

## Using ancient DNA to examine genetic continuity at the Mesolithic-Neolithic transition in Portugal

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### Resumo

São dois os principais mecanismos que têm sido propostos para a transição do Mesolítico ao Neolítico em Portugal: adopção e colonização. Do ponto de vista da Genética, as duas alternativas podem ser colocadas em termos de continuidade ou descontinuidade entre os dois lados da transição. Este estudo descreve uma comparação genética entre populações do Mesolítico e do Neolítico antigo usando ADN antigo.

Extraiu-se ADN mitocondrial (ADNmt) de restos humanos provenientes de diferentes sítios mesolíticos do estuário do Sado e de grutas-necrópole neolíticas, e realizou-se uma análise filogenética dos resultados, com base na região hipervariável 1 mitocondrial (HSVI) e na comparação com populações europeias modernas. A ausência do haplogrupo J na amostra neolítica portuguesa indica que ela não representa derivação directa a partir de agricultores do Próximo Oriente; mas a existência de descontinuidade genética entre os grupos mesolíticos e neolíticos implica que a transição para o Neolítico em Portugal terá envolvido algum tipo de colonização.

### Abstract

Two main mechanisms for the introduction of agriculture at the transition from the Mesolithic to the Neolithic in Portugal have been proposed: indigenous adoption and colonisation. Distinguishing between these mechanisms can be regarded as a question of genetic continuity or discontinuity at the transition. A genetic comparison of late Mesolithic and early Neolithic populations at the transition using ancient DNA is described here.

Mitochondrial DNA (mtDNA) was extracted from human remains collected in several Mesolithic sites of the Sado estuary and from Neolithic cave sites. Phylogenetic analysis, based on the mitochondrial hypervariable region 1 (HVSI), and comparison with DNA from modern European populations was performed. The absence of mtDNA haplogroup J in the ancient Portuguese Neolithic sample suggests that this population was not derived directly from Near Eastern farmers. The Mesolithic and Neolithic groups show genetic discontinuity implying colonisation at the Neolithic transition in Portugal.

## INTRODUCTION

### The Neolithic transition in Portugal

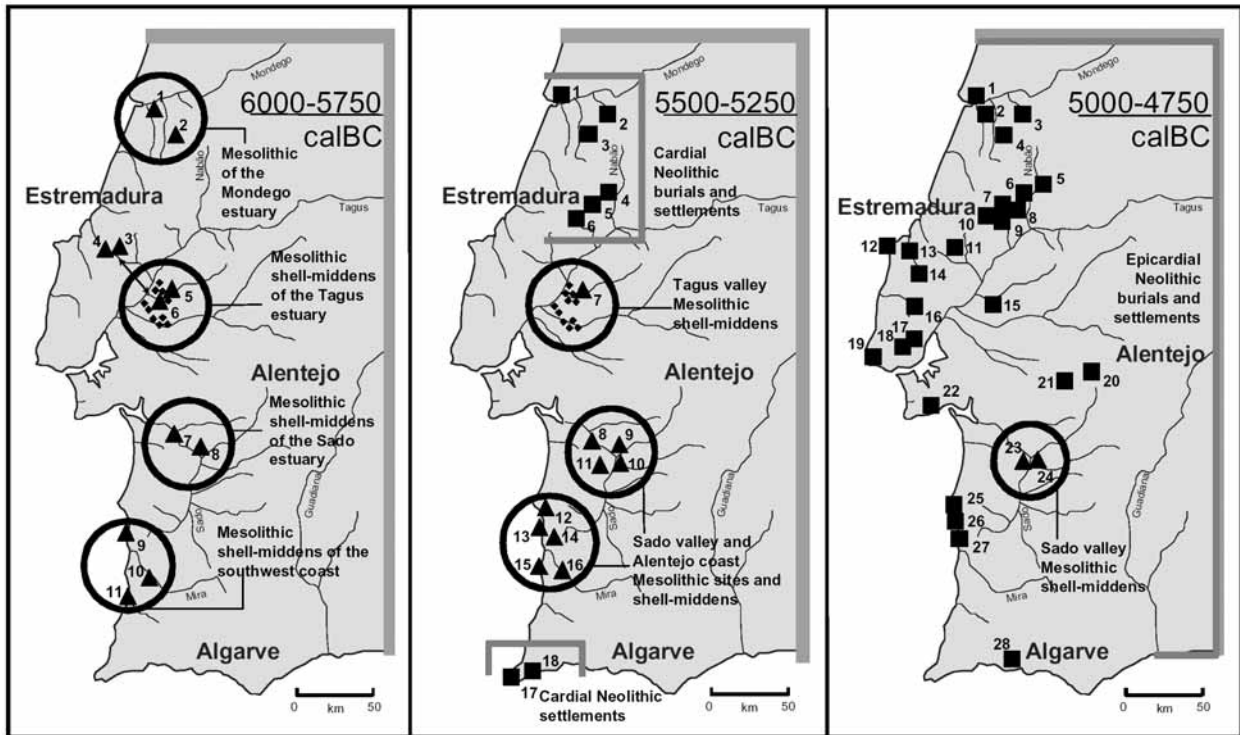
One of the central questions in understanding the transition from the Mesolithic to the Neolithic in Portugal is whether the introduction of agriculture was a result of colonisation or indigenous adoption (Zilhão 2000). While much archaeological evidence has been amassed in addressing this issue, it has remained unresolved. This is due in part to the limitations of answering what is essentially a biological question using observations of material culture alone. The central question of colonisation versus indigenous adoption can be reduced to a genetic one: Were the first farming groups of the Neolithic in Portugal genetically continuous with the preceding Mesolithic hunter-gatherers, implying indigenous adoption of farming? Or were the first farmers a genetically distinct population, implying some form of colonisation?

The Mesolithic in Portugal, began *ca.* 9500 BP and is represented mainly by shell midden sites in estuarine and coastal regions, where resources were varied and abundant (Bicho 1994, Clark 2000). In the later part of the period, after 7500 BP, many of these Mesolithic sites, namely the major shell-middens in the inner part of the estuaries of the Sado and Tagus rivers, were utilised all year round and reflect a semi-sedentary settlement pattern (Arnaud 1989). The further inland areas of interior Portugal were composed mainly of pine and oak forest, and show no evidence of Mesolithic settlement after 8000 BP; they seem to have been abandoned with the onset of the Atlantic climactic optimum (Zilhão 2000).

Farming practices spread across Europe after the domestication of plants and animals in the Near East around 10000 BP. The geographic distribution and chronology of the earliest Neolithic sites in Portugal show that the arrival of farming was sudden and intrusive, with agro-pastoral economies appearing in Portugal

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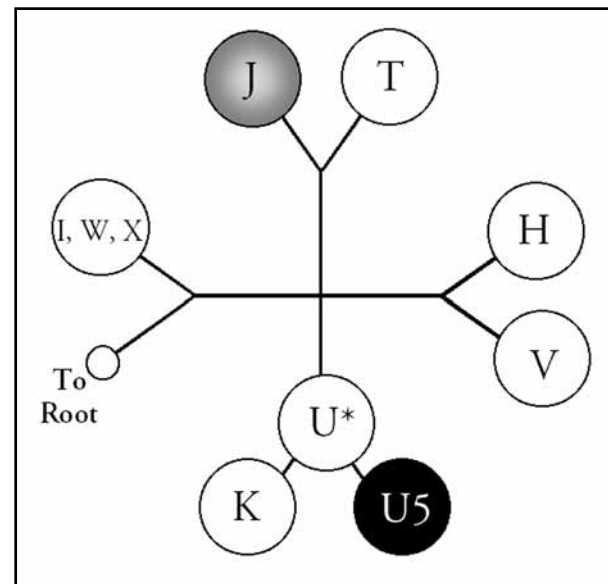
**Figure 1.** Geographic distribution of late Mesolithic (▲) and early Neolithic (■) sites in south-central Portugal between 6000 and 4657 cal BC.

at essentially the same moment as their appearance across the western Mediterranean (Zilhão 2001). The Neolithic is characterised by inland settlements, forming enclaves based on an agricultural economy, geographically separated from the hunter-gatherer settlements based in the coastal and estuarine regions. The earliest farming sites show evidence for a complete agricultural package, including domesticates, pottery and lithic technology which distinguishes these groups from the Mesolithic hunter-gatherers of the same region. Radiocarbon dating of sites throughout Portugal shows that the Neolithic and Mesolithic groups co-existed for 500 years (Zilhão 1993, Zilhão 2000). Figure 1 shows the changing settlement patterns of southern Portugal at the Neolithic transition.

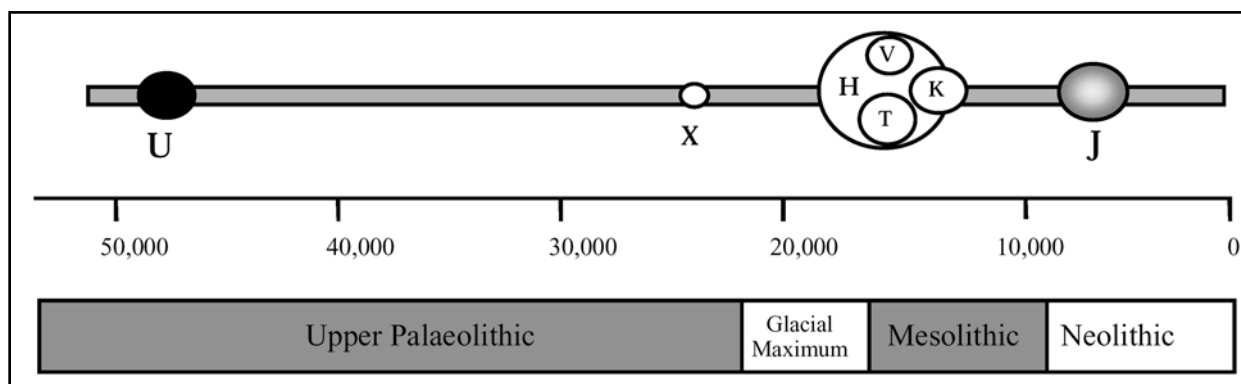
Stable isotope analysis of carbon and nitrogen in bone of human remains from Mesolithic and Neolithic burials in Portugal has shown that the Mesolithic groups had a diet comprising 50% marine foods, while the Neolithic farming groups had an entirely terrestrial diet (Lubell *et al.* 1994). This difference in diet demonstrates that the two different settlement patterns observed, that is, the shell middens and the agricultural enclaves, do not represent seasonal movements of a single population, but rather two distinct populations.

Information from skeletal morphology, however, has been contradictory. Different studies have claimed evidence for population replacement based on skeletal differences across the transition (Fox 1996), or alternative-

ly evidence for continuity of morphological traits (Jackes *et al.* 1997). The evidence from skeletal morphology has thus not been able to resolve whether the observed cultural, economic and dietary discontinuities reflect a genetic discontinuity. Given these circumstances, a direct study of the genetics of these popula-



**Figure 2.** A stylised simplified European mtDNA median network. The major haplogroups are designated by capital letters.



**Figure 3.** Ages of mtDNA clusters in Europe. Areas of circles are proportional to the frequency of the haplogroup in the modern European population. Haplogroup J is the youngest major haplogroup in Europe, but is also common in the Near East.

tions is clearly desirable, and ancient DNA analysis on human remains from both Mesolithic and Neolithic sites in Portugal was thus performed.

#### Modern European population genetics

Certain properties of the human mitochondrial genome mean that the majority of modern population genetics studies compare the diversity within a section of the mitochondrial DNA (mtDNA) known as the hypervariable region I (HVSI). The mtDNA is separate from the nuclear DNA and is strictly maternally inherited. Thus, recombination, which occurs in chromosomal DNA, does not occur in mtDNA. In addition, mtDNA is present at approximately 1000 copies per cell and has a high mutation rate. The large number of studies carried out using mtDNA in modern Europeans means that a large comparative data set exists.

The relationship between mtDNA sequence types can be represented on a median network diagram (Bandelt *et al.* 1999, Bandelt *et al.* 1995). Studies of modern Europeans have led to the development of a European mtDNA network that shows the presence of several different clusters of sequence types, known as clades or haplogroups. These have been given letter designations (Macaulay *et al.* 1999, Richards *et al.* 1998). The major European haplogroups are summarised in figure 2.

Based on the variability observed within each of the different clusters it is possible to calculate the length of time that each haplogroup has been present in Europe. The ages of the major clusters are shown in figure 3.

The ages of the clades show that most Europeans have an ancestry in Europe going back to the last glacial maximum with some dating back to the initial colonisation of Europe approximately 45 000 years ago. Around 15–20% of Europeans however, have mtDNA belonging to the haplogroup J which has been present in Europe for only around 8000–10000 years and which is derived from populations in the Near East. The results of modern mtDNA work thus imply a moderate input of population from the Near East with the beginning of farming

in Europe, with haplogroup J being the main marker for the farming migration (Richards *et al.* 2000).

#### METHODS

Human remains from three Neolithic and seven Mesolithic sites in Portugal were subjected to ancient DNA analysis. Strict measures were followed to prevent contamination of ancient samples with modern DNA and all experimental work was carried out in a dedicated DNA clean room facility. DNA was extracted from long bones or teeth by means of a phenol/chloroform extraction, followed by concentration and purification (Colson *et al.* 1997). The mtDNA HVSI was then amplified by PCR from the DNA extracts. Amplification products were sequenced and the sequences assigned to clades according to Macaulay *et al.* (1999).

Mesolithic sites were primarily sampled from the Sado valley estuary, including Arapouco, Vale de Romeiras, Poças de São Bento, Cabeço de Pez and Cabeço das Amoreiras (São Romão) (Arnaud 1989, Cunha and Umbelino 1995–1997). The two sites sampled outside the Sado valley were the early Mesolithic site of Toledo in coastal Estremadura (Araújo 1998) and the coastal Algarve site of Fiais (Araújo 1995–1997, Morales and Arnaud 1990). Two of the three Neolithic sites represented, Gruta do Caldeirão (Zilhão 1992) and Algar do Bom Santo (Duarte 1998) are cave burial sites. The other, Perdígões (Lago *et al.* 1998), is a much larger and slightly later development, including a necropolis, settlement area and megalith.

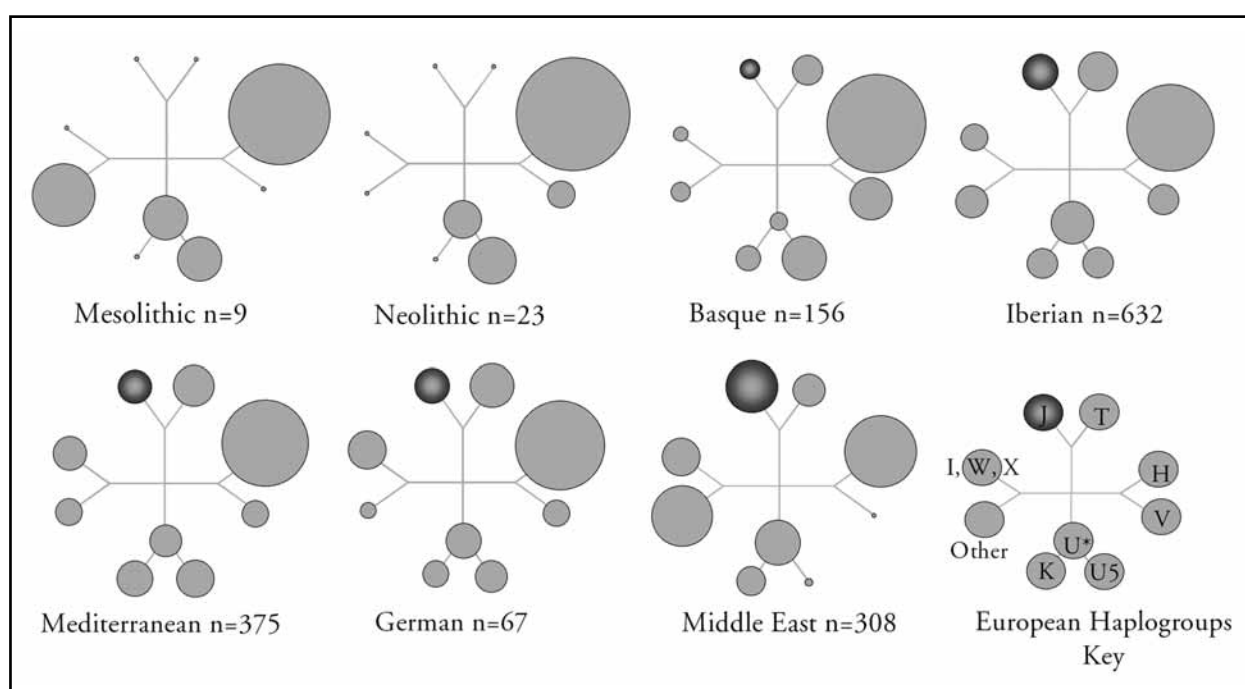
#### RESULTS

The results of the extraction and sequencing are summarised in table 1. The percentage of successful DNA recovery is typical for ancient DNA work.

Sequences obtained from the bone samples were allocated to haplogroups and the proportion of each mtDNA haplogroup was compared for the Mesolithic

	Neolithic			Mesolithic						
	ABS	Caldeirão	Perdigões	Ara	CdA	CdP	PSB	Tol	Fia	VdR
Samples extracted	49	17	9	10	5	7	5	1	2	6
No DNA	31	0	1	7	2	1	4	0	0	2
Failed or inconsistent	9	4	0	1	2	1	0	0	0	4
One sequence only	5	0	1	0	0	1	0	0	1	0
Reproducible	4	13	7	2	1	5	1	1	1	0
Percentage recovery	8	77	78	20	20	71	20	100	50	0

**Table 1.** Summary of extraction and sequencing results for Mesolithic and Neolithic sites. ABS - Algar do Bom Santo; CdA - Cabeço das Amoreiras; CdP - Cabeço de Pez; PSB - Poças de São Bento, Tol - Toledo; Fia- Fiais; VdR - Vale de Romeiras.

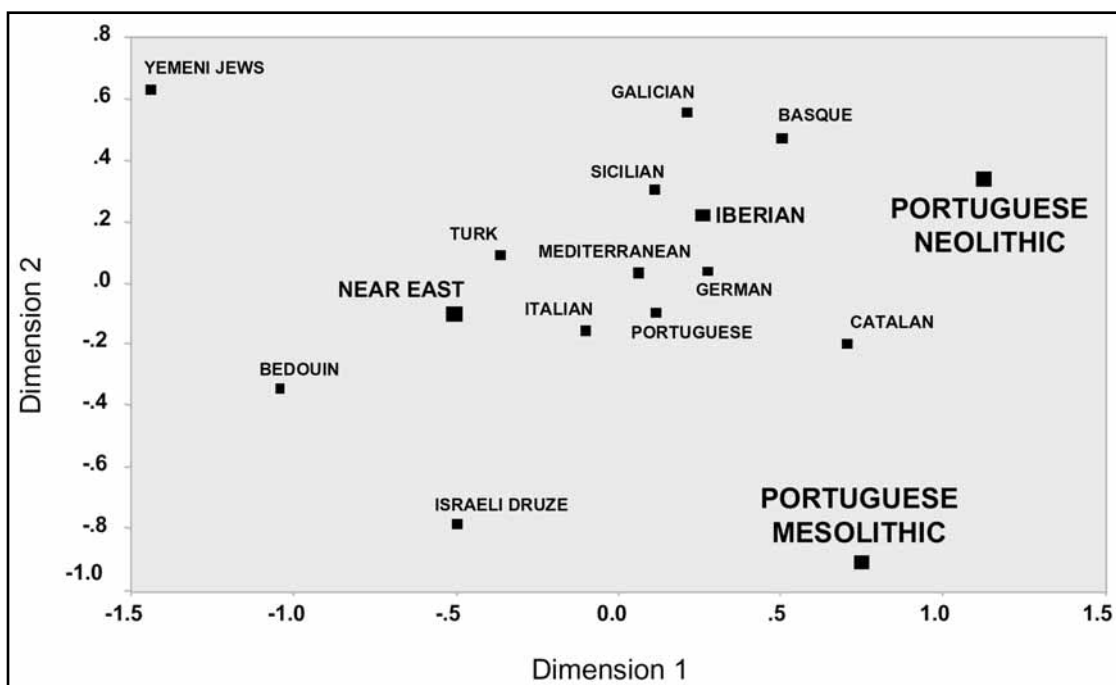


**Figure 4.** Haplogroup frequencies for selected ancient and modern populations. The relative frequencies of the haplogroups are represented by the sizes of the nodes on a simplified European mtDNA network.

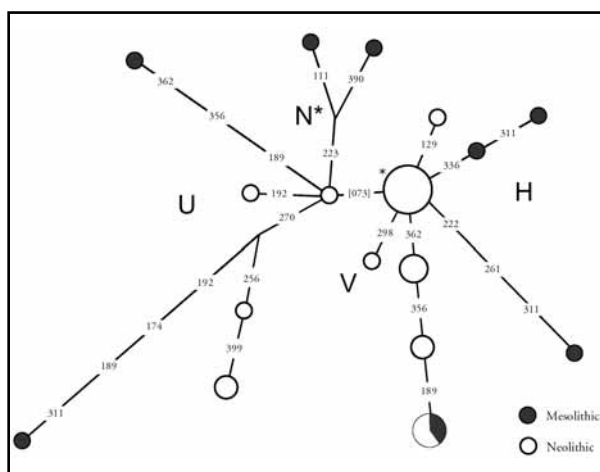
and Neolithic populations studied and other published European and Near Eastern populations. Figure 4 shows the haplogroup frequencies for the ancient samples when compared with other populations. The diagram clearly shows that the ancient Portuguese haplogroup frequencies are more closely related to Iberian and Mediterranean populations than to Near Eastern populations. This is true even for the early Neolithic population. It is also notable that the Portuguese Mesolithic and Neolithic samples contain no haplogroup J, that is, no marker of Near Eastern population input.

The genetic distances between populations were calculated from mean pairwise differences using the Arlequin

2.0 software package (Schneider *et al.* 2000) and the resulting genetic distances were then converted to two-dimensional plots using multidimensional scaling (MDS) in SPSS. This allows the genetic distances to be rendered as physical distances on a two-dimensional plot. The MDS plot in figure 5 shows that the ancient Portuguese samples are closer genetically to Iberian populations than Near Eastern ones. They show the closest similarity with the Basques, Galicians and Catalans. It appears that there is no Near Eastern component to either the Mesolithic or Neolithic populations. However, the MDS plot shows that the Mesolithic and Neolithic populations are not themselves closely related. This comparison indicates that they are genetically distinct populations.



**Figure 5.** Multidimensional scaling plot of the genetic distances between ancient Portuguese and modern European and Near Eastern populations.



**Figure 6.** Reduced median network of HVSI of the ancient Portuguese samples. Node size is proportional to haplotype frequency. An asterisk marks the position of the Cambridge Reference Sequence (CRS). Numbers refer to mutated sites relative to the CRS. HVSI numbering.

The sequences obtained for these ancient samples were used to draw a mtDNA network diagram (Bandelt *et al.* 1995), shown in figure 6. The network shows that while the Mesolithic and Neolithic sequences are not hugely divergent, they share only one haplotype. A single inter-breeding population would be expected to share many more haplotypes.

## DISCUSSION

Haplogroup frequencies and genetic distances show that the ancient Portuguese populations studied here, both Mesolithic and Neolithic, are most closely related to the modern Basque and Galician populations of the Iberian Peninsula.

The Mesolithic and Neolithic populations show no evidence for the presence of mitochondrial DNA haplogroup J. This haplogroup is considered one of the strongest markers for Neolithic population input into Europe from the Near East at the arrival of farming. Its absence in the ancient Portuguese populations implies that these populations are not derived directly from a Near Eastern source population. The Portuguese Neolithic sample, containing no J haplotypes in 23 samples, indicates that agriculture in Portugal was not brought directly by migrating farmers from the Near East.

An analysis of genetic distances indicates that the Portuguese Neolithic and Mesolithic groups studied here represent different genetic populations. While it must be noted that this observation is based on a very small Mesolithic sample size, this finding implies genetic discontinuity between the hunter-gatherer and early farming populations. A discontinuity at the Neolithic transition is consistent with the Maritime Pioneer Colonisation model for the arrival of farming in Portugal (Zilhão 1993, Zilhão 2001). In this model, agricultural enclaves were formed by groups of leap-frogging sea-faring colonists who moved around the Mediterranean coast. The source population however is not Near Eastern, as demonstrated both by the

absence of haplogroup J in the Portuguese Neolithic population and by the genetic distance observed between the Neolithic Portuguese and Near Eastern populations. More likely, a Mediterranean group which itself had adopted farming through exchange or only limited migration moved into the uninhabited parts of Portugal's coastal regions to pursue an agricultural subsistence strategy.

Larger sample sizes, particularly for Mesolithic era sites, are required to confirm genetic discontinuity at the Neolithic transition in Portugal. However, the low resolution of mtDNA means that a source population for the Neolithic farmers may never be conclusively identified.

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