Supplemental Data

The Derived *FOXP2* Variant of Modern Humans Was Shared with Neandertals

Johannes Krause, Carles Lalueza-Fox, Ludovic Orlando, Wolfgang Enard, Richard E. Green, Hernán A. Burbano, Jean-Jacques Hublin, Catherine Hänni, Javier Fortea, Marco de la Rasilla, Jaume Bertranpetit, Antonio Rosas, and Svante Pääbo

Supplemental Experimental Procedures

Description of El Sidrón Site and Bone Samples

The El Sidrón is a young karstic system located in the Asturias region in northern Spain (X: 311.572,815; Y: 4.806.338,042; Z: 167,89) [S1]. Around 1323 Neandertal remains and 358 lithic artifacts have been recovered through systematic excavation since the year 2000. Three human samples were dated by accelerator mass spectroscopy ^{14}C at Beta Analytic (Miami, Florida); the average calibrated age was 43,129 ± 129 cal BP (CalPal2005) [S2, S3]. The fossils were discovered in a small gallery (Galería del Osario), about 28 m length and 12 m at its widest, which is presently blocked at its southern end. The gallery is located around 220 m into the cave system. Fossil bones were recovered from the central area of the gallery, an area no larger than 6 m^2.

The Galería del Osario was connected to the earth's surface at some point during the Pleistocene. The original deposition of the bones was located outside the cavity. A potential collapse of a doline might have resulted in the deposition of the Neandertal remains into the cave [S1, S3]. After the refitting of several bone fragments and 17 flakes, evidence of limited displacement appears. Some skeletal remains, such as a partial thoracic cage or foot and hand bones, have been found anatomically connected.

The site's depth provides very stable temperature conditions throughout the year (10–12°C). The exceptional taphonomic conditions and the fact that the individuals had been cannibalized [S3] probably explain the excellent preservation of the endogenous DNA in remains from the site.

The bones 1253 and 1351c are fragments of long bones that came from mature individuals with Neandertal morphological features (discussed in [S3]) and that were deliberately fragmented, presumably to retrieve the bone marrow. They were found in 2006, removed under sterile conditions from the sediment in the cave, and stored and transported at −20°C. They were subjected to computer tomography in a frozen state and subsequently sampled in our clean-room facility.

High-Frequency-Derived Alleles in *FOXP2* Intron

If the high-frequency-derived alleles are removed from the data sets [S4], the excess of high-frequency-derived alleles used to date the sweep disappears (H = 2.98, p = 0.37 from simulations). The value of H upon removal of the nine high-frequency-derived alleles and the resulting statistical significance was computed with DNAsp. The p -value was determined from coalescent simulations assuming a fixed value of segregating sites and no recombination, for 10,000 iterations.

Experimental Procedures in Leipzig

A detailed overview of all results for all four multiplex PCRs carried out on the two Neandertal DNA extracts can be found in Table S1. The individual clone sequences of all PCR products obtained in Leipzig can be found in Table S2. Primer sequences can be found in Table S5.

Positioning of basal Y chromosomal SNPs in the Y-DNA haplogroup tree that were amplified with control primer pairs Y1–Y5 are illustrated in Figure S1.

Experimental Procedures in Lyon

The extraction was conducted in a laboratory exclusively dedicated to ancient-DNA analysis. A mock extract and 400 mg of powder from a cave bear bone were extracted at the same time as the Neandertal specimen, to act as extraction controls. The Neandertal bone El Sidrón 1253, which is kept frozen, was sampled in the clean room in Leipzig, and then 388 mg was sent on dry ice to Lyon. There, the sample was reduced to powder with a hammer in a sterile enclosed plastic bag and incubated at 37°C for 48 hr in 7.5 ml of extraction buffer (EDTA 0.5 M, pH = 8.5, N-lauryl sarcosyl 0.5%, proteinase K 0.5 mg/ml). The phenol/chloroform/isoamylic alcohol extraction and centricon-30 (Amicon) concentration procedures were completed as described in [S5]. The final ~150 μl recovered from the extract were distributed in 5 μl aliquots and stored at −20°C. Because the powder was not completely digested by the extraction procedure, the pellets were re-extracted at 55°C for 48 hr in order to recover the remaining genetic material. The ~110 μl recovered from this re-extraction were aliquoted in 5 μl and stored at −20°C. In order to remove *Taq* polymerase inhibitors, the extract (or re-extract) was partially purified, concentrated, and eluted in elution buffer with MinElute (Invitrogen) spin columns before each PCR session. Two-step multiplex-PCR reactions were performed in a total volume of 20 μl in a Mastercycler apparatus (Eppendorf), according to the procedure described in [S6]. For the first step of amplification, PCR tubes contained 0.5 units of shrimp nuclease (Biotec Pharmacon), 4 mM MgCl2, 1 mg/ml BSA, 250 μM of each dNTP, and 150 nM of each primer. Before the amplification process was started, the PCR tubes were incubated for 10 min at 25°C to remove every trace of dsDNA by the shrimp-nuclease activity. The nuclease was then heated to 70°C for 60 min in order to inactivate it. Four microliters of the purified extract (or re-extract or mock extract or elution buffer) and 2 units of *Taq* Gold polymerase (Perkin-Elmer) were then added to the PCR tubes. A 10 min activation step at 94°C was followed by 30 cycles of denaturation (94°C, 20 s), annealing (60°C, 30 s), and elongation (72°C, 30 s). The final elongation step lasted 4 min. The same PCR conditions were used for the second step of amplification, except that (1) 2 μl of the first-round amplification products were used after a 16× dilution in sterile water, and (2) *Taq* and primer concentrations were changed to 0.25 unit and 1.5 μM, respectively. Two independent blanks (one mock extract and one PCR blank) were also subjected to this process in order to monitor contamination during each PCR round. All PCR products (19 for the extract and 10 for the re-extract; Table S3) were cloned with the Topo TA cloning kit (Invitrogen) according to the manufacturer's instructions. Colonies positive for insertion were screened by PCR into a 20 μl reaction mix using M13 universal primers (5′-GTT TTCCCAGTCACGACGTTG-3′) and REV (5′-TTTCACACAGGAAACA GCTAT-3′) and 40 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and elongation (72°C, 40 s) (final elongation step: 72°C, 5 min). PCR products were further sequenced by a service provider (Cogenics). Cave bear DNA was successfully amplified from the cave bear extract (primers: H16143-H2 and PCR conditions described in [S7]; data not shown). Similarly, Neandertal DNA was recovered after 60 cycles of a simplex amplification using the procedure and primers 16262#80-16230#81 described in [S8]. Eleven clones of the amplicon were sequenced, and the consensus was identical to the El Sidrón HVR-I sequence published in [S9] (GenBank accession number DQ859014). Overall, this suggests that authentic ancient DNA was recovered after extraction. The consensus sequence of the products obtained and the individual clone sequences can be found in Table S4.

In summary, two extracts were prepared from individual 1253, and two-step multiplex amplifications using (1) all the seven controls or (2) two Y controls (Y2, Y4) with primer pairs for the two FOXP2 substitutions were performed. Although 16 amplification products all yielded ancestral alleles, two PCR products from one multiplex

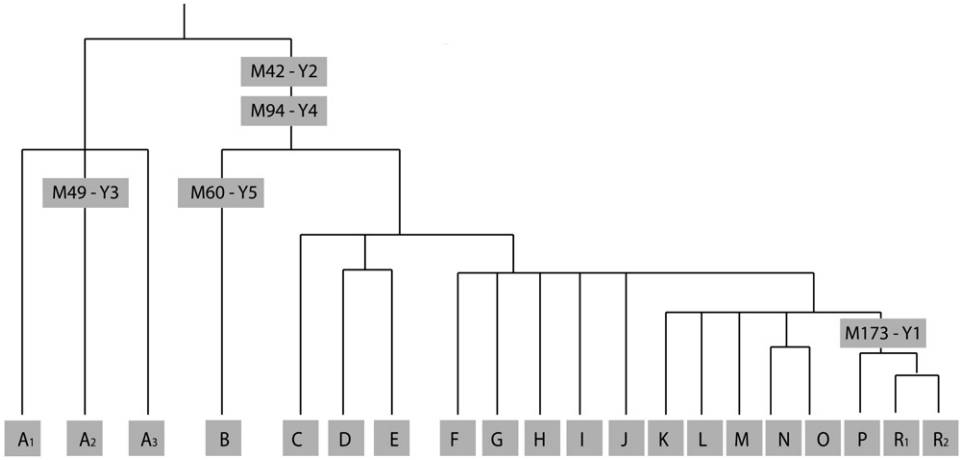

Figure S1. Schematic View of Human Y Chromosome Variation, Modified after Y-DNA Haplogroup Tree 2007 [S10], to Illustrate Position of Y-Chr SNPs used as controls Y1–Y5. SNP names were retrieved from [S10].

PCR (multiplex A, Y2 chromosomal control, Table S3) showed the derived and a mixture of the derived and ancestral state, respectively. Given that seven other amplification products from this amplification and all the other controls (nine PCR products) from the other four multiplex PCR yielded exclusively ancestral alleles, this suggests very low levels of contamination. For position 911 in the FOXP2 gene, eight products showed the derived state, and for position 977, three products were analyzed and all showed the derived state.

Experimental Procedures in Barcelona

DNA was extracted in Barcelona from about 0.5 g from the Sidrón 1253 bone, which was powdered, incubated with a proteinase K-containing lysis buffer, and extracted with silica extraction prior to concentrating as described in [S2]. For the initial attempt to amplify nuclear DNA, three primer pairs were used: Y2 as control primer pair, as well as F911-1F+R and F977-1F +R. The PCR reaction conditions used were 2 U AmpliTaq Gold (ABI), 1× AmpliTaq Gold buffer (ABI), 4 mM $MgCl_2$ (ABI, USA), 500 μM of each dNTP, and 1 μM of each primer. Products were obtained for each of the three primer pairs used and cloned with the Topo TA cloning kit (Invitrogen) according to the manufacturer's instructions. Colonies were subjected to PCR with M13 universal primers; inserts with the right size were sequenced with an Applied BioSystems 3100 DNA sequencer. At least ten clones were sequenced for each of the three products obtained. All clones from the Y chromosomal control (as well as the *FOXP2* products) and from the first extract were found to be derived, suggesting contamination with modern human DNA in this extract. Therefore, a second extract was produced from ~400 mg removed separately from the Sidrón 1253 bone. From this extract, products were obtained for the Y2 control (through the use of newly synthesized shorter primers Y2aF TTAATCAGATTTAGGACAC and Y2aR AAGTCACCAGCTCTCTTTTTC) and F911-1 and F977-1 with the same conditions as described above. All 16 clones sequenced for the F911-1 and F977-1 showed the derived state, whereas all 16 clones from the Y2 control displayed the ancestral state. This suggests that genuine Neandertal DNA was amplified from the second extract. As in Leipzig and Lyon, for the two FOXP2 positions, only the derived state was observed.

In summary, from a first bone sample of the first Neandertal (1253), one Y chromosomal control was found to be contaminated with modern human DNA showing only the derived state. When two additional bone fragments from the first and second Neandertal were further tested with primers designed to amplify a shorter fragment for amplicon Y2, both individuals showed the ancestral allele in all clones. Additionally, the two *FOXP2* substitutions 911 and 977 were retrieved from the uncontaminated first Neandertal sample, showing the derived alleles in all clones.

Supplemental References

S1. Fortea, J., de la Rasilla, M., Martinez, E., Sánchez-Moral, S., Cañaveras, J.C., Cuezva, S., Rosas, A., Soler, V., Julià, R., de Torres, T., et al. (2003). La Cueva de El Sidrón (Borines, Piloña, Asturias): Primeros resultados. Estudios Geológicos *59*, 159–179.

S2. Lalueza-Fox, C., Sampietro, M.L., Caramelli, D., Puder, Y., Lari, M., Calafell, F., Martínez-Maza, C., Bastir, M., Fortea, J., de la Rasilla, M., et al. (2005). Neandertal evolutionary genetics: Mitochondrial DNA data from the iberian peninsula. Mol. Biol. Evol. *22*, 1077–1081.

S3. Rosas, A., Martínez-Maza, C., Bastir, M., García-Tabernero, A., Lalueza-Fox, C., Huguet, R., Ortiz, J.E., Julià, R., Soler, V., de Torres, T., et al. (2006). Paleobiology and comparative morphology of a late Neandertal sample from El Sidron, Asturias, Spain. Proc. Natl. Acad. Sci. USA *103*, 19266–19271.

S4. Enard, W., Przeworski, M., Fisher, S.E., Lai, C.S., Wiebe, V., Kitano, T., Monaco, A.P., and Pääbo, S. (2002). Molecular evolution of FOXP2, a gene involved in speech and language. Nature *418*, 869–872.

S5. Orlando, L., Darlu, P., Toussaint, M., Bonjean, D., Otte, M., and Hänni, C. (2006). Revisiting Neandertal diversity with a 100,000 year old mtDNA sequence. Curr. Biol. *16*, R400–R402.

S6. Krause, J., Dear, P.H., Pollack, J.L., Slatkin, M., Spriggs, H., Barnes, I., Lister, A.M., Ebersberger, I., Pääbo, S., and Hofreiter, M. (2006). Multiplex amplification of the mammoth mitochondrial genome and the evolution of Elephantidae. Nature *439*, 724–727.

S7. Orlando, L., Bonjean, D., Bocherens, H., Thenot, A., Argant, A., Otte, M., and Hänni, C. (2002). Ancient DNA and the population genetics of cave bears (*Ursus spelaeus*) through space and time. Mol. Biol. Evol. *19*, 1920–1933.

S8. Serre, D., Langaney, A., Chech, M., Teschler-Nicola, M., Paunovic, M., Mennecier, P., Hofreiter, M., Possnert, G., and Pääbo, S. (2004). No evidence of Neandertal mtDNA contribution to early modern humans. PLoS Biol. *2*, E57.

S9. Lalueza-Fox, C., Krause, J., Caramelli, D., Catalano, G., Milani, L., Sampietro, M.L., Calafell, F., Martínez-Maza, C., Bastir, M., García-Tabernero, A., et al. (2006). Mitochondrial DNA of an Iberian Neandertal suggests a population affinity with other European Neandertals. Curr. Biol. *16*, R629–R630.

S10. International Society of Genetic Genealogy. (2007). Y-DNA Haplogroup Tree 2007. Vol. Version: 2.06. 25 July 2007. http://www.isogg.org/tree/.