

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 (Torroni 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.

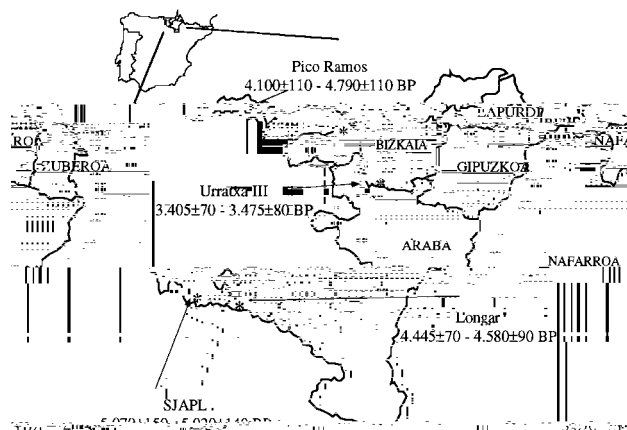


Figure 1 Geographic map of Basque Country, showing locations and radiocarbon dates of periods of occupation of prehistoric sites.

Before analysis, the samples were cleaned and the DNA was extracted using the method of Birnboim and Doly (1979). The DNA was amplified by PCR using the primers described by Harpending et al. (1993). The PCR products were digested with *Hpa*I and *Hha*I and the fragments were separated by electrophoresis on 8% polyacrylamide gels. The bands were stained with ethidium bromide and visualized under short wave UV light. The results were analyzed using the software program GeneAmp 3.0 (Applied Biosystems).

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	8761634376700(B)9314107719207D(97)216722071607110(A)302703T10D)59641069)116(61)39076(65)15 u
--------------------------	---

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

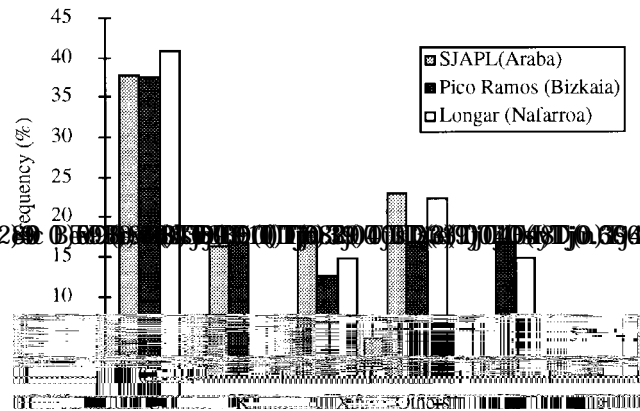


Figure 2 Distribution of frequency (%) of mtDNA haplogroups detected at three prehistoric sites in the Basque Country: SJAPL (Araba), Pico Ramos (Bizkaia), and Longar (Nafarroa).

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) (n = 63)	23	...	10	14	11	3	...	2
Pico Ramos (Bizkaia) (n = 24)	9	...	4	4	3	4
Urratxa (Bizkaia) (n = 5)	2	...	1	...	2
Longar (Nafarroa) (n = 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			

gene frequencies of several nuclear markers, proposed a demic diffusion model (Tjebk et al. 2001).

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 archa (S119074400T1a

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.

References

- Aguirre A, Vicario A, Mazón LI, Estomba A, Martínez de Pancorbo M, Arrieta Picó V, Perez Elortondo F, et al (1991) Are the Basques a single and a unique population? *Am J Hum Genet* 49:450-458
- Ammerman AJ, Cavalli-Sforza LL (1984) *The Neolithic transition and the genetics of populations in Europe*. Princeton University Press, Princeton
- Armendariz J, Irigarai S (1995). La arquitectura de la muerte: el hipogeo de Longar (Viana, Navarra), un sepulcro colectivo de 2.500 AC. Centro de Estudios Tierra-Estella, Lizarraldeko Ikastetxea, pp 1-36
- Bertranpetit J, Sala J, Calafell F, Underhill PA, Moral P, Comas D (1995) Human mitochondrial DNA variation and the origin of the Basques. *Ann Hum Genet* 59:63-81
- Calafell F, Bertranpetit J (1994) Principal component analysis of gene frequencies and the origin of Basques. *Am J Phys Anthropol* 93:201-215
- Calderón R, Vidales C, Peña JA, Pérez-Miranda A, Dugoujon J-M (1998) Immunoglobulin allotypes (GM and KM) in Basques from Spain: approach to the origin of the Basque population. *Hum Biol* 70:667-698
- Calderón R, Wentzel J, Roberts DF (1993) HLA frequencies in Basques in Spain and in neighbouring populations. *Ann Hum Biol* 20:109-120
- Cavalli-Sforza LL, Menozzi P, Piazza A (1994) *The history and geography of human genes*. Princeton University Press, Princeton
- Cavalli-Sforza LL, Minch E (1997) Paleolithic and Neolithic lineages in the European mitochondrial gene pool. *Am J Hum Genet* 61:247-251
- Clark GA, Lindly JM (1991) On paradigmatic biases and Paleolithic research traditions. *Curr Anthropol* 32:577-587
- Côrte-Real HB, Macaulay VA, Richards MB, Hariti G, Issad MS, Cambon-Thomsen A, Papiha S, et al (1996) Genetic diversity in the Iberian Peninsula determined from mitochondrial sequence analysis. *Ann Hum Genet* 60:331-350
- DeGusta D, White TD (1996) On the use of skeletal collections for DNA analysis. *Ancient Biomol* 1:89-92
- de la Rúa C (1995) La historia del poblamiento del País Vasco desde una perspectiva antropológica. In: Bertranpetit J, Vives E (eds) *El passat dels pirineus des de'una perspectiva multidisciplinaria*. Muntanyes I Poblacio, Andorra, pp 301-316
- Etxeberría F, Vegas JI (1988) ¿Agresividad social o guerra durante el Neo-eneolítico en la cuenca media del Valle del Ebro? a propósito de San Juan Ante Portam Latinam (Rioja Alavesa). *Munibe (Antropol-Arkeol)* 6:105-112
- Fox JC, Ait-Khaled M, Webster A, Emery VC (1991) Eliminating PCR contamination: is UV irradiation the answer? *J Virol Methods* 33:375-382
- Ginther C, Issel-Tarver L, King MC (1992) Identifying individuals by sequencing mitochondrial DNA from teeth. *Nat Genet* 2:135-138
- Hagelberg E (1994) Mitochondrial DNA from ancient bones. In: Herrmann B, Hummel S (eds) *Ancient DNA: recovery and analysis of genetic material from paleontological, archaeological, museum, medical, and forensic specimen*. Springer-Verlag, New York, pp 195-204
- Hagelberg E, Clegg JB (1991) Isolation and characterization of DNA from archaeological bone. *Proc R Soc Lond B Biol Sci* 244:45-50
- (1993) Genetic polymorphism in prehistoric Pacific islanders determined by analysis of ancient bone DNA. *Proc R Soc Lond B Biol Sci* 252:163-170
- Handt O, Krings M, Ward RH, Pääbo S (1996) The retrieval of ancient human DNA sequences. *Am J Hum Genet* 59:368-376
- Handt O, Richards M, Trommedorf M, Kilger C, Simanainen J, Georgiev O, Bauer K, et al (1994) Molecular genetic analysis of the Tyrolean Ice Man. *Science* 264:1775-1778
- Harpending H, Sherry ST, Rogers AR, Stoneking M (1993) The genetic structure of ancient human populations. *Curr Anthropol* 34:483-496
- Horai S, Hayasaka K, Kondo R, Tsugane K, Takahata N (1995) Recent African origin of modern humans revealed by complete sequences of hominoid mitochondrial mtDNAs. *Proc Natl Acad Sci USA* 92:532-536
- Howell N, Kubacka I, Mackey DA (1996) How rapidly does the human mitochondrial genome evolve? *Am J Hum Genet* 59:501-509
- Howell N, Mackey D (1997) Reply to Macauley et al. *Am J Hum Genet* 61:986-990
- Iriondo M (1998) Análisis de la estructura genética de la población. *Am J Hum Genet* 63:105-112

- Pääbo S (1989) Ancient DNA: extraction, characterization, molecular cloning and enzymatic amplification. *Proc Natl Acad Sci USA* 86:1939–1943
- Pääbo S, Higuchi RG, Wilson AC (1989) Ancient DNA and the polymerase chain reaction: the emerging field of molecular archaeology. *J Biol Chem* 264:9709–9712
- Parr RL, Carlyle SW, O'Rourke DH (1996). Ancient DNA analysis of Fremont Amerindians of the Great Salt Lake wetlands. *Am J Phys Anthropol* 99:507–518
- Parsons TJ, Muniec DS, Sullivan K, Woodyaty N, Alliston-Greiner R, Wilson MR, Berry DL, et al (1997) A high observed substitution rate in the human mitochondrial DNA control region. *Nat Genet* 15:363–368
- Penny D, Steel M, Waddell PJ, Hendy MD (1995) Improved analyses of human mtDNA sequences support a recent African origin for *Homo sapiens*. *Mol Biol Evol* 12:863–882
- Pesole G, Sbisà E, Preparata G, Saccone C (1992) The evolution of the mitochondrial D-loop region and the origin of modern man. *Mol Biol Evol* 9:587–598
- Prince AM, Andrus L (1992) PCR: how to kill unwanted DNA. *Biotechniques* 12:358–360
- Ribeiro-dos-Santos AKC, Santos SEB, Machado AL, Guapindala V, Zago MA (1996) Heterogeneity of mitochondrial DNA haplotypes in pre-Columbian natives of the Amazon region. *Am J Phys Anthropol* 101:29–37
- Richards M, Côrte-Real H, Forster P, Macaulay V, Wilkinson-Herbots H, Demaine A, Papiha S, et al (1996) Paleolithic and Neolithic lineages in the European mitochondrial gene pool. *Am J Hum Genet* 59:185–203
- Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Mol Biol Evol* 9:552–569
- Ruvolo M (1996) A new approach to studying modern human origins: hypothesis testing with coalescence time distributions. *Mol Phylogenet Evol* 5:202–219
- Sajantila A, Lahermo P, Anttinen T, Lukka M, Sistonen P, Savontaus MJ, Aula P, et al (1995) Genes and languages in Europe: an analysis of mitochondrial lineages. *Genome Res* 5:42–52
- Slatkin M, Hudson RR (1991) Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics* 129:555–562
- Stone AC, Stoneking M (1993) Ancient DNA from Pre-Columbian Amerindian population. *Am J Phys Anthropol* 92:463–471
- (1998) mtDNA analysis of a prehistoric Oneota population: implications for the peopling of the New World. *Am J Phys Anthropol* 105:101–116