

## Supporting information

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### SI Materials and Methods

**El Sidrón cave: evidence for a synchronic accumulation.** The El Sidrón cave lies in the Surco of Oviedo-Infiesto. It is a karstic system that has developed in Tertiary carbonate conglomerates alternating with fine to medium-grained sandstones. The main axis of the cave measures ca. 600 meters of length (of a total of 3,700 m mapped), with a difference in height of 4.67 m. It can be considered a pressure tube of a subterranean river, aligned in a NNW to SSE axis. The lateral ramifications, that include the Ossuary Gallery, where the neandertal bones are, run mainly N-S. This gallery is located 220 m from the nearest entrance to the karstic system, called the Tomb Entrance (1).

The sedimentary infill of the Ossuary Gallery has been studied with a geological mapping based on stratigraphic cross-sections every 33 cm. Five main units corresponding to five events with specific hydrodynamic sedimentary traits can be defined. The unit that holds most of the bones and lithic remains is unit III (2). This unit consists of a massive debris flow deposit, composed by a chaotic mixture of gravels, mud and water. The entrance of these materials is associated to a high energy event. The current data indicate that the neandertal remains were dragged down from a higher level, either from the surface or from an undiscovered upper gallery. A mass of loose material situated in that higher level went downstream into the cave in a single occurrence due to a violent storm followed by a collapse. It seems that the skeletal and archaeological material entered the gallery through one of the vertical shafts located in the G/H-9/10 sections of the Ossuary Gallery, which subsequently became blocked with a filler of clay and pebbles (2). The spatial projection of the numerous lithic refittings (Fig. S1) confirms this hypothesis and demonstrates the existence of a debris-flow cone that caused the current distribution of both the archaeological and the paleontological materials. Some other evidence confirms this scenario for the origin of El Sidrón assemblage. First, the osteological preservation of the bone surface is excellent, with little or no evidence of weathering and erosion. Secondly, the bones do not display large carnivorous toothmarks or other evidences of scavenging activities, suggesting they were exposed on the surface for a very short time. Thirdly, there is an almost complete absence of other materials, such as animal bones, apart from the neandertal bones and the lithic artefacts. And fourthly, some skeletal specimens, such as partial thoracic cage or foot bones, are still in anatomical connection, indicating limited *post mortem* displacement of the remains.

The artefacts recovered from the El Sidrón site have been dated using a number of different methods, including AMS  $^{14}\text{C}$  (Accelerator Mass Spectrometry Carbon-14), U/Th (Uranium Thorium), OSL (Thermoluminescence), ESR (Electron Spin Resonance) and AAR (Amino Acid Racemisation). Although initial  $^{14}\text{C}$  ages were likely underestimated due to contamination of the samples by modern carbon, subsequent dating incorporating filtration of the contaminant carbon with the ninhydrin protocol produced more reliable  $^{14}\text{C}$  dates of circa 49,000 years ago (3), that are in agreement with those obtained with the other dating methods (3). Dates from different materials along the site are concordant with a synchronic accumulation event of the neandertal bones within the site.

**Bone sampling.** Fifteen bone flakes with no morphological entity extracted at El Sidrón site were originally selected for paleogenetic analysis. Some of them were retrieved with the anticontamination protocol implemented at the site; however, some others come from the surface of the stratigraphic cut, and were exposed to water filtration at the beginning of the excavation season. The samples have the following inventory numbers: SDR-170, SD-1252, SD-1253, SD-1343, SD-1351a, SD-1351b, SD-1351c, SD-1351d, SD-1351e, SD-1415, SD-1416, SD-1420, SD-1434, SD-1435, SD-1504. The bone flake samples mainly comprise material from the 2006-2009 field seasons, although one sample (SDR-170) was retrieved in 1994. From a morphological point of view, the small size of these samples only allows inference that they belong to adults or adolescents, with the probable exception of sample SD-1351e that could belong to a juvenile because of its thin cortical tissue. Additionally, sample SD-1343 can be identified as belonging to an adolescent because of partial epiphyseal plate fusion, although at present it is impossible to know to which one. Although it is impossible to discern whether the remaining bones represent a random sample among all the individuals, the scattering of the remains and differences in size and robustness indicates that several adult individuals are also represented.

Partial or complete mtDNA hypervariable region 1 and 2 sequences have in the past been retrieved by PCR and cloning from all these samples. The results show that there are only three polymorphic positions among El Sidrón bone samples: 200, 204 and 16124 (Vi33.16 reference sequence). Additionally, all El Sidrón mtDNA haplotypes differ from Vindija 33.16 by an A to C substitution at position 16,177. Samples SD-1253, SDR-170, SD-1415, SD-1420, SD-1504 had the C lineage (200G-204T-16124G); samples SD-1351a, SD-1351b, SD-1351c, SD-1351d, SD-1351e, SD-1435 the A lineage (200A-204T-16124A), and sample SD-1416 the B lineage (200G-204C-16124G). At present it is impossible to know with certainty to which mtDNA lineage the samples SD-1343 and SD-1434 belong to, due to limited sequencing.

Although only one complete El Sidrón mitogenome (C lineage) has been retrieved so far, it is extremely unlikely that identical HVR1 and 2 haplotypes could harbour differences in the mtDNA coding region, because the HVR1 and 2 mutation rate is at least ten times higher than the rest of the mtDNA genome and also because the small effective population size of neandertals does not favour the existence of parallel mutations within the population.

We noticed that the El Sidrón bone specimens showed two discrepancies in positions 16173 and 16177 to a previously published bone sample, SD-1252, in a section that was amplified by an independent laboratory (4). We sequenced this section *de novo* from SD-1252 in four different amplifications using different primer sets, and found only the nucleotides T in 16173 and A in 16177, like in all other El Sidrón bones analysed. Therefore, the SD-1252 sample also carries the majority El Sidrón mtDNA lineage (A haplotype), although at present it is not possible to know exactly to which individual it belongs to.

**Individual sampling.** A minimum number of 12 individuals (MNI) have been identified in the El Sidrón neandertal fossil assemblage. These correspond to six adults, three adolescents, two juveniles and one infant. Dental evidence is mainly used for this determination together with specific postcranial elements in the juveniles and the infant. Teeth are the most represented skeletal parts in the paleoanthropological record, both because of its relative abundance within the organism (32 pieces in a hominoid), as well as its relative hardness and durability. The sex of the individuals was assigned following standard traits of robustness in mandibles and maxillae, when preserved, but also from the dimensions of the most sexually

dimorphic tooth, the canine (Table S1, Figures S2 and S3). The sex of the adolescent 2 cannot be ascertained from canines, due to the lack of these teeth in this individual.

The El Sidrón teeth were not selected *a priori* for ancient DNA analysis due to their anthropological value, and thus they were handled and washed after the excavation with no special anti-contamination precautions. Moreover, some of the most complete dental specimens were discovered in the Ossuary Gallery in 1994, six years prior to the regulated excavation that started in 2000, and before the implementation of the anti-contamination protocol, in 2005. Therefore, we faced the problem of limited sampling availability, and in many cases, heavily contaminated specimens for the individual genetic analysis. Following the recommendations for preserving hominin remains from invasive sampling (5), no samples were obtained by drilling into the teeth. The fragmentation of some teeth during the drying process after excavation produced small pieces of dentine that were too small for allowing the reconstruction of the original dental root. These dentine fragments were selected for DNA analysis. The dental samples weighed between 20 and 100 mg.

In one case, a broken mandible fragment (SDR-011) was included in the analysis, and in two other cases, postcranial bone fragments (a femur, SD-1634, and a finger bone, SD-634) that could be attributed to particular immature individuals due to its age, as deduced from bone size and epiphyseal closure. These skeletal samples were slightly larger, weighing 230, 440 and 150 mg, respectively.

**DNA extraction and amplification.** DNA extraction in Barcelona was performed as described elsewhere (6). Specifically, samples were ground; the powder was subjected overnight to a decalcification with 5 mL of 0.5 M EDTA, at 37°C. After centrifugation, the liquid phase was discarded and the remaining pellet was incubated overnight at 50°C in 10 mL of lysis buffer (0.5% SD-S, 50 mM TRIS, and 1 mg/mL of proteinase K in H<sub>2</sub>O). Subsequently, the samples were extracted in three successive steps, with phenol, phenol-chloroform and chloroform-isoamyl alcohol. The extract was finally concentrated to a volume of 50 µl with centricon filters (Millipore, Billerica, MA, USA) with a 30,000 Dalton molecular cut-off.

For the amplification of the mtDNA HVR1, a two-step PCR protocol was used (7). Both steps included 2 U AmpliTaq Gold (ABI, USA), 1X AmpliTaq Gold buffer (ABI, USA), 2.5 mM MgCl<sub>2</sub> (ABI, USA), and 500 µM for each dNTP. A set of blocking primers, designed to match the majoritary CRS contamination in the samples, were designed along the HVR1 (Table S2). The blocking primers have their 3' end modified with a C3 spacer that prevents the TaqDNA polymerase from extending it once they are annealed to the contaminant DNA (8). In the first multiplex step, 150 nM of each primer pair (both standard and blocking primers) and 5 µl of DNA extract were included, in a final reaction volume of 20 µl.

First step amplification consisted in a 12 min activation step at 94°C, followed by 27 cycles at 94°C for 20 s, 50°C for 20 s, and 72°C for 20 s. Five µl of a 1 to 20 dilution of the primary amplification product were used as a template for the second PCR. Conditions were as described for the first step, except that the standard primer concentration was increased to 1.5 µM and the blocking primer concentration was maintained at 150 nM. The PCR programme was the same as the first step, except that 33 cycles were performed, followed by a final step of 11 min at 72°C. Blank and mock PCR controls were included in each amplification to monitor against contamination.

After visualization of the products on 2% low melting point agarose gels, amplification products of the expected size were excised from the gel with a sterile razor blade. DNA was purified with a GeneClean silica method, and cloned using the Topo TA cloning kit (Invitrogen, The Netherlands). Insert-containing colonies were subjected to 30

cycles of PCR with M13 universal primers, and subsequently sequenced with an Applied BioSystems 3100 DNA sequencer, at the Servei de Seqüenciació of the University Pompeu Fabra (Barcelona). Some PCR products were pooled together and sequenced with the 454 pyrosequencing GS-FLX (Life Sciences) at the Center for Genomic Regulation (CRG) of Barcelona, following manufacturer's instructions.

For the mtDNA HVR1 analysis, previously published primers were used. For the mtDNA HVR2 analysis, two sets of primers were used. The highly-specific neandertal H235 primer (5'-TGCAGACATTTAGTCGTTATTAT-3', with 3 mismatches to the human reference sequence) was combined with L188 (5'-ACGTTCAATATTACAGGCGAG-3') or L198 (5'-TTACAGGCGAGCATACTTAC-3') to produce amplicons of 98 bp and 79 bp respectively.

Some HVR1 amplifications, specially the short L16119-H16149 and L16104-H16135 fragments (Table S4), designed to check the diagnostic position 16124, yielded only contaminant sequences. This can be attributed to the fact that, in these cases, only one blocking primer per reaction (H16135 or H16149) could be incorporated. These problematic amplifications were repeated with a higher blocking primer concentration in both steps (between 1 and 1.5  $\mu$ M, depending on the sample), which increased the endogenous sequences ratio in the product. In the HVR2, the haplotype 200G-204C defining lineage B is not neandertal-specific, although it is rare among modern humans and has only been described among the African L3 haplogroup and the Eurasian W haplogroup. At least in one specimen (SD-763a), corresponding to adolescent 1, it was possible to retrieve those positions in a long amplicon, encompassing also the neandertal 243G position (using the H245 primer). Thus, the resulting haplotype 200G-204T-243G is neandertal-specific. Although we cannot exclude that the HVR2 sequence in the SDR-011 sample could be due to some form of modern human contamination, the fact that i) no W haplogroup carriers are present among the archaeologists, paleontologists and the molecular biologists from Barcelona involved in the project, ii) the contamination should have to be present only on this particular sample and not the others that were excavated and manipulated by the same people, and iii) the H235 primer is highly specific for neandertals, makes this possibility less parsimonious.

An attempt was made to retrieve the HVR1 with the primer extension capture (PEC) (9) and 454 pyrosequencing approach in one of the El Sidrón contaminated samples, SD-1343, at the CRG of Barcelona. Although approximately 40% of the 109,705 sequences generated were identified as mitochondrial, only twenty of these were compatible with a potential neandertal origin, while the remainder reflect contaminant modern human DNA. In contrast, from the same extract, it was possible to retrieve by PCR+blocking primers one Neandertal-specific mtDNA fragment almost free of contaminants (primers L16225-H16257). These results suggest that the massively parallel sequencing approaches capture more efficiently the overwhelming contaminant background on these problematic samples, while the specific PCR-based approaches can prevent the amplification of at least part of these contaminants.

Primers for the Y-chromosome marker were the same previously used in Barcelona and described in (7). A single forward blocking primer, designed to anneal to the putative human contaminant sequences (5'-AATCAGATTTAGGACACAAAAGCT-3') was also included in the PCR.

**Complete HVR1 in four samples.** Four El Sidrón samples, corresponding to the three adult males and the juvenile 1 (SD-441, SD-753, SD-1240 and SD-1634, respectively) were better preserved than the rest, yielding a majority of neandertal sequences even in those mentioned problematic mitochondrial regions. Therefore, the complete HVR1 was generated by using

multiple overlapping fragments and neandertal-specific primers. SD-441, SD-1240 and SD-1634 were retrieved in 14 overlapping fragments and SD-753 in 18 fragments (both between nt positions 16031 and 16396) (Table S4). The three adult male samples carry the El Sidrón mtDNA haplotype A, while the juvenile 1 carries the haplotype C.

The combination of severe DNA degradation, limited sampling and high levels of modern human contamination precluded the retrieval of additional mtDNA fragments from the rest of the individual samples. An attempt to shotgun sequence DNA from one of the most problematic teeth samples (SD-331c) by 454/FLX pyrosequencing produced negative results because of insufficient DNA concentration for the genomic library preparation, even after primer-adaptor amplification.

Future genotyping of nuclear markers in bone samples with individual attribution could help elucidate the precise genealogical relationships among the El Sidrón individuals. Because of the aforementioned problems associated to teeth samples, any further analyses will have to be carried out on well preserved bone samples that could be related to specific individuals, such as the sample SD-1253. However, due to the low mtDNA variation within the group, it is likely that not all the individuals will have indisputably associated bone samples.

**Independent replication in four specimens.** Seven subsamples, corresponding to adults 1 (SD-441), 3 (SDR-011) and 5 (SD-1327h), all three adolescents (SD-763a, SD-566 and SD-500) and juvenile 1 (SD-1634) were sent to the Centre for GeoGenetics, Natural History Museum of Denmark for independent replication. Primers designed to retrieve the diagnostic HVR1 16,124 position and the HVR2 200 and 204 positions were used to generate PCR products that were subsequently cloned and sequenced. For the first fragment PCR amplification was performed in 25  $\mu$ L volumes, using 1 $\times$  PCR buffer, 2 mM of  $MgSO_4$ , 1.6 mg/ml Bovine Serum Albumine (BSA), 0.2  $\mu$ M of each primer, 2  $\mu$ M of blocking primers, 0.2  $\mu$ M of dNTPs, 0.5U of High Fidelity Platinum Taq (Invitrogen, Carlsbad, CA) and 2  $\mu$ L of template. Cycling conditions were: 94  $^{\circ}C$  for 2 min; 60 cycles of 94  $^{\circ}C$  for 30 s, 50 $^{\circ}C$  for 30 s and 72  $^{\circ}C$  for 30 s followed by 72  $^{\circ}C$  for 7 min. The two step PCR protocol was used for the amplification of the HVR2 fragment. PCR products were diluted 1:10 and subsequently cloned using TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). A minimum of 32 clones was sequenced for each sample. Four samples, corresponding to SD-441, SD-1327h, SD-500, and SD-763a yielded neandertal sequences, the first three for the HVR1 fragment and the later for the HVR2. All haplotypes were identical to those found in the Barcelona laboratory.

## References

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**Table S1:** Dimensions of the El Sidrón canines for the individual sex attribution.

**Table S2. List of the L and H blocking primers used for the mtDNA HVR1 retrieval.** The blocking primers match the human reference sequence and carry a C-3 spacer at the 3' end that prevents the extension by the Taq DNA polymerase.

**Table S3: Diagnostic mtDNA positions genotyped at El Sidrón individualised samples (Vi33.16 reference sequence).** For SD-441, SD-753, SD-1240 and SD-1634 the complete HVR1 was also retrieved (between positions 16031-16397) (see Table S4). Individual adult 6 is represented by two different samples.

**Table S4: HVR1 and 2 mtDNA sequences of clones derived from PCR products in all El Sidrón samples with individual attribution.** In cases where many (>20) clones have been generated, only 10-15 are displayed for clarity. Primers and clones are numbered according to CRS (modern human reference sequence) for clarity. B: Barcelona; C: Copenhagen.

**Figure S1: Horizontal and vertical projection of all the refitted lithic industry within the Ossuary Gallery at El Sidrón.** The distribution shows that the lithic tools were specifically made *in situ* for the anthropic activities associated to the deposit, and also illustrates the existence of the debris-flow cone that entered the remains into the gallery in a single collapse. In grey, there is the area removed by the Civil Guard, after the accidental discovery of the site in 1994.

**Figure S2:** Representation of the bucco-lingual and mesio distal diameter of the mandibular El Sidrón canines, for each individual as well as the mean average (A: Adult, Ad: Adolescent).

**Figure S3:** Representation of the bucco-lingual and mesio distal diameter of the maxillar El Sidrón canines, for each individual as well as the mean average (A: Adult, Ad: Adolescent).

Table S1

	Mandibular Canine						Maxillary Canine					
	Right			Left			Right			Left		
	MD	BL	LABEL	MD	BL	LABEL	MD	BL	LABEL	MD	BL	LABEL
Adult 1	8.5	9.3	SDR-006e				8.8	10.2	SD-1010	8.3	10.2	SD-1202
Adult 2							8.7	10.7	SD-1427i	8.8	10.7	SD-1427g
Adult 3										8.5	9.6	SD-1220
Adult 4	7.9	9.0	SD-209				8.3	9.7	SD-1661	8.3	9.4	SD-1018
Adult 5				7.4	8.9	Sd-1327e						
Adult 6				8.3	9.2	Sd-753				8.7	10.1	SD-1161
Adolescent 1							8.9	10.6	SD-221			
Adolescent 3				8.4	9.3	Sd-61				9.2	10.9	SD-1107
El Sidrón Mean	MD= 8.1			BL= 9.1			MD= 8.6			BL= 10.2		



Table S2

L16073 AAGTATTGACTCACCCATCAA  
L16164 CCACCTGTAGTACATAAAAACCC  
L16177 AAACCCAATCCACATCAAAC  
L16204 CCCCATGCTTACAAGCAAGT  
L16229 GTACAGCAATCAACCCTCAACTATCAC  
L16258 ATCACACATCAACTGCAACTCCAAAGCCACCCC  
L16295 CCAACAAACCTACCCACCCTTA  
L16315 CAGTACATAGTACATAAAGC  
L16358 ACAGTCAAATCCCTTCTCGT  
H16135 TTATGTACTACAGGTGGTCAAGTAT  
H16149 GATTGGGTTTTTATGTACTA  
H16164 GGGTTTTGATGTGGATTG  
H16218 GCAGTTGATGTGTGATAGTTGAG  
H16251 GTTTGTGGTATCCTAGTGGGTGAGGGGTGG  
H16295 TAAATGGCTTTATGTACTATGTACTGTT  
H16358 TATCTGAGGGGGGTCATCCATGGGGA  
H16396 GATTCACGGAGGATGGTGG

Table S3

Ind	Sample	200	204	16124	16134	16143	16229	16239	16251	16253	16257	16259
Adult 1	SD-441	A	T	A	T	T	T	A	A	G	T	A
Adult 2	SD-1240	A	T	A	T	T	T	A	A	G	T	A
Adult 3	SDR-011	G	C	G	T	T	T	A	A	G	T	A
Adult 4	SD-331c	G	T	G	T	T	T	A	A	G	T	A
Adult 5	SD-1327h	A	T	A	T	T	T	A	A	G	T	A
Adult 6	SD-753	A	T	A	T	T	T	A	A	G	T	A
Adult 6	SD-1161	?	?	A	T	T	T	A	A	G	?	?
Adolescent 1	SD-763a	G	T	G	T	T	T	A	A	G	T	A
Adolescent 2	SD-566	A	T	A	T	T	T	A	A	G	T	A
Adolescent 3	SD-500	A	T	A	T	T	T	A	A	G	T	A
Juvenile 1	SD-1634	G	T	G	T	T	T	A	A	G	T	A
Juvenile 2	SD-763b	A	T	A	T	T	T	A	A	G	T	A
Infant	SD-634	G	T	G	T	T	T	A	A	G	T	A





























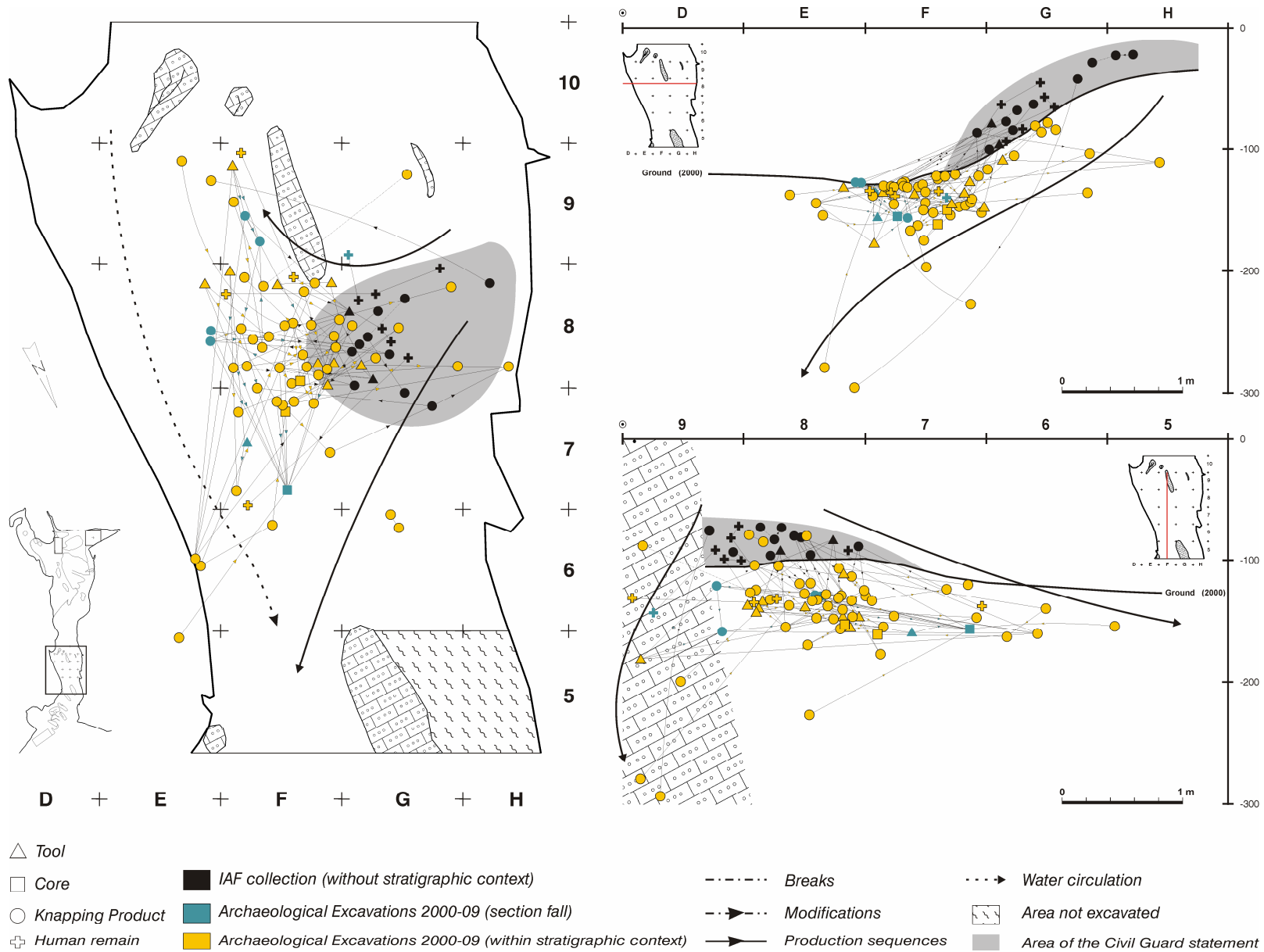


Figure S1



Figure S2

