Longar (Nafarroa) (<i>n</i> = 29)	11	6	4	4	2	2

^a Haplotypes not corresponding to any of the nine Caucasian haplogroups described by Torroni et al. (1996).

Table 3**Frequency of Haplogroup V in Three Samples from Present-Day Basque Population and in Prehistoric Basque Sample**

Sample	Frequency ^a (%)	Marker	Reference
Present-day Basques:			
Gipuzkoa:			
Gipuzkoa (<i>n</i> = 50)	20.0	RFLPs	Torroni et al. (1998)
Gipuzkoa (<i>n</i> = 45)	11.1	D-loop	Bertranpetit et al. (1995)
Bizkaia and Araba (<i>n</i> = 61)	3.3	D-loop	Côrte-Real et al. (1996)
Prehistoric Basques	.0	RFLPs	Present study

^a Calculated by Torroni et al. (1998).

iation in the reported frequencies for this haplogroup (see table 3), the range being 3.3%–20%, depending on the sample considered. This point is discussed below, in the context of the absence of this haplogroup in our prehistoric samples (121 teeth and 92 femurs).

The levels of genetic diversity in the prehistoric samples are similar to those described in modern populations, excluding the modern Basques, which show the lowest values (table 4). This, apart from its own intrinsic value, also corroborates our results from prehistoric remains and decreases the possibility that the same individual will be analyzed more than once, as might happen if an error were made in the classification of teeth.

Discussion

Contamination by exogenous DNA represents a major limitation when one is dealing with aDNA, since, as a result of its higher concentration and quality, its amplification is favored over that of the endogenous DNA in the sample (Pääbo et al. 1989; Hagelberg 1994). Some authors have dealt with this problem by trying to establish a series of measures to double-check their results (Krings et al. 1997). The test that confers the greatest level of robustness is duplicate analysis by two independent laboratories. However, this is not always feasible (especially in view of the need to obtain additional funding). Thus, in most of the works published to date, confirmation is obtained by analysis of different samples from the same individual (Pääbo 1989; Handt et al. 1994, 1996; Stone and Stoneking 1998), although even then it is not always possible.

Another frequent problem when one is working with degraded DNA is the appearance of nonspecific PCR products that may induce errors in the determination of both the size of the amplified product and the presence/absence of cleavage. This is more serious when typing is determined on the basis of absence of digestion, because if the amplified product does not match the specific fragment there will never be a positive digestion. In this study, we therefore considered it necessary to determine the haplotype of each sample (i.e., the pattern of absence/presence of cleavage at each of the seven polymorphic

sites analyzed) before considering a result as valid, a procedure described by Torroni et al. (1996, table 3).

The reliability of the results described here is supported by several points:

1. All the dental prehistoric samples analyzed yielded positive restriction of the 4577 *Nla*III site. Although the absence of a target sequence for this site (an absence that characterizes haplogroup V) could be explained by post-mortem modification of any of the nucleotides defining it, the opposite (the creation of a restriction site by post-mortem modification) is considerably less likely.
2. None of the bone samples analyzed in parallel belonged to haplogroup V.
3. We have been able to detect haplogroup V in bone samples from the present-day Basque population (in samples 50–60 years old).
4. The levels of genetic diversity detected in the prehistoric populations are similar to those described in modern populations.

Therefore, we can consider that the absence of haplogroup V in the prehistoric Basque samples cannot be attributed to methodological problems.

The genetic data on nuclear markers and mtDNA sequences of present-day populations suggest two possible explanations for the history of European populations. Ammerman and Cavalli-Sforza (1984), analyzing the

Table 4**mtDNA Diversity, on the Basis of Frequencies of Haplogroups in Modern European Populations and in Three Prehistoric Basque Samples**

Source	Genetic Diversity ^a
Modern populations:	
Tuscans (Italy)	.769
Finns (Finland)	.785
Basques (Gipuzkoa)	.713
Swedes (Sweden)	.762
Prehistoric Basque samples:	
SJAPL (Araba)	.756
Pico Ramos (Bizkaia)	.793
Longar (Nafarroa)	.762

^a Calculated according to the method published by Nei (1987).

gene frequencies of several nuclear markers, proposed a demic diffusion model (i.e., a slow expansion of Neolithic farmers into smaller groups of local Mesolithic populations in Europe), whereas other authors (Sajantila et al. 1995; Richards et al. 1996; Sykes et al. 1996) have suggested that the origin of the diversity of mtDNA sequences in present-day Europeans lies in the Upper Paleolithic, coinciding with the expansion of anatomically modern humans, who, according to genetic data, did not mix with Neanderthals (Richards et al. 1996; Krings et al. 1997). Most mtDNA data are explained in the context of one of these two viewpoints, but some authors have suggested other colonizations to explain the distribution of some mtDNA haplogroups; thus, Torroni et al. (1998) have suggested an expansion of haplogroup V ~10,000–15,000 YBP, from a point of origin in southwestern Europe. This last hypothesis clashes with the aDNA data obtained in the present work, in which, in a sample of 121 subjects from various prehistoric sites in the Basque Country, we have found no subjects belonging to haplogroup V.

We can invoke different hypotheses to explain this discrepancy between the described frequency for haplogroup V in modern Basques and its absence in prehistoric samples:

1. The presence of heterogeneity in extant Basques is a debated point. Although some authors propose the existence of heterogeneity (Aguirre et al. 1991; Calderón et al. 1993; Manzano et al. 1996; Iriondo 1998), others have argued against this hypothesis, claiming an excessive fragmentation in the sampling of this population (Calafell and Bertranpetit 1994). With regard to mtDNA, the data available are scanty. As for mtDNA RFLPs, we have available, for comparison, data from only 50 individuals from the province of Gipuzkoa (Torroni et al. 1998), in which haplogroup V displays a frequency of 20%. However, we can increase the sample to a certain extent, if we infer, from the D-loop sequence data, the corresponding associated RFLPs, as has been proposed by Torroni et al. (1996). If we consider such inferred RFLPs, then we have available to us two more Basque samples, in which we can observe a substantial variation in the frequencies for haplogroup V (table 3): thus the sample analyzed by Bertranpetit et al. (1995), composed of 45 individuals from Gipuzkoa, displays a frequency of 11.1% for haplogroup V, and the sample of 61 individuals from Bizkaia and Araba that was studied by Côte-Real et al. (1996) shows a frequency of 3.3%.

However, in our opinion these data are insufficient to support the hypothesis of heterogeneity in modern Basque populations. First, the correlation procedure employed to infer RFLPs from D-loop sequence data, which has been proposed by Torroni et al. (1996), may be misleading: on one hand, the correlation is established on the basis of a small number of sequences (in the case of haplogroup V, only eight samples were jointly ana-

lyzed for D-loop sequence and general RFLP haplotypes) (Torroni et al. 1998); on the other hand, we have detected inconsistencies in the correlation—for instance, not all the samples that, according to RFLP analyses, belong to haplogroup H carry, in the sequence of the control region, the mutation (i.e., nucleotide A in position 73 in segment II of the control region) that, according to Torroni et al. (1996), defines this haplogroup. Second, we must bear in mind that mtDNA is just a single nonrecombining locus. Thus, in our opinion, a more robust procedure as well as more-extensive sampling and comprehensive study would be required to come to grips with Basque heterogeneity.

If Basque heterogeneity has ever existed, we might think that genetic drift has played such a role that haplogroup V has reached a high frequency in one or a few subpopulations from the Basque Country and that it is absent in others. This would agree with what we have found in our study, but it would make Torroni et al.'s (1998) hypotheses on the expansion of haplogroup V difficult to sustain.

Torroni et al. (1998) also have found support for their hypothesis in the archeological interpretation proposed by Otte (1990) for the Upper Paleolithic in Europe. Otte claims that the similarity of the Magdalenian industry (10,000 YBP) in southwestern France and other regions, such as Belgium, the Rhine and the Swiss regions, Moravia, and Poland, is explained by a major population expansion from southwestern to central Europe during the end of the Second Pleniglacial. However, attempts to explain the similarities, in both art and technology, between the northern and the southern bounds of the Magdalenian culture come up against serious drawbacks when migration/diffusion scenarios are considered, because humans do not seem to expand from one region to another unless there is a cause (Clark and Lindly 1991). In this context, in the case of the alleged “late Paleolithic population expansion from southwestern to northeastern Europe,” we see neither any potential “push” factors in the proposed haplogroup V homeland nor any “pull” factors (attractors) in the northeastern European destination. What most likely happened was a more generalized process of occupation of the territory from southern to northern Europe, related to the ecological changes that took place at the end of the Pleistocene and the Paleolithic (~12,000–10,000 YBP). In the postglacial period—that is, after 10,000 YBP—further changes in both the landscape and ways of subsistence enabled humans to adapt to a wide variety of habitats and to establish extensive social networks throughout wide areas, especially middle and northern Europe (Straus 1996), areas that until then had been sparsely inhabited by small groups of Paleolithic hunters scattered throughout vast territories. At that moment, Magdalenian culture was thriving throughout Europe, with close similarities between the northern and the southern regions of the continent.

2. A major limitation in mtDNA-based phylogenetic analyses lies in the lack of an accurate estimate of the divergence rates. Currently, divergence rates of

7%–22%/million years (Myr) are being used for the noncoding region (Pesole et al. 1992; Tamura and Nei 1993; Horai et al. 1995). This wide range of divergence rates leads to a high degree of variability in estimates of divergence times; a clear example is the calculation of the divergence of modern humans from an African ancestor, which shows values in the range of 0.2–0.6 Myr (Penny et al. 1995; Wills 1995; Ruvolo 1996).

Studies based on families (Howell et al. 1996; Parsons et al. 1997) lead to estimated divergence rates (260%/Myr [Howell et al. 1996]) much higher than those inferred by reference to the divergence between humans and chimpanzees. However, although substitution rates and mutation rates can be equated from a strictly neutral point of view, selection may play a role, by eliminating some of those mutations detected in pedigrees, before such mutations become fixed in the population (Howell et al. 1996; Howell and Mackey 1997). Therefore, in evolutionary terms, the figure could be lower. These discrepancies require the analysis of a greater number of families, which would allow the identification of hot spots, the estimation of mutation rates for specific nucleotide positions, and the effect of heteroplasmy in both the appearance and the fixation of new mutants. On the other hand, it is also important to take into account the noise produced by high mutation rates, as well as demographic aspects such as differential migrational models for men and women, when it comes to establishing the evolutionary history of genes and populations (Cavalli-Sforza and Minch 1997).

Therefore, the date of origin of haplogroup V might be more recent than that proposed by Torroni et al. (1998), which would account for its absence in ancient samples and would cast doubt on the idea that these authors have proposed with regard to the Paleolithic expansion.

3. The last explanation that could be used to account for the discrepancy between the described frequency for haplogroup V in modern Basques and its absence in prehistoric samples is that immigration of people bearing haplogroup V occurred <4,000 YBP (i.e., the age of the youngest site analyzed in the present work [Pico Ramos, Bizkaia]).

In this regard, a recent hypothesis argues for a process of replacement to explain the origin of the Basques (Calderón et al. 1998). It suggests that the hunter-gatherers who lived in what is now the Basque region were replaced ~5,000–5,500 YBP by a small Neolithic group from the northern Caucasus. However, this population movement from the Caucasus fails to explain the existence of haplogroup V in the present-day Basque population, since the frequency of this haplogroup in the present-day population of the northern Caucasus is 0% (Torroni et al. 1998). It has been claimed that, after the Neolithic, subsequent expansions and migrations into Europe seem to have had only minor genetic impact (Cavalli-Sforza et al. 1994).

ancient samples is a unique and valuable tool for checking the conclusions based on genetic analysis of modern populations. In light of our results concerning mtDNA variation in prehistoric Basques, we consider Torroni et al.'s (1998) hypothesis to be hasty; on the one hand, the mutation-rate issue demands caution when divergence times are being estimated; on the other, the samples from extant populations analyzed up to the present show certain limitations: for instance, no results are available for haplogroup V in southwestern France, and, with regard to the Basque samples from the Iberian Peninsula, frequencies vary within the range of 3.3%–20% (table 3). Besides, in our opinion, the interpretation of the archaeological record from a typological point of view clashes with more-acceptable views of culture as a complex system whose interpretation should be framed in an ecological perspective. From this point of view, the “colonization” of the northern environments in Europe after the climatic amelioration that took place after the end of the Second Pleniglacial can be better described as part of a spatially generalized process of niche expansion that resulted in the settlement of a wide range of distinct environments.

In light of the presently available data on the distribution of mtDNA haplogroups in extinct and extant human populations, mutation rates and archaeological findings (references here cited), a plausible explanation for the genetic data here reported might be that genetic drift is responsible for the significantly different frequencies described for haplogroup V in the different samples from the Basque Country that have been analyzed, with the figure for Gipuzkoa (20%) being one of the highest in Europe. In this context, this mutation's rise to significant frequency must have taken place at a time when the effective population size was small enough to allow genetic drift to have a significant effect. This would date to pre-Mesolithic periods (~11,000 YBP), because from then onward there is a considerable widening of the settled areas in the Basque Country, along with both a diversification in the subsistence patterns and a climatic amelioration, leading to a substantial demographic change versus earlier periods (de la Rúa 1995). This point of view is compatible with the attributed date for the origin of the mutation that defines haplogroup V (4577 *NlaIII*), but it does not need to resort to migrationist models to explain this mutation's distribution in Indo-European populations.

Acknowledgments

This work was supported by project “Aportación de la biología molecular al estudio de la evolución biológica de la población del País Vasco” (Diputación Foral de Vizcaya, Diputación Foral de Alava, Fundación Caja Vital, and BBK) and

This work proves that direct analysis of the DNA of

by project GN 154.310-0001-94 (Gobierno de Navarra and U.P.V./E.H.U.). We also thank two anonymous reviewers for their comments on an earlier version of the manuscript.

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