Ancient DNA suggests the leading role played by men in the Neolithic dissemination

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The impact of the Neolithic dispersal on the western European populations is subject to continuing debate. To trace and date genetic lineages potentially brought during this transition and so understand the origin of the gene pool of current populations, we studied DNA extracted from human remains excavated in a Spanish funeral cave dating from the beginning of the fifth millennium B.C. Thanks to a "multimarkers" approach based on the analysis of mitochondrial and nuclear DNA (autosomes and Y-chromosome), we obtained information on the early Neolithic funeral practices and on the biogeographical origin of the inhumed individuals. No close kinship was detected. Maternal haplogroups found are consistent with pre-Neolithic settlement, whereas the Y-chromosomal analyses permitted confirmation of the existence in Spain approximately 7,000 y ago of two haplogroups previously associated with the Neolithic transition: G2a and E1b1b1a1b. These results are highly consistent with those previously found in Neolithic individuals from French Late Neolithic individuals, indicating a surprising temporal genetic homogeneity in these groups. The high frequency of G2a in Neolithic samples in western Europe could suggest, furthermore, that the role of men during Neolithic dispersal could be greater than currently estimated.

The Neolithic transition was a crucial step in the history of European settlement, but the exact modalities of its dissemination are still not totally understood. In western Europe particularly, despite the abundance of archeological data, the real importance of the Mesolithic substrate and of the Neolithic migrants in the first farmers' origin is a crucial point still debated among the scientific community (1). In this context, access to ancient DNA data seems to be a good way to trace and date the dispersal of European genetic lineages and better understand the origin of current populations.

Presently, few ancient data are available on the Neolithic period, and most of them consist of mitochondrial DNA data, which are only informative for the maternal origin. These have revealed a particularly high frequency of haplogroup N1a, a haplogroup quite rare currently in central European (2, 3) and in Atlantic coast Neolithic specimens (4), whereas this last was never found in southern European samples (5–7). These furthermore suggested a probable genetic continuity between ancient southern Neolithic specimens and current populations located in the same areas (6, 7), whereas the ancient central European plains samples would share a greater affinity with the modern-day Near East and Anatolia (2). The findings deduced from the study of maternal genetic lineages seemed consistent with the archeological evidences of the existence of two distinct routes of neolithization: one along the central plains of Europe and another along the Mediterranean coasts.

Concerning paternal lineages, because of the bad preservation of nuclear DNA in ancient samples, few analyses have been performed to date on the Y-chromosome of Neolithic specimens, thus few paternal lineages existing at this period have been characterized. The study of only three male specimens associated with the Linear Pottery Culture, a Neolithic culture found in the central European plains (2), and of 22 men buried in a late Neolithic French necropolis (6) permitted data to be obtained on the paternal lineages existing before the Cooper and Bronze age migrations. Interestingly, they all revealed the importance of the G2a haplogroup, which is rare in modern European populations. Of course, these works do not provide a complete overview of the Neolithic male diffusion. Additionally, no data are currently available on the paternal lineages existing in the early Mediterranean Neolithic.

In this context, to improve the knowledge of the neolithization of southwestern Europe, we studied DNA extracted from human specimens excavated in the Avellaner cave, an ancient funeral cave of northeastern Spain. According to ^{14}C dating performed on bones and charcoals found in the cavity, this funeral cave was used during the first part of the fifth millennium B.C. (8), which corresponds to the end of the establishment of the Neolithic cultures in Spain (Epicardial Culture). The study of this funeral site is thus particularly interesting to access directly the gene pool of the first farmers in Spain and to understand the particular funeral practices of this transition period, which are still poorly understood (9).

Because most of bones found in the cave were fragmented and partially burned, the first challenge of this work was to identify individuals buried. Afterward, we analyzed different and complementary genetic markers, located on autosomes and Y-chromosomal and mitochondrial DNA to characterize any kinship between individuals and to trace their biogeographical origin.

Through these data, the main objectives of this work were to genetically characterize early farmers from northern Spain and to compare the genetic lineages found with those previously obtained from Neolithic specimens and those currently present in European populations, to understand the complexity of the Neolithic dispersal and its heritage in southern Europe.

Results

Autosomal Results. Of the 27 samples studied, 14 permitted acquisition of unambiguous partial or complete autosomal profiles, which can be related to seven individuals (Table 1). Of the seven individuals clearly identified, six were male and one was female. No close familial relationship could be highlighted between these individuals. Estimation of the nuclear DNA concentration per

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Table 1. Consensus short tandem repeat (STR) autosomal profiles of the seven ancient Avellaner individuals

Name	Cavity	No. of samples	[DNA] (ng/μL)	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
Ave01	II	3	4.62E-03	13/(14)	30/31.2	10/11	11/12	18/18	9.3/9.3	8/11	11/11	17/23	12/13	15/16	—	17/20	X/Y	13/13	21/24
Ave02	I	4	1.39E-02	11/13	28/32.2	10/12	10/11	15/18	6/(9.3)	10/(11)	8/10	17/19	15/15.2	16/19	8/(11)	16/17	X/Y	11/12	23/26
Ave03	III	3	8.16E-03	11/13	28/29	12/12	9/12	17/18	9.3/9.3	8/12	11/12	17/24	12/13	17/19	—	14/18	X/Y	10/11	24/(25)
Ave04	III	1	Und	13/(15)	(31.2/32.2)	(11/11)	(11/11)	16/(18)	(7/7)	(11)/12	11/14	(24/24)	12/14	16/16	(8)/9	(16/19)	X/X	13/13	(24/24)
Ave05	I	1	9.45E-03	14/14	—	(12/13)	(11/11)	18/18	(7/7)	11/11	(11/11)	(20/24)	(14/14)	(15/16)	(8/8)	(17/21)	X/Y	(11/11)	—
Ave06	II	1	Und	(14/15)	29/29	—	11/12	(14)/17	—	(11/11)	(11/11)	(16/20)	(13/13)	(15/15)	(8/8)	12/(14)	X/Y	(11)/13	(20/24)
Ave07	II	1	8.27E-03	13/15	28/31	8/10	10/12	15/16	9.3/9.3	11/(12)	(8/11)	17/25	(12/13)	(16/17)	(8/11)	(16/16)	X/Y	10/12	21/22

Dashes denote that alleles could not be clearly amplified for the locus in question. Alleles in parentheses were just observed once. Consensus allelic profiles were built after two amplifications performed on each DNA extract. [DNA], average quantity of nuclear DNA obtained; Und, undetermined.

sample ranged from below the detection capability of the kit to 34.2 pg/μL (Table 1).

Mitochondrial Results. Mitochondrial HVS-I sequences were obtained for the seven individuals and can be classified into four different haplotypes (Table 2). All are still frequent in current European populations (Table S1), and three of them were also found in ancient Neolithic samples (Table S2). These haplotypes permitted the determination that the individuals ave01, ave02, and ave06 belonged to K1a, ave04 and ave05 to T2b, ave03 to H3, and ave07 to U5 haplogroups.

For all samples, typing of mitochondrial SNPs in the coding region permitted confirmation of the haplogroup determination previously inferred from the haplotypes (Table 2).

Y-Chromosomal Results. For the six male samples, two complete and four partial Y-STRs haplotypes were obtained (Table 3). They allowed classification of individuals into two different haplogroups: G2a (individuals ave01, ave02, ave03, ave05, and ave06, which seem to share the same haplotype) and E1b1b1 (individual ave07). The four markers chosen to confirm belonging to these haplogroups (Y-E1b1b1-M35.1, Y-E1b1b1a1b-V13, Y-G2-M287, and Y-G2a-P15) were typed with a rate of 66%, which permitted confirmation that four males were G2a and one was E1b1b1a1b (Table 3).

Analysis of shared haplotypes showed that the G2a haplotype found in ancient specimens is rare in current populations: its frequency is <0.3% (Table S3). The haplotype of individual ave07 is more frequent (2.44%), particularly in southeastern European populations (up to 7%). The Ave07 haplotype was also compared with current Eb1b1a2 haplotypes previously published (10–14). It appeared identical at the seven markers tested to five Albanian, two Bosnian, one Greek, one Italian, one Sicilian, two Corsican, and two Provence French samples and are thus placed on the same node of the E1b1b1a1b-V13 network as eastern, central, and western Mediterranean haplotypes (Fig. S1).

Lactase Persistence Result. The LP-13910-C/T SNP associated with lactase persistence was successfully typed for all ancient samples tested. The mutated position would have appeared during the dissemination of the Linear pottery culture in central Europe (15). All our ancient samples from Spain were homozygous C/C for this marker.

Discussion

Results Authenticity. The main difficulty in ancient DNA analyses is to produce authentic data. In this study, drastic precautions previously described (6, 16) were taken to avoid contaminations, and a multimarkers approach was used to validate the accuracy of the produced data. For the seven individuals presented here, despite the fact that all of the authenticity criteria could not be fully respected, results obtained are in favor of endogenous and reliable outcomes: extraction controls, PCR blanks, and amplifications from DNA extracted from sheep or goat remains with human primers were always negative. The nuclear DNA quantity recovered and the inverse relationship between the amplification efficiency and length of the amplification obtained were characteristic of a degraded ancient DNA. Results acquired from the different amplifications and from cloning were always consistent between each other, and results of SNP typing were also 100% concordant with mitochondrial and Y-chromosome haplotypes previously deduced. The absence of the polymorphism associated with the lactase persistence is also coherent with results previously published from ancient Mesolithic and Neolithic samples (6, 17, 18).

Avellaner Genetic Diversity. Regarding the biogeographical origin of Avellaner individuals, mitochondrial and Y-chromosomal

Table 2. MtDNA haplotypes and haplogroups inferred for each Avellaner specimen

Name	Cavity	No. of samples	Mitochondrial haplotypes	H-C7028T	T2-A14233G	T2B-G930A	U5-T3197C	H1-J1-G3010A	H3-T6776C	HV-C14766T	K-A10550G	K1-A10398G	K1A-C497T	T-A4917G	U-A12308G	Haplogroup
Ave01	II	3	16093C; 16224C; 16311C	T	A	G	T	G	T	T	**G**	**G**	**T**	—	**G**	K1a
Ave02	I	4	16093C; 16224C; 16311C	T	A	G	T	G	T	T	**G**	**G**	**T**	A	**G**	K1a
Ave03	III	3	CRS	**C**	A	G	T	G	**C**	**C**	A	A	C	A	A	H3
Ave04	III	1	16126C; 16294T; 16296T; 16304C	T	**G**	**A**	T	G	T	T	A	A	C	—	A	T2b
Ave05	I	1	16126C; 16294T; 16296T; 16304C	T	**G**	**A**	T	AG	T	T	A	A	—	—	A	T2b
Ave06	II	1	16093C; 16224C; 16311C	T	A	G	T	AG	T	T	—	—	**T**	A	**G**	K1a
Ave07	II	1	16051G; 16189C; 16270T	T	A	G	**C**	G	T	T	A	A	C	A	**G**	U5

SNPs in bold are variants at concerned positions.

results suggest different origins of maternal and paternal lineages. Mitochondrial haplogroups found (U5, K1a, T2b, and H3), which are quite common in western Eurasia, are relatively uninformative for identifying clear genetic affiliations with demographic movements. However, they suggested a fairly diverse origin of Avellaner maternal lineages, consistent with an early Neolithic group: a Paleolithic origin from Middle East for haplogroup K1a, T2b, and U5 and a relation with the postglacial expansion from southwestern Europe for the H3 haplogroup (19, 20). Additionally, haplotypes identical to those assigned to H3, K1a, and T2b haplogroups were previously found in samples related to central and Mediterranean Neolithic cultures (Table S2). Only the U5 haplotype was never found in ancient specimens, but it differs at only one position (np 16051) from another Mesolithic haplotype (21). This finding also observed in other late Neolithic samples could indicate a greater diversity of the U5 cluster at the Neolithic period, consistent with an ancient origin of this lineage (6).

Concerning paternal lineages, only two different haplogroups were identified: G2a and E1b1b1a1b.

G2a is common in modern populations of Caucasus (10) but is quite rare in current western European populations. It represents only approximately 4% of the haplogroups found in the Spanish population (22). It seems nevertheless much more common in Neolithic samples because it was previously found in ancient Neolithic samples from the linear pottery culture in Germany, as well as in late Neolithic French samples (2, 6). Thus, it represents one of the main Y-haplogroups found in Neolithic individuals. Of course, there are still too few data to confirm the overrepresentation of the G2a haplogroup among western Neolithic populations, but this G2a in early Spanish Neolithic samples is strong evidence of a link previously suggested between Neolithic migration and G2a dispersion in Europe (6). Additional G2a haplotypes will, however, be needed to determine whether the G2a found in the male individual associated with dispersal of Neolithic in central Europe and those linked with the Mediterranean route shared a same Neolithic Middle Eastern origin.

For E1b1b1a1b, the link between this haplogroup and the Neolithic expansion could also be made. This haplogroup, which is the main European clade of haplogroup E, has been described as having spread into western Europe from the southern Balkans (10, 14, 23), but the exact period at which this expansion would be held is still debated. It has been previously related to several demographic events, such as the Neolithic dispersal in direction of the eastern Adriatic (10) or along the Vardar-Morava-Danube rivers into central Europe (24) or the migrations during the Bronze age (13, 23, 25). The presence of this haplogroup in an early Neolithic sample in Spain confirms, therefore, that this marker may be related to the Mediterranean Neolithic expansion, even if it does not permit quantification of the real importance of the Neolithization contribution in the spread of this haplogroup in western Europe. It confirms, furthermore, that the Neolithic dispersal was not a uniform movement from the Middle East but that it was more probably an arrhythmic phenomenon punctuated by rapid expansion phases and periods of breaks related to cultural changes like the one previously identified by archeologists in the Balkans area (26–28).

Unusual Neolithic Burial Cave. Because of the fragmented state of skeletons, few data could be previously obtained from the physical analysis of remains. Thanks to ancient DNA data, we prove that among the seven individuals clearly identified, six were male. No close relationship could be demonstrated, but five of the six males buried seem to belong to the same paternal lineage, which suggests a funeral recruitment of individuals from the same group. According to archaeological evidence, few burial places were found at the recent phases of the Early

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Table 3. Y-haplogroups inferred from Y-STR haplotypes and NRY-SNPs typing results for the male specimens

			Y-STRs																Y-SNPs				
Name	Cavity	No. of samples	DYS456	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385	DYS393	DYS391	DYS439	DYS635	DYS392	GATA-H4	DYS437	DYS438	DYS448	Y-E1b1b1-M35.1	Y-E1b1b1a1b-V13	Y-G2-M287	Y-G2a-P15	Haplogroups
Ave01	II	3	15	12	(23)	(29)	18	15	(14/14)	13	10	11	(21)	—	12	16	10	—	G	G	—	**T**	G2a
Ave02	I	4	15	12	23	—	18	15	14/14	13	10	11	21	(11)	12	16	10	22	G	G	—	**T**	G2a
Ave03	III	3	15	12	23	29	18	15	14/14	13	10	11	21	11	12	16	10	(22)	G	G	—	**T**	G2a
Ave05	I	1	15	12	(23)	29	(18)	(15)	(14/14)	13	(10)	11	—	—	12	16	—	22	G	—	—	**T**	G2a
Ave06	II	1	15	12	—	—	18	(15)	—	13	(10)	(11)	—	—	12	(16)	—	—	—	G	—	—	G2a 99.6%
Ave07	II	1	16	13	24	(31)	16	13	16/19	13	(10)	11	22	11	10	(14)	10	20	**C**	**A**	—	C	E1b1b1a1b

Dashes denote that alleles could not be clearly amplified for the locus in question. Consensus Y-STR profiles were built after two amplifications from each DNA extract. Alleles in parentheses were observed just once. For the sample for which the Y haplogroup could not be confirmed by the typing of SNP, the determination of the haplogroup was obtained solely from the haplotype. The percentage of probability is shown in the last column. SNPs in bold are variants at concerned positions.

Neolithic compared with the putative population density at this period. Epicardial burials were furthermore usually individual in natural cavities like the Gazel cave, Aude, France, or in open-air sites like Ca l'Estrada, Catalonia (1). Collective burials appeared later during the Late Neolithic period (IV and III millennia B.C.). In this context, the funeral recruitment of the Avellaner cave seems very unusual for the early Neolithic period (1, 29). The absence of any significant grave goods in the cavities is not in favor of funeral recruitment according to potential social elite. According to archaeological hypotheses, it could rather reflect a continuance of funerary rites from the late Mesolithic period (1). The presence of individuals carrying paternal lineages linked with the Neolithic expansion in a funeral cave where Mesolithic funeral practices were used could suggest the maintenance of the Mesolithic traditions during the early stages of the western Europe Neolithic. No genetic data from Mesolithic grave specimens are currently available to be compared with the genetic structure obtained. Further genetic analyses of ancient southern European graves will be necessary to validate this hypothesis and potentially provide a better idea about the origin of this particular burial.

Avellaner and the Neolithic Transition. All of the results obtained are concordant with those previously published for ancient Neolithic specimens. The mitochondrial lineages found are consistent with a genetic continuity in southern Europe, at least since Neolithic times, and the absence of the N1a haplogroup widespread in central Europe confirms furthermore the archaeological evidence of the existence of two routes of Neolithic dispersal in Europe. Moreover, the paternal lineages characterized and their current repartition along the Mediterranean coasts confirm this assumption.

The sex-specific diversity observed in Avellaner specimens is finally broadly similar to that previously observed in the samples of the Treilles cave, a French funeral cave 2,000 y more recent than the one presented here. In both studies, the Paleolithic origin of most of the maternal lineages coupled with a more recent paternal ancestry as well as the G2a presence could thus suggest a common origin of the two Neolithic groups and a probable genetic continuity in the western Mediterranean area from 5000 to 3000 B.C.

The high frequency of G2a haplogroup in Neolithic specimens, whereas this haplogroup is very rare in current populations, also suggests that men could have played a particularly important role in the Neolithic dissemination that is no longer visible today. This would imply that intra-European migrations related to the metal ages may have strongly affected the modern gene pool.

Conclusion

This work offers a direct overview of the genetic lineages of the first northern Spanish farmers. It reveals the complexity of the implementation of agriculture in Spain and the probable high level of heterogeneity of the Neolithic dissemination in Europe. It highlights furthermore that maternal and paternal lineages could have had different histories, which complicates even more the different scenarios issued on the Neolithic transition in Europe. This study, which was performed on only seven individuals, is of course not sufficient to estimate the real importance of the arrival of men in the Neolithic transition, and new investigations on ancient southern specimens will be necessary to improve our knowledge on this crucial period.

Materials and Methods

The Avellaner cave is a small burial cave located in Catalonia, in northeastern Spain. The main cavity is in fact divided into three independent areas separated by stone walls, each containing several successive primary or secondary burials. Skeletons found were not in anatomic connection, which did not

permit formal individualization of each individual; however, the minimum number of individuals could be estimated at 19 (8). Because of the fragmentation and the mix of bones within each sepulchral place, we decided to work from teeth still fixed on the four mandible fragments available (two or three teeth for each fragment), on the most well-preserved scattered teeth, and three femoral shafts. In all, we analyzed 27 human samples taken in the three cavities, as well as two nonhuman material (two sheep teeth) taken at the same time with human remains to detect possible contamination events during excavation.

Sample Preparation and DNA Extraction. Bones samples were first abraded with sterile equipment before UV exposure and grinding into a liquid nitrogen environment. Teeth were also crushed after decontamination with bleach and UV exposure 30 min on each side. DNA was then extracted according to a protocol previously described (30). Four to five extractions were realized on each sample, according to the powder quantity recovered.

Nuclear DNA Analysis. For at least one extract per sample we determined the nuclear DNA quantity extracted using the Quantifiler Human DNA Quantification Kit (Applied Biosystems).

Autosomal profiles were determined using both AmpFlSTR Identifiler Plus and the MiniFiler PCR Amplification Kits (Applied Biosystems) on a 3500 Genetic Analyzer. STRs profiles were analyzed with GeneMapper 4.1 software. Two amplifications were performed on each DNA extract.

Haplotype Determination. Mitochondrial haplotypes were obtained by the sequencing of 381 base pairs of the HVS-I region of the mtDNA in two overlapping fragments, as previously described (16). To meet the authenticity criteria, we also cloned the amplicons obtained during the analyses of 5 of the 27 samples. Cloning was performed using the pGEM-T Easy Vector system II kit (Promega), according to the manufacturer protocol. Between 16 and 28 clones were analyzed for each sample. All sequences obtained were used to deduce mitochondrial haplogroups according to the latest mtDNA phylogeny (31).

For male individuals, Y-chromosomal haplotypes were obtained from the analysis of 17 Y-STRs loci using the AmpFlSTR Yfiler PCR Amplification Kit (Applied Biosystems). They were used to estimate Y-haplogroups thanks to the Haplogroup Predictor software (32).

Haplogroup Assignment and Typing of an SNP Associated with Lactase Persistence. To clarify the haplogroup status inferred from HVS-I sequences and Y-chromosomal haplotypes, we analyzed supplemental SNPs localized on the mitochondrial coding region and the nonrecombining region of the Y-chromosome (NRY). SNP typing was performed using iPLEX Gold technology (Sequenom), which seems to be a very sensitive and effective typing technology for degraded DNA analyzes (30). Two multiplexes containing a total of 17 SNPs located on mtDNA, the NRY, and the *MCM6* gene (SNP associated with the lactase persistence) were designed with MassArray Assay design software (version 4.0). The typing reactions were performed twice on two different DNA extracts per sample.

Statistical Analysis. The putative genetic relationships were investigated from autosomal STR profiles with DNA•VIEW Software (33).

To compare ancient Spanish genetic lineages obtained and those current in European populations, analyses of shared haplotypes were performed thanks to two personal databases comprising 14,645 mitochondrial HVS-I sequences and 14,166 Y-haplotypes. Mitochondrial profiles obtained were also compared with ancient Neolithic haplotypes previously published. Detailed compositions of the different datasets are available in Table S2 and Table S4. To allow maximum comparability among all populations, Y-shared haplotype analyses were performed on only seven Y-STRs markers (DYS19, DYS390, DYS391, DYS392, DYS393, DYS389I, and DYS389II).

A haplotype network was generated for NRY haplogroup E-V13 via the median joining algorithm of Network, version 4.5.1.6. To obtain the most parsimonious networks the reticulation permissivity was set to zero. Datasets were preprocessed using the star contraction option in Network, version 4.5.1.6 (5). The seven Y-STR loci used were weighted according to the observed STR allelic variance, as described by Qamar et al. (34).

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