Genetic evidence for patrilocal mating behavior among Neandertal groups

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The remains of 12 Neandertal individuals have been found at the El Sidrón site (Asturias, Spain), consisting of six adults, three adolescents, two juveniles, and one infant. Archaeological, paleontological, and geological evidence indicates that these individuals represent all or part of a contemporaneous social group of Neandertals, who died at around the same time and later were buried together as a result of a collapse of an underground karst. We sequenced phylogenetically informative positions of mtDNA hypervariable regions 1 and 2 from each of the remains. Our results show that the 12 individuals stem from three different maternal lineages, accounting for seven, four, and one individual(s), respectively. Using a Y-chromosome assay to confirm the morphological determination of sex for each individual, we found that, although the three adult males carried the same mtDNA lineage, each of the three adult females carried different mtDNA lineages. These findings provide evidence to indicate that Neandertal groups not only were small and characterized by low genetic diversity but also were likely to have practiced patrilocal mating behavior.

patrilocality | kinship | demography | human evolution

Demography, the study of survival, fertility, and population dynamics, is crucial for understanding human evolution (1). Two properties of the El Sidrón site (Asturias, northern Spain) (2) make it unique for learning about the demographic characteristics of a Neandertal group: the synchrony of the accidental assemblage of 12 Neandertal individuals, and the exceptional taphonomic conditions that favor DNA preservation.

El Sidrón is a 3,700 m-long karst system formed by a main gallery orientated approximately west to east and several small transverse galleries. Within one of these galleries (the Ossuary Gallery, located 220 m from the main entrance) excavation of Neandertal remains has been ongoing since 2000. The excavations to date have yielded >1,800 hominin skeletal fragments and ~400 Mousterian stone tools made in situ (3), but faunal remains are very scarce. The Neandertal bones are in a secondary position, and the original deposit, worn out by erosion, is thought to have been placed either on the surface or in an upper karst level (2). The present assemblage occurred by the collapse of an upper gallery into the Ossuary Gallery shortly after the death of the individuals, a collapse triggered by a natural event, probably a violent storm that also dragged down pebbles and clay (Fig. S1). Given that (*i*) ≈18% of the lithic industry can be refitted, and (*ii*) the widespread spatial distribution of these refitted artifacts, it may be surmised that they result from a single and brief cultural activity. This likelihood lends even more support to the synchrony of the whole assemblage (2, 3), dating to around 49,000 y ago (4). Some evidence, such as skeletal parts still in anatomical articulation, indicates little site disturbance since formation. *Ex hypothesis*, the fact that all types of skeletal remains show evidence of anthropic activities associated to cannibalism (2) could indicate that the assemblage corresponds to a Neandertal group processed by other Neandertals on the surface. Although it is impossible to be sure that the individuals represent a contemporaneous group, alternative explanations, such as recurrent accumulation over time of cannibalized individuals that were closely related through the female line, seem less plausible. The inaccessibility and the low temperature of the Ossuary Gallery, along with the anticontamination protocol implemented during the excavation (5), provide excellent conditions for endogenous DNA preservation and retrieval.

The group of at least 12 individuals includes six identifiable adults, three adolescents, two juveniles, and one infant (Table 1). Morphological sex attribution can be achieved for the adults through traits of robustness in the mandibles and dentition, indicating the presence of three females and three males (Table 1). Furthermore, analysis of canine size suggests that two adolescents probably are males (Table S1). The sex of the juveniles and one adolescent could not be determined with certainty by morphological traits. A group size of 12 individuals at El Sidron is consistent with a previous estimate of 8–10 individuals per Neandertal group, based on the size of sleeping and combustion activity areas in the long-occupied rock shelter of Abric Romaní (Barcelona, Spain) (6). However, because the original external deposit cannot be studied, we cannot exclude the possibility that the El Sidrón group was larger and that some original members are not represented in the current assemblage.

In this study we analyze the mtDNA and test for presence or absence of the Y chromosome in the El Sidrón individuals to obtain information about the kinship, sex, and diversity of a Neandertal group.

Results and Discussion

We used a Y-chromosome assay (*Materials and Methods*) to evaluate the sex identification based on morphological traits. Four samples (corresponding to adults 1, 2, and 6 and adolescent 1) yielded Y-chromosome products, confirming their previous male attribution (Table 1). The remaining samples, including those putatively classified as adult females, yielded mtDNA PCR

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Table 1. Age and sex structure mitochondrial DNA lineage attribution of the 12 Neandertal individuals present at the El Sidrón site

Individual	Sample	Age	Morphological sex	Genetic sex	Mitochondrial lineage
Adult 1	Tooth	Young adult	Male	Male	A
Adult 2	Tooth	Young adult	Male	Male	A
Adult 3	Mandible	Adult	Female	?	B
Adult 4	Tooth	Young adult	Female	?	C
Adult 5	Tooth	Adult	Female	?	A
Adult 6	Tooth	Adult	Male	Male	A
Adolescent 1	Tooth	12–15 y	Male	Male	C
Adolescent 2	Tooth	12–15 y	Male?	?	A
Adolescent 3	Tooth	12–15 y	Male	?	A
Juvenile 1	Femur	5–6 y	?	?	C
Juvenile 2	Tooth	8–9 y	?	?	A
Infant	Phalanx	2–3 y	?	?	C

Lineage A has the 200A-204T-16124A haplotype, lineage B the 200G-204C-16124G haplotype, and lineage C the 200G-204T-16124G haplotype (Vi33.16 reference sequence). In addition, all lineages differ from the Vi33.16 Neandertal by an A to C transversion at position 16177. Vi33.16 has the following haplotype at El Sidrón polymorphic positions: 200G,-204T-16124A.

products but not Y-chromosome products. Thus, the results of the Y-chromosome assay are consistent with the sex identification based on morphological traits. However, the sexing method used can generate false-positive results for females, because the absence of amplification can indicate either absence of the Y chromosome or insufficient genomic coverage in a particular sample.

Previous analyses have determined the complete mtDNA genome of one postcranial bone sample (SD-1253) (7) as well as complete or partial hypervariable regions 1 and 2 (HVR1 and HVR2) from a number of other bone fragments, yielding three closely related but distinct mtDNA haplotypes, A (200A-204T-16124A), B (200G-204C-16124G), and C (200G-204T-16124G). All three haplotypes differ from the Vindija 33.16 reference sequence by a C to A transversion at position 16177 (8). However, the sequences show a close phylogenetic relationship with those from Neandertals at Vindija and Feldhofer (sharing with them the 16073G-16149C haplotype), suggesting there was very little population stratification in this species across Western Europe (9). In previous analyses, meaningful inferences about the demographic characteristics of the El Sidrón Neandertal group were precluded by the fragmentary nature of the remains. However, dental-association analyses now have enabled us to determine that at least 12 distinct individuals are represented in the remains. For the most part, teeth were not subjected to destructive drilling in order to obtain DNA samples. They had not been specifically identified as potential DNA sources during the excavation and were thus heavily contaminated. However, we were able to select one sample per individual by relying on small dentine fragments that has dislodged naturally, one mandible bone fragment (adult 3), and in the cases of juvenile 2 and the infant, postcranial bones that could be indisputably associated based on their size and epiphyseal closure.

Conventional PCR (Table S2) and cloning were used to sequence of 11 phylogenetically informative Neandertal mtDNA positions in all 12 El Sidrón Neandertals [positions 200, 204, 16124, 16134, 16143, 16229, 16239, 16251, 16253, 16258, and 16259 (8)] (Table S3) as well as the complete HVR1 in the three adult males and juvenile 1 (Table S4). Diagnostic mtDNA fragments were replicated in an independent laboratory in four specimens (Table S4). Although it is possible that additional, previously unidentified polymorphisms may remain undetected in unsequenced sections of the mtDNA genome of these individuals, the resulting haplotypes nonetheless would stem from the already-described El Sidrón A, B, and C lineages and thus should not affect significantly the pattern of diversity observed in this study. We were able to infer that the most studied El Sidrón bone sample, SD-1253 (7, 10–15), corresponds to adolescent 1 because of the unique combination of mtDNA lineage C and male sex (7, 12).

Table 1 shows that all but 1 of the 12 individuals in the El Sidrón group carry mtDNA lineages A (seven individuals) and C (four individuals). Assuming that Neandertal groups were kin structured like other hominoid species, it seems reasonable to assume further that these two lineages represent two groups of very close relatives in the female line. The probability of recovering such a configuration from a large sample of HVR1 and HVR2 sequences from unrelated modern humans (16) by chance is very low ($P < 0.0001$ and $P = 0.013$, respectively). Previous estimates of mean pairwise differences within the HVR1 mtDNA suggested that Neandertals had an effective population size similar to that of the modern Europeans (17). For the El Sidrón sample, this value is 1.23 substitutions, significantly lower ($P < 0.0001$) than the mean pairwise differences (6.78; minimum and maximum pairwise values are 2.47 and 11.30, respectively) esti-

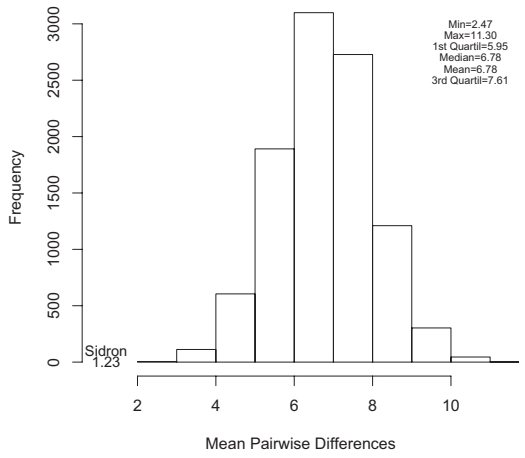

Fig. 1. Distribution of pairwise nucleotide differences among modern Europeans. Pairwise nucleotide differences were calculated from 10,000 iterations of 12 sequences randomly extracted without replacement from a HVR1 and HVR2 mtDNA European dataset. The value obtained for the 12 El Sidrón individuals (1.23) falls outside the current pairwise distribution.

mated from 10,000 random sets of 12 sequences extracted without replacement from a large European dataset (Fig. 1). Thus, the El Sidrón mtDNA diversity is significantly lower than any random subsample of sequences from unrelated modern Europeans, lending support to the taphonomically derived hypothesis that El Sidrón represents a family group and suggesting that mtDNA genetic diversity was low within such Neandertal groups.

Interestingly, each of the three adult females belongs to a different mtDNA lineage, whereas the three adult males all carry lineage A (Table 1). Although we have only fragmentary HVR1 and HVR2 sequences for the females, we know they carry different haplotypes because they differ in the three key positions that determine A, B, and C lineages. In the three males the complete HVR1 and partial HVR2 sequences are identical. We note that this finding may indicate patrilocal mating behavior among Neandertal groups. Patrilocality is present in about 70% of modern societies (18) and is expected to result in greater diversity of mtDNA lineages among females in social groups than among males—as we found in this study. Patrilocality also is expected to result in a wider geographical dispersal of mtDNA lineages relative to those of the Y chromosome. This expectation is consistent with the finding of minimal phylogeographic structure among the mtDNAs of Western European Neandertals (7). Further research will reveal whether Neandertals exhibit greater geographic patterning for the Y chromosome.

Based on the ages of the El Sidrón group members and their mtDNA lineages, we speculate that juvenile 2 is the offspring (or close matrilineal relative) of female adult 5 and that juvenile 1 and the infant are the offspring of female adult 4. If correct, the latter relationship would indicate an interbirth interval of around 3 y for Neandertals. This period fits with the average 3.4-y interbirth interval reported for several modern hunter-gatherer groups (19). The length of lactation is the main factor in determining female fertility in human populations, and therefore this information could help in modeling Neandertal population dynamics. In conclusion, the results obtained in this study of a putative social Neandertal group provide tantalizing clues about the demography and behavior of the species that once was our closest living relative and could be used to help understand the factors that contributed to their extinction.

Materials and Methods

Identification of Individuals. A minimum number of 12 individuals has been identified in the El Sidrón Neandertal fossil assemblage (Table 1). The El Sidrón individuals have been identified mainly by their dentition, except for the juveniles and the infant that have specific postcranial elements associated. As of the 2009 field season, the El Sidrón dental sample is composed of 170 teeth. There are 88 lower teeth (from the 51 left side, 37 from the right side), of which 43 are in situ and 45 are isolated. Upper dentition is represented by 82 teeth (45 from the left side, 37 from the right side), of which 21 are in situ and 61 are isolated. Two nearly complete mandibles, two hemimandibles, and two more small mandibular fragments are preserved also.

In addition, a complete maxilla, which included 13 teeth in situ, and two fragments of maxillae with four teeth each are preserved. A dental set (SD-1327a–i) found with the teeth in anatomical contact was found also, but the bony support (mandible) had dissolved away. Individuals were assigned to one of five age categories (20, 21): "infant," "child," "juvenile," "adolescent," and "adult." The age at death for the nonadult individuals was estimated based on the accelerated dental formation chronology previously proposed for Neandertal populations (22). Among the adults, different categories have been distinguished (young adult, adult, and mature) based on the differences of the degree of occlusal dental wear following wear stages (23). For postcranial immature remains, age at death was estimated following methods of bone growth (24). The sex of the individuals was estimated using measurements of the mandibular corpus and differences in the canine tooth size in the context of the sample and other European Neandertals (Table S1 and Figs. S2 and S3). No canines are yet available for adolescent 2, although the general size of the dentition suggests it could be a male (Table 1). However, the accuracy of sex attribution of the immature skeletal material decreases significantly, because secondary sex traits are not discernible before puberty (25, 26).

Different criteria have been used to secure the association of the teeth for each particular individual. In order of importance, these criteria are anatomical connection, fitting of a tooth in its alveolar socket, matching interproximal wear facets, degree of occlusal dental wear, dental developmental age and eruption rates, crown and root morphology of the teeth, presence of enamel hypoplasia, presence and distribution of dental calculus and oral pathologies, taphonomic features (e.g., color), chipping enamel features, and pattern and abundance of cultural striations. Ten of the samples are either in anatomical connection to the remaining individual's dentition or in situ in the mandible itself. The two remaining samples (those corresponding to adult 2 and adolescent 1) have been assigned to the respective individuals because the degree of wear and tooth size and morphology are identical to the rest of the individual's dentition.

Mitochondrial DNA Extraction, Amplification, and Sequencing. Dentine powder samples (usually <100 mg) and some bone samples were extracted as described (15), and mtDNA was amplified using a two-step PCR protocol (12). In each PCR, one or two blocking primers, designed to anneal to the anatomically modern contaminant sequences (Cambridge Reference Sequence haplotype) and prevent their amplification (27), were added. Amplification products were cloned with the TOPO TA cloning kit (Invitrogen) and a number of clones were sequenced with an ABI3730 capillary sequencer (Applied Biosystems). DNA sequences from subsamples of seven of the specimens, corresponding to adults 1, 3, 4, and 5 and the three adolescents, were replicated independently in a second laboratory. Blocking primer sequences, clone sequences, and detailed experimental procedures are described in *SI Materials and Methods*.

Genetic Sex. Molecular sexing of the El Sidrón individuals was performed through PCR amplification and sequencing of an ancestral Y-chromosome marker (12). A Neandertal diagnostic mtDNA fragment was included as an amplification control. The phylogenetic tree of the modern human Y chromosome is well established (28), with several studies estimating the age of the most recent common ancestor of all modern human Y chromosomes to be about 90,000 y ago (29). Because Neandertal and human lineages diverged much earlier (11), it is assumed that Neandertal individuals will have the ancestral state in the deepest nodes that define the human Y-chromosome genealogy. Therefore, we used a previously designed primer pair (12) to determine a SNP, Y2-M49 (12), that typifies the divergence of the most basal African lineages (A1, A2, and A3) in the Y-chromosome tree. Along with Neandertals, only a small minority of modern humans carry an A at this SNP, all of whom are members of Y-chromosome haplogroup A, which is found almost exclusively in sub-Saharan Africans. (The majority of Y chromosomes found in modern humans carry a T allele at the Y2-M49 SNP.) Y chromosomes belonging to haplogroup A are absent in the Iberian Peninsula and North Africa, based on two large samples of 1,708 and 361 Y chromosomes, respectively (30, 31). It is expected that male individuals will yield Y-chromosome and mtDNA sequences and that females will yield only mtDNA sequences. When only mtDNA samples are yielded, however, the possibility that the Y-chromosome amplification simply has failed cannot be rejected.

Phylogenetic Analysis. Sequence alignments of 473 modern European HVR1 and HVR2 mtDNA sequences (between positions 70–370 and 16,036–16,401) were retrieved from the Human Mitochondrial Genome database (http://www.genpat.uu.se/mtDB) (16). The mean pairwise differences for the 12 El Sidrón individuals were estimated with the MEGA3.1 software, and the value obtained was compared with the distribution generated by randomly sampling 12 sequences without replacement 10,000 times (Fig. 1). Although there is no overlap between the observed and simulated values, we acknowledge that the diversity of modern mtDNA genetic datasets generally is exaggerated because of the intentional sampling of maternally unrelated individuals.

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