Pig domestication and human-mediated dispersal in western Eurasia revealed through ancient DNA and geometric morphometrics

Supplementary text

The DNA analyses reported in this study were carried out in three ancient DNA facilities: Durham University (UK), Mainz University (Germany), and Leuven University (Belgium). Specific details about the analytical procedures carried out in each lab are described below.

1.0 Ancient DNA procedures and sample preparation

Leuven. Genetic analyses were performed in the aDNA facilities of the Laboratory of Forensic Genetics and Molecular Archaeology in Leuven (Department of Human Genetics, University of Leuven, Belgium). Pre- and post-PCR procedures were carried out in physically separated laboratories. Access to the pre-PCR laboratory was restricted to only two people (CO and NV) and only after wearing clean overalls, gloves, over-shoes, surgical facemasks, plastic spectacles, and following an irreversible sequence of work steps to avoid contamination. Entry was not permitted if PCR products had been handled the same day.

The aDNA facilities were routinely cleaned with bleach and RNAse Away (Molecular BioProducts, San Diego, CA, USA). Dedicated equipment was used in the pre-PCR laboratory, laboratory plastic-ware was irradiated in a cross-linker (four hours with ultraviolet (UV) light at 254nm, 5cm distance), and every item entering the room was extensively washed with bleach or RNAse Away and subsequently UV-irradiated. Various reagents including nuclease-free water (Promega, Fitchburg, WI, USA), dNTPs (Promega), and PCR Gold Buffer (Applied Biosystems, Foster City, CA, USA), were filtered through 100 kDa Centricon micro-concentrators (Millipore, Billerica, MA,

USA) and stored in small volume aliquots. Extractions were performed in a UV-irradiated workstation while preparation of amplification reactions was carried out in a UV-irradiated laminar flow cabinet (Esco, Breukelen, Netherlands).

For each ancient individual at least two extractions were undertaken at different time points. At least three amplifications for each extraction were performed and both strands of the DNA were sequenced in order to assess the reproducibility of the results. When possible, independent extractions of each individual were carried out from anatomically distant samples. To detect potential contamination by exogenous modern DNA, extraction and amplification blanks were used as negative controls.

To extract DNA from teeth and bone, one sample was prepared at a time. Samples were subjected to the following decontamination procedures. The outer surface of bone and teeth samples was removed through sterile blades or by sanding with a Dremel drill (Dremel, Racine, WI, USA). Additionally, the surfaces of the teeth were gently wiped with 10% bleach and rinsed with bi-distilled water. Bone and teeth samples were then UV-irradiated (254nm wavelength, 12W and 5cm distance) in a cross-linker on each side for 60 minutes and subsequently ground into a fine powder in a 6750 Freezer Mill (SPEX CertiPrep, Metuchen, NJ, USA) and stored at 4°C. Grinding vials were decontaminated using RNAse Away (Molecular BioProducts, San Diego, CA, USA) and subsequent UV-irradiation (254nm in cross-liker). To test for potential cross contamination in the grinding vials, hydroxyapatite powder was used as blank control in each grinding batch.

Durham. DNA extraction was performed in a dedicated ancient DNA laboratory in the Archaeology department at Durham University following strict laboratory procedures as according to commonly applied guidelines (Cooper and Poinar 2000; Gilbert et al. 2005). All equipment and work surfaces were cleaned before and after each use with a dilute solution of bleach (5-10%)

followed by ethanol (99%). Pipettes and plastic racks were subsequently UV-irradiated in a dedicated cross-linker (254 nm wavelength) prior to and after use. Pre- and post-PCR laboratories are physically isolated and access to the pre-PCR laboratories is restricted to Ancient DNA lab users only; access is also prohibited if the lab user had entered post-PCR areas the same day. Ancient DNA lab users wear clean lab coats, double set of gloves (nitrile and latex) and over-shoes in order to avoid introducing contamination from post-PCR areas.

The ancient pig remains were prepared for DNA extraction by removing an approximately two-millimeter layer of the outer bone surface by abrasion using a Dremel drill with clean cut-off wheels (Dremel no 409), targeting compact cortical bone or dental dentine. The bone was then pulverized in a Micro-dismembrator (Sartorious-Stedim Biotech), followed by collection in 15ml Grainer tubes. Milling containers and grinding balls were subsequently suspended and cleaned in 1% virkon and rinsed in absolute ethanol.

Mainz. The samples were analyzed in the facilities of the Institute of Anthropology (AG Palaeogenetics) in a laboratory dedicated for ancient DNA-work (free of molecular work). Laboratory rooms for Pre- and Post-PCR-work were strictly separated and persons were not allowed to enter the Pre-PCR-laboratory after working in the Post-PCR-rooms (including offices) the same day. All samples were subjected to the same procedures for ancient DNA analysis. The work was carried out while wearing clean overalls, disposable facemasks, face shields, gloves and over-shoes. All benches and rooms were routinely treated with soap and bleach or DNA-Exitus® (AppliChem) and UV-irradiated overnight. Additionally, the majority of the steps were carried out in special UV-irradiated work stations. The surface of all equipment entering the clean rooms was intensively washed and UV-irradiated (10h). The HPLC-water (Acros Organics) used for extraction and PCR was also UV-irradiated for at least 15h with a special waterproof UV-bulb. To detect contamination, extraction and amplification blanks were routinely used as negative controls.

The ancient specimens were prepared for DNA extraction as follows. Every sample was UV-irradiated for at least 1h (30min each side) before the outer surface was removed with sand blasting equipment (Harnisch and Rieth) and the samples were cut into pieces with a diamond drill (Dremel). The small pieces were UV-irradiated again and finally ground to a fine powder using a mixer mill (Retsch).

1.1 Assessing the authenticity of ancient DNA data

We can exclude contamination and demonstrate the authenticity of mtDNA results on the following grounds:

1. The analyses were undertaken in dedicated aDNA laboratories under strictly controlled conditions. A selection of samples (supplementary table S1) was processed in two independent laboratories (Leuven and Durham) and those samples generated identical haplotypes.

2. The molecular behavior of the PCR amplifications agrees with what we expect from the analysis of ancient samples. Younger samples were more likely to produce a greater proportion of successful amplifications (supplementary fig. S1b) while all DNA amplifications failed in the oldest samples (Aceramic Neolithic). Interestingly, despite variation in success rates of recovery between sites (supplementary table S1, supplementary fig. S1a) the recovery rate is nearly linear through time (supplementary fig. S1b). It is worth mentioning that the slight increase in success rate in Late Bronze Age (BA) layers compared to the Iron Age (IA) layers could be the result of mistaken contextual dating at Lidar Höyük, the site from which the majority of samples in this temporal bin come from. The re-assignments from LBA to IA of nearly all directly radiocarbon-dated samples from this site/time bin (supplementary table S1) support this observation.

Furthermore, in all instances, sequences were reproduced in multiple experiments, at least in two (and up to three) independent PCR experiments from up to two independent extracts. In some instances, particularly in the oldest samples, several amplification attempts (up to seven) were necessary to reproduce sequences. When this was the case, data were reproduced in a third extraction.

3. Results of the cloning experiments in six specimens (Leuven) confirmed the haplotypes determined through direct sequencing of the PCR products, with consistency of mutations ranging from 78% (Bad86) to 100% (supplementary table S3). The pattern of variation of the cloned sequences showed single substitutions (mostly C→T and G→A transitions) that were interpreted as artifacts due to misincorporations during the amplification or miscoding lesions. The latter is likely the result of *post-mortem* hydrolytic deamination that is common and characteristic in ancient samples (Hofreiter et al. 2001; Briggs et al. 2007; Gilbert et al. 2007). Average rate of C→T and G→A transitions ranges from 1% to 8%. Significantly, consistency of artifacts was higher in the oldest samples (e.g. Bad47 and Bad52, dated to Early Neolithic), compatible with a higher level of damage of nucleic acids and a lower number of template molecules initiating the amplification reaction.

A similar pattern was observed in a subset of the available 'sub-clonal' data set from Durham (supplementary table S3). Out of ~450-1000 randomly drawn ANC1 sequences (first 45bp forward read) from four specimens (LG281, LG459, LG477 and LG495) a total of 59 haplotypes were observed (N=12, 12, 10 & 25 respectively, supplementary table S3). C→T/G→A transitions (Type 2 transitions) are more common than other types of substitutions, including Type 1 transitions (83% and 17% respectively), and are interpreted to mainly represent post-mortem damage-derived miscoding lesions (C→U deamination). The other types of

substitutions, including Type 1 transitions, are sporadic and most likely derive from nucleotide misincorporations or sequencing errors. In support for this argument is the lack of consistency of other types of substitutions in between clones as compared to Type 2 transitions (supplementary table S3). The average rate of Type 2 transitions, calculated as the total number of transitions over C/G bases in the total extracted sequences (not accounting for identical haplotypes that might be derived from a single template molecule) ranged in between 1% to 14% with an average of ~5%.

4. The phylogenetic consistency between sequences produced independently in three different laboratories, and the phylogeographic consistency observed in the total data set (temporal and geographic), again consistent in all three laboratories, strongly indicate that the observed data are authentic.

These findings together with the above-mentioned laboratory procedures make it highly unlikely that the haplotypes observed in our samples arose from contamination or post-mortem damage, and lend credibility to the molecular results obtained in this study.

2. Molecular analyses

Leuven. Aliquots of 0.3-0.4 g powder were incubated overnight in a water bath at 56°C, followed by 24h at 37°C in a digestion solution of 0.5 M EDTA pH 8 (Invitrogen, Carlsbad, CA, USA), 0.5% SDS (USB Affymetrix, Santa Clara, CA, USA) and 0.1mg/mL Proteinase K (Roche, Penzberg, Germany). DNA was extracted through silica-based spin columns (Yang et al. 1998) and re-suspended in 100µL TE. Each independent extraction batch contained not more than eight samples, including two blank controls and one hydroxyapatite control.

Amplifications of the first and the second ~120bp fragments in the mtDNA control region (ANC1 and ANC2, (Larson et al. 2007a; Larson et al. 2007b)) were performed in a final volume of 50µL, containing 1x PCR Gold Buffer (Applied Biosystems), 2.5mM $MgCl_2$ (Applied Biosystems), 0.2mM dNTPs mix (Promega), 0.1µM each primer (Eurogentec, Seraing, Belgium – IDT, Leuven, Belgium), 0.05% BSA (Sigma Aldrich, St. Louis, MO, USA), 2.5 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), 5-10µL of aDNA extract. The following cycle conditions were used: 94°C for 10 min, 45 cycles of 94°C for 45 sec, 56°C for 1 min, 72°C for 1 min, and a final step of 72°C for 5 min.

All the amplification reactions were carried out on a GeneAmp PCR System (Applied Biosystems). The amplification products were visualized on a microchip electrophoresis system (MCE-202 MultiNA, Shimadzu Biotech).

Positive amplification products were purified with Microcon filter concentrators (Millipore) or through ExoSAP-IT (USB Affymetrix), according to manufacturer's specifications. The purified amplicons were directly sequenced by means of ABI Prism BigDye Terminator Cycle Sequencing Kit (ver3.1, Applied Biosystems) according to the manufacturer's specifications. Dyed products were ethanol precipitated and sequence reactions were performed on each strand by using 5'-tailed sequencing primer (Binladen et al. 2007a). The products were detected by capillary electrophoresis on ABI PRISM™ 3130XL Genetic Analyzer (Applied Biosystems). The two ~120bp fragments in the control region of the mtDNA were successfully amplified in 93 out of the 153 specimens from Anatolia, except for one (Bad4) in which the second fragment could not be amplified.

Cloning of the ANC1 products was carried out in six individuals (Bad17, Bad47, Bad52, Bad86, M46, M96) using the TOPO TA Cloning kit (Invitrogen), according to the manufacturer's instructions. Up to 10 colonies from amplification products of two independent extracts were picked

into 25µL nuclease free water (Promega), of which 1µL was used for PCR amplifications in a 25 uL volume of 1x PCR Master Mix (Qiagen, Hilden, Germany), 0.5µM each of vector M13R and M13F primers. Amplification products were purified and sequenced as previously described and the sequences were aligned, analyzed for artefacts induced by *post-mortem* miscoding lesions and the presence of contaminant DNA sequences (supplementary table S3). Sequences from independent experiments were aligned by using BioEdit v5.0.9 (Hall 1999).

Overall, 21 out of total 927 blank controls produced positive amplification (2.3%). After sequencing, the positive blank controls always revealed a European haplotype, and in one instance an East Asian haplotype. To determine whether the PCR success rate of the archeological samples is significantly different from the amplification rate due to potential contaminants in the reagents (Leonard et al. 2007; Champlot et al. 2010), and to ensure authenticity of the sample amplification with a 95% confidence level, we used the Fisher's exact test (Champlot et al. 2010). Blank control data obtained over many experiments with a given reagent batch were pooled, and after Bonferroni correction, only sequences with a 95% confidence interval were validated and considered authentic. Of the 153 samples analyzed, 60 resulted in unsuccessful genetic analyses (39% of the total individuals), 56 of which did not produce any amplification products after multiple attempts, whereas four gave low success rate which turned out to be non-significant to the Fisher's exact test, likely because of poor DNA preservation.

Durham. Bone powder (100-400mg) was digested in 0.425 M EDTA, 0.05% SDS, 0.05 M Tris-HCI and 0.333 mg/mL proteinase K and incubated overnight (18-24 hours) on a rotator at 50°C, or until fully dissolved. The digestion buffer, excluding proteinase K, was UV-irradiated (254 nm wavelength) for an hour in a dedicated cross-linker prior to use. 2mL of extract solution was then concentrated in a Millipore Amicon Ultra-4 30 KDa MWCO (Millipore) to a final volume of 100µL. The concentrated extract was purified using silica spin-columns (QIAquick PCR

Purification Kit, Qiagen) following manufacturers recommendations, except that the final elution step was performed twice to produce a final volume of 100µL. One in five or one in ten negative extraction controls were performed alongside the ancient bone samples. All extraction blank controls were negative when screened for the ANC1 fragment.

PCRs were setup in 25µL reactions using 1.25U Taq GOLD (Applied Biosystems), 1x Gold buffer (Applied Biosystems), 2.5mM $MgCl_2$, 0.5µg/µL BSA (Bovine Serum Albumine), 200µM of each dNTP, 0.8µM of each forward and reverse primers, and 2µL of aDNA extract. We used PCR primers ANC1 (Larson et al. 2007a), and the two primer pairs:

- U15697 (5'-CATATYATTATTGATCGTACATAGCACA-3')
- L15787 (5'-AAGAGGGATCCCTGCCAAG-3'), and
- U15775 (5'-AAYTACCATGCCGCGTGAAA)
- L15864 (5'GGTGAGATGGYCCTGAAGTAAGAAC-3') (Geörg, this study),

that target two fragments overlapping the ANC2 fragment amplified in Leuven. One PCR negative control was included for every 5-8 aDNA template PCRs. PCR cycling conditions were 95°C for 5min, 50 cycles of 94°C for 45 sec, 54°C for 45 sec and 72°C for 45 sec, followed by 72°C for 10 min. PCR products were stored at -20°C.

An initial PCR using the ANC1 primers was performed in order to screen the extracts for preserved DNA. Successful amplifications were Sanger sequenced on the Applied Biosystems 3730 DNA Analyser at the DNA sequencing service in the School of Biological and Biomedical Sciences at Durham University. Once preserved samples were identified we used 5bp 5'-tagged PCR primers (Binladen et al. 2007b) to re-amplify the ANC1 fragment and, in addition, the fragment corresponding to ANC2. In both instances PCR products were visualized on agarose gel and stained with GelRed, and then pooled by eye into approximately equimolar concentrations using a reference series of PCR products previously quantified on the Qubit fluorometer; approximately 12µg/µL of

each PCR product was used for the final pool. The pooled 5' tagged PCR products were then concentrated using an Amicon Ultra-4 30KDa MWCO filter column to a final volume of 100µL. The concentrated amplicon pool was subsequently purified using the QIAquick PCR Purification Kit following manufacturers recommendations, except that the final elute volume was 80µL. The concentrated PCR amplicon pool was then built into a paired-end library (Paired-End DNA Sample Prep Kit, Illumina) following manufacturers guidelines and subsequently sequenced on the Illumina GAII platform at the Department of Biology at Copenhagen University.

Illumina's Genome Analyzer Sequencing Control Software (SCS) v2.4 was used for base calling. A custom written PERL script (Rasmussen, M., University of Copenhagen) was used to filter out sequences containing the 5' tag label and to mate paired-end reads into single lines containing both forward and reverse 5' tag label information. A second custom written PERL script (Frantz, L., Wageningen University) was used to write a single fasta file for each tag label/amplicon. The resulting fasta files were assembled into contigs against a reference sequence (EU333163) in Geneious Pro 5.4.3 (Drummond et al. 2011). Assembly was performed using total quality score to call the best base (any base with a quality <20, equivalent to PHRED scores, was called as N and subsequently excluded from further analysis). All resulting haplotypes corresponded to the ANC1 fragment previously sequenced using Sanger sequencing at Durham University and we observed consistency in the extended ANC2 haplotype with sequences produced in Leuven and Mainz. At least one hundred sub-clones per sample were obtained for each re-sequenced PCR product, although we reached an average of several thousand copies per PCR amplicon. Nucleotide positions that could not be resolved despite the deep coverage were discarded from further analysis and called according the IUPAC nucleotide code.

Mainz. For each specimen two independent extractions were carried out. Aliquots of 0.3-1g of bone powder were incubated in a decalcifying and digestive solution containing 0.5M EDTA (pH 8.3,

Applied Biosystems), 30-60µL Proteinase K (Roche) and 1/10 volume of 0.5M N-lauryl sarcosine (Merck) on a rotary mixer over night at 37°C. DNA was extracted using phenol / chloroform-isoamylalcohol (Roth). The supernatant was transferred to an Amicon Ultra-15 filter unit (50kDa, Millipore) and washed with at least 5ml of UV-irradiated water before concentrated to a final volume of 100-200µL. Extracts were stored at -20°C. Each extraction contained at least two blank controls to detect contamination. PCR was performed in a final volume of 50µL containing 2.5U AmpliTaq Gold® DNA Polymerase (Applied Biosystems), 1x Gold Buffer (Applied Biosystems), 2.5mM $MgCl_2$ (Applied Biosystems), 20µg BSA (Roche), 0.2mM dNTP-Mix (Quiagen), 0.2µM of each primer (Biospring) and 2-8µL of bone or teeth aDNA extract. The PCR thermal cycling conditions were 94°C for 6min and 50 cycles of 94°C for 30sec, 57°C for 30sec and 72°C for 40sec. The products were stored at -20°C. All PCR reactions were carried out on a Mastercycler (Eppendorf). The primers used overlapped the ANC1 and ANC2 fragments amplified in Leuven and Durham: U15516/L15620 and U15697/L15787 (Geörg, this study, see primer sequences above). At least one negative control was included for every 10-15 amplified templates.

Positive PCR products were visualized on an agarose gel (Ultra Pure™, Invitrogen) stained with bromophenol blue (Fermentas). Purification of the amplified products were done with MSB Spin PCRapace Kit (Invitrogen) according to manufacturer´s specification or trough enzymatic digesting using 0.5U SAP and 2U EXO (30min incubation at 37°C followed by 15min of denaturation at 80°C). The purified fragments were directly sequenced using ABI Prism® Big DyeTM Terminator Cycle Sequencing Kit (vers. 3.1, Applied Biosystems) on a ABI Prism® 3130 Genetic Analyzer (Applied Biosystems) with dye products purified using Sephadex G50-fine (GE Healthcare) on Multiscreen-plates (Millipore) following manufacturer´s instructions.

None of the blank controls produced a positive amplification. In 25 of 43 samples it was possible to reproduce at least four sequences out of two independent extractions for every fragment analyzed.

One sample from Ulucak (Ulu28) failed in one part of the 80bp fragment. The resulting sequences were edited using Lasergene software (DNASTAR Lasergene, Version 7.1, GATC Biotech AG).

Modern sequences. Modern wild boar mtDNA sequences were processed at the Department of Animal Sciences and Aliments, University of Barcelona (Spain). We used previously described methods (Alves et al. 2009) except for the use of a reverse primer (5'-GTAACCATTGACTGAATAGCACCT-3') to avoid amplifying NUMTs.
There are few published reports on *Sus* NUMTs (Fang et al. 2011) and no database readily available for screening putative NUMT sequences (if not undertaking NUMT identification through previously published pig nuclear genomic sequences). By applying the strict authentication criteria described in section 1.1 we have minimized the confounding effect of co-amplifying NUMTs.

3. Phylogeographic analyses

Haplotype assignment for each specimen was based on the ANC1 variation using the same terminology proposed in Larson et al. (Larson et al. 2007a). It is worth noting that we have defined the variable site diagnostic for haplotype Arm1T as an insertion (15567.1T in ANC1), which is a more parsimonious phylogenetic marker. Fragment ANC2 is less variable and possesses less discriminating power because of some recurrent mutations (e.g. np 15714) (supplementary table S4).

All the ANC1 sequences in this study matched haplotypes previously described in Larson et al. (Larson et al. 2007a). Only three novel ANC1 sequences were observed (Bad9, M123 and M56), but they were only one mutation distant from the motif of Arm1T (Bad9 and M123) and A (M56), and were therefore assigned to these haplotypes. Overall, 141 specimens possessed Near Eastern

ancestry (haplotypes Arm1T, Y1, Y2, Yellow star) and 51 possessed European ancestry (haplotypes A, C, LDomBritSaddle01 and LDomGermanyAngler).

We constructed a Maximum-Likelihood tree based on 661 bp sequences of the mtDNA control region of 267 modern wild boar (present study, (Larson et al. 2005; Larson et al. 2007a) from Near-Middle East, Europe, and East Asia (supplementary fig. S2a) using PhyML (Guindon and Gascuel 2003) in Geneious (Drummond et al. 2011). The same topology was obtained using different substitution models (GTR, HKY85) and support values were calculated using a chi-squared test. The same topology of the main clades was obtained in three Bayesian trees run for up to 5,000,000 iterations using MrBayes (Ronquist and Huelsenbeck 2003) in Geneious. The general topology of the tree confirmed the clades that were observed in previous phylogeographic analyses (Larson et al. 2007a). Within the large European clade (fig. 2a) two main sub-clades were observed including the Italian-specific sub-clade (supplementary fig. S2a). The Near Eastern sequences are structured in two main sub-clades that we termed NE1 and NE2. Genetic variation contained in the fragment ANC1 allowed for the assignment of all the ancient Near Eastern haplotypes observed in this study to the NE2 clade. Crucially, the topology of the tree within clade NE2 shows that the mutations 15567.1T and 15592 contained in the ANC1 provide strong phylogenetic support to the definition of the two main Near Eastern ancient haplotypes that we encountered in our study, Arm1T and Y1 (supplementary fig. S2a, supplementary table S4).

To assess the phylogeography of the mtDNA clades, the place of origin and genetic signature of the modern wild boar used in this study (supplementary table S2) were plotted on a map (supplementary fig. S2b). The geographic distribution of mtDNA clades NE1 and NE2 in the Near East shown in figure 2b was designed after calculating the spatial distribution of their absolute frequencies in modern wild boar mtDNA haplotypes (as in supplementary fig. S2b) with Surfer 6

(http://www.goldensoftware.com/) using the Kriging method. Regions that were not sampled (*e.g.* the Arabian peninsula) were removed from the analysis.

4. Morphometric analyses

We used 2D landmarks and sliding-semi-landmarks based GMM approaches to describe the molar size and shape variation. Photographs were taken using a reflex camera (Nikon D90) coupled with a 60mm micro-length (AF-S Nikkor) to obtain images of the teeth in their occlusal view. Images were standardized for position and parallax. Two-dimensional coordinates of landmarks within the occlusal surface and sliding-landmarks along the outline of the teeth were recorded (Cucchi et al. 2011), as well as traditional measurements (maximum length and widths) using TpsDig (http://life.bio.sunysb.edu/morph/, (Rohlf 2010b). We recorded 9 landmarks and 66 sliding semi-landmarks for the lower M2 and 12 landmarks and 87 sliding semi-landmarks for the lower M3 (supplementary fig. S3b). The coordinates of the semi-landmarks were recorded using the "Draw background curves" tool of TpDIG that allows for the positioning of equidistant points. The outline of the lower M2 was divided into two anterior and posterior curves composed of 28 and 38 points respectively plus two landmarks in between.

The outline of the lower M3 was divided into four curves (anterior (28 points), posterior (28 points), labial (18 points), lingual (13 points) plus 4 landmarks in between). We used TpsRelw (http://life.bio.sunysb.edu/morph/ (Rohlf 2010a) to slide the semi-landmarks along their respective curves with the Procustes distance minimization criteria (Bookstein et al. 2002). The aligned coordinates and the centroid size, as well as the traditional measurements, were then analyzed using R v2.13.1 (R Development Core Team 2011) and the "Rmorph" library (Baylac 2012). The first components of the Principal Component Analysis (PCA) realized on the coordinates after superimposition were analyzed instead of the original dataset to minimize the number of variables compared to the number of specimens. Before discriminant analyses were carried out, a

dimensionality reduction was applied on the scores of the PCA with the Baylac & Friess procedure (Baylac and Friess 2005) that selects the N firsts components that maximize the variability between the groups. Traditional measurements of maximum lengths and widths (supplementary fig. S3b) were analyzed using the isometric size and shape parameters calculated following the Mosimann log shape ratio approach (Mosimann 1970; Mosimann and James 1979).

It is worth noting that using traditional metrics, the only significant result that was obtained was the difference in the isometric size of the lower M3 between the pigs with European and Near Eastern mtDNA lineages (supplementary table S6, fig. 2b). In this analysis, the pigs with European mtDNAs show smaller lower M3 than the pigs with Near-Eastern lineages.

5. Brief descriptions of key archeological sites

Ancient pig specimens analyzed in this study were excavated from 48 archeological sites in modern day Armenia, Cyprus, Georgia, Iran, Syria and Turkey (supplementary fig. S1a). Supplementary tables S1 and S4 list additional details and statistics regarding the results of the genetic analyses in each archeological site. Below we provide background information for key sites mentioned in the main text.

Turkish sites

Bademağacı Höyük is located in the south of the Lake District (Pisidia), southwest Anatolia, about 50 Km north of Antalya. The mound lies at an altitude of 780m above sea level (asl) just north of the pass (ancient Klimax) in the Taurus Mountains, which links this region with the coastal plain of Pamphylia. The site was excavated from 1993 until 2010 (Duru and Umurtak 2011) and a study of

the faunal material was published (De Cupere et al. 2008). A total of 41 ancient pig specimens were genetically analyzed dated to the Early Neolithic and the Early Bronze Age on the basis of AMS dates or the associated archeological context from which the bones were unearthed. Fourteen specimens were identified as wild boars on the basis of metric analyses. Radiocarbon dating previously carried out on animal bones unearthed from the levels 3 and 4A of the Early Neolithic, in which some of the pig specimens of this study were also found (supplementary table S1), provided an age ranging in 6,450-6,240 BC (De Cupere et al. 2008). Of the 41 analyzed specimens, 19 yielded reproducible amplifications.

Lidar Höyük & Hassek Höyük. Hassek Höyük and Lidar Höyük are located in the Karababa Basin in the Urfa and Adiyaman provinces respectively (southeastern Anatolia). Altitudes range between 400 and 600m asl. Studies of the faunal material has been published (Kussinger 1988; Stahl 1989; Boessneck 1992). Based upon stratigraphical sequencing of the sites and AMS radiocarbon dating, pig samples from Hassek Höyük were dated to the Calcholithic and Early Bronze Age, and those from Lidar Höyük to a large time frame spanning the Early Bronze Age to the Middle Ages. Nine specimens from Lidar Höyük were directly AMS dated (supplementary table S1). In two instances (M51 and M76) the AMS dates did not support the chronological assignment based on stratigraphy, whereas in the other samples a total or partial agreement within the range of two-sigma calibrated results was observed. A total of 25 specimens from Hassek Höyük were analyzed, of which four yielded positive and reproducible amplifications of DNA. Sequences from 57 out of 77 specimens from Lidar Höyük were successfully generated.

Sagalassos & Düzen Tepe. The antique site of Sagalassos is located 7km north of the small city of Ağlasun on a steep, south-facing slope of the Ağlasun Dağları (Western Taurus range, Southwest Turkey) at an altitude of 1,450 to 1,650m asl. In Imperial times it was the main city of ancient Pisidia, and was continuously inhabited from the 5th century BC until the earlier 13th century. On a

lower plateau, ~1.8km southwest of Sagalassos, lies the Classical/Hellenistic proto-urban site of Düzen Tepe (5th-later 2nd century BC). Excavations of both sites are ongoing (e.g. www.sagalassos.be (Degryse and Waelkens 2009; Vanhaverbeke et al. 2010; Vyncke 2012) and the fauna is being studied (De Cupere 2001). Based upon AMS dating and stratigraphical sequencing of the site, pig specimens from Sagalassos and Düzen Tepe range from the 5th century BC to the 12th century AD (Ricaut and Waelkens 2008; Ottoni et al. 2011). A total of 24 ancient specimens were analyzed in this study all of which generated reproducible results.

Gordion was the capital of the Kingdom of the Phrygians (10th-early 7th century BC), located along the Sakarya River, on a mound (700m asl) known as Yassıhüyük, ca 80km west-southwest of Ankara. The mound was rebuilt and refortified several times and contained mainly 'megara' used as audience halls, shrines and storage buildings. The mound is surrounded by dozens of tumuli. The major periods represented here are the Bronze Age (~2500–1200 BCE) and the Early Iron Age (~1200–550 BC), when, at the latest from the 10th century BC, it became the capital of the Phrygian kingdom, but from the early 7th century BC onward was subjected first to the kingdom of the Lydians, and subsequently to that of the Achaemenid Persians (or "Late Phrygian") period (546-333 BC). After its conquest by Alexander the Great (333 BC), the site declined becoming a village during the Hellenistic period (3rd century to 25 BC), the Roman Imperial (25 BC – mid 6th century AD), the Byzantine (mid 6th century – early 15th century), and the Ottoman period (early 15th century– 1923). At the time of the formation of the Republic of Turkey in 1923, the mound was no longer inhabited. Genetic analyses were successful in six out of seven ancient pigs, recovered in layers associated to the Late Bronze Age, the Late Phrygian and the Late Hellenistic period.

Çamlıbel Tarlası is located on the Anatolian Plateau, approximately 1,000m asl. It is located on a ridge overlooking a river in what was once a heavily forested area. Excavations focused on a rural Late Chalcolithic settlement dated to 3,590-3,470 BC (Schoop 2009). The faunal material of this

site was studied and pigs are found in much greater frequency than goats and sheep (Bartosiewicz and Gillis 2011). Genetic analyses were carried out on 15 pig specimens, 13 of which were successful.

Çayönü Tepesi is an Early Neolithic site located in the upper Tigris valley, in Southeast Turkey, dated to 10,000-6,500 BC. It has been considered one of the oldest pig domestication sites in Western Asia and possesses evidence that the *Sus* population around Çayönü lived in an intermediary relationship with humans between 'wild' and 'domestic' (Ervynck et al. 2001). A total of 14 pigs were genetically analyzed though only one was successfully amplified and sequenced.

Ulucak Höyük Ulucak Höyük is favorably situated along one of the main arteries between the Aegean coast and inland Anatolia in the Izmir province. This settlement mound covers about 3 ha and rises about 6m above the plain. Well-preserved Neolithic deposits represent a material culture akin both with the Lake District and the Greek Neolithic (Çilingiroğlu 2012). Altogether 19 specimens were analyzed, 17 of which associated with levels IV and V that date from 6,400-5,900 BC (Çakırlar 2012). Six of these samples, all identified as domestic pigs, were successfully analyzed.

Malkayası. This cave, discovered in 2001, is located upon the northern fringe of the Beşparmak Mountain (ancient Latmos) in Ionia on the coast of Western Anatolia. In prehistoric times, Mount Latmos was a holy place. The Malkayası cave and several other sites in that area show similarity to the Hacilar-culture (Plain of Burdur, territory of classical Sagalassos) and were therefore dated to the Chalcolithic period. AMS-dates of two wild boar analyzed in this study support this classification (5,000-4,500 BC). These two specimens provided successful amplification of their DNA though five others did not.

Menteşe. This tell is located in the northern part of Western Anatolia next to the dried-out lake Yenişehir, about 25km south of the archeological site of Ilıpınar. The mound is four meters in height and has a diameter of ~150m. It encompasses three different strata. The youngest layer is associated with the Roman Imperial period, the other two date to the Bronze Age and the Chalcolithic. The oldest stratum of this latest phase was dated to 6,400 BC and is older than Ilıpınar. The three analyzed specimens, all identified as domestic pig on the basis of bone traditional metrics, were recovered from the Chalcolitic layer and date around 6,000 BC. Two samples yielded reproducible products.

Sirkeli Höyük. This is one of the biggest settlement mounds in Cilicia at the interface between Syria, Cyprus and Anatolia. The site is located approximately 40km east of the modern city of Adana, close to the Ceyhan River, which represented a trade route between Syria and the Central Anatolian Plateau. The site was occupied from the Chalcolithic throughout the Bronze and Iron Ages but was abandoned in Hellenistic times. A total of 12 Iron Age individuals were genetically analyzed, four of which generated reproducible data.

Göbekli Tepe and Gürcütepe. The Early Neolithic site of Göbekli Tepe is located on a limestone ridge overlooking the Harran plain, northeast of the town of Sanhurfa, in Southeastern Turkey (Schmidt 1995,2000), at an altitude of 770 m above sea level (asl). Twelve specimens dating back to the 10th millennium BC (Aceramic Neolithic), of which at least four were identified as wild boar, were analyzed in the present study, resulting in unsuccessful DNA amplification. Gürcütepe is located south to Göbekli Tepe, in the Harran plain (Schmidt 1995), at the altitude of about 450 m asl. A total of 9 pig specimens from Gürcütepe, dated to the 8th millennium BC, were analysed in the present study, providing no successful amplification of DNA.

Syrian sites

Samples from four archeological sites were analyzed (see supplementary table S1). DNA sequences were obtained from **Tell Leilan** located in Northeast Syria in the Khabur River Basin at an altitude of ~390-400m asl. It is one of the largest archeological sites in Syria and was one of the most important cities in Northern Mesopotamia during the second and third millennia BC (http://leilan.yale.edu/index.html). A total of eight ancient pig specimens were genetically analyzed. Samples were collected from two different areas: the Acropolis Northwest and the Lower Town South dated to 2,600-2,200 BC when the settlement was abandoned following an abrupt aridification. Two out of eight samples were successfully analyzed.

Armenian sites

Samples from six archeological sites were analyzed (supplementary table S1) and we obtained aDNA from four of these (Areni-1, Tsakaektsi, Sevkar-4, Lchashen-2).

Areni 1 (Wilkinson et al. 2012) is a cave located in south-central Armenia in the Vayots Dzor district (the Arpa River valley), on the border with Nakhijevan (Azerbaijan). The main focus of the excavations in 2007 were the Late Chalcolithic layers dating to the late 5th - Mid 4th Millennium BC, though later medieval intrusions were also present. The only pig that yielded DNA from Areni 1 (supplementary tables S1 and S4) has an uncertain date (Pinhasi et al. 2010).

Tsakaektsi is a settlement located in north-east Armenia in the Tavush district, 5 km east from the Sarigyugh village (the Aghstev River valley). The main focus of the excavations in 1983-1985 (Yesayan 1992) were the medieval constructions dating to 12 - 13th centuries AD (High Medieval period).

Sevkar 4 (Surb Nahatak) is a fortified settlement located in north-east Armenia in the Tavush district, south from the Sarigyugh village (the Aghstev River valley). The main focus of the excavations between 1960-1972 were the Iron Age structures dating to VII - VI centuries BC (Yesayan 1976).

Lchashen is a village close to Lake Sevan in the Gegharkunik Province (supplementary fig. S1a). A large, 55-hectare complex of archeological remains possesses dates ranging over several millennia with the oldest belonging to the Neolithic, is located close to the village (Smith et al. 2009). We successfully extracted DNA from one specimen belonging to the Bronze Age.

Shengavit settlement is located in south-central Armenia (in the northern proximity of Ararat valley in the Ayrarat province) and the inhabited phase is dated to between the 4th millennium BC to the 2nd century BC. The town covered approximately six hectares and was probably the commercial and cultural center for a number of satellite settlements in the region. Through cultural objects including ritual obelisks in particular, the town of **Mokhrablur** is closely connected to Shengavit (http://www2.widener.edu/~msrothma/shengavitweb2.html).

Georgian sites

Aruchlo is located ~50km southwest of the town Tiflis, the capital city of Georgia, and close to the small village Nachiduri. The rivers Chrami and Masavera converge in sight of the mound. The Neolithic tell is 6m high and is made up of several phases of later occupations and has been assigned to the "Sulaveri-Somutepe" group of settlements typical for the region. The AMS dates for the Neolithic layers provide an age ranging from 6,000-5,300 BC. The nine analyzed samples date

to ~5,600-5,300 BC, a time frame associated with the Early Neolithic in Caucasia. Seven domesticated animals from this site were successfully analyzed.

Tachti Perda. The multilayered site of Tachti Perda lies in a settlement chamber in the centre of the southern Caucasus and is surrounded by the Great and Small Caucasus Mountain ranges. This area functions as an important travel route connecting the Eurasian steppe with Asia Minor and Central Asia. The mound is ~20m in height and encompasses several layers dated to the Iron and Bronze Age. Eight domestic pigs dating back to the Late Bronze Age (1,400-1,200 BC) were analyzed and all yielded reproducible products.

Iranian sites

Haftavan Tepe was excavated in the 1970s by Charles Burney. It is one of the largest sites in the Province of Azerbaijan and was occupied from the Late Bronze Age (Trans Caucasian) to the Early Islamic period. The site is located north of Lake Urmia. The fauna of this site has been recently studied as part of a PhD Thesis (Mohaseb Karimlu 2012). The six AMS dates were performed on animal bones and range between 2,000 cal BC and 730 AD.

Kohneh Tepesi. The site, near the city of Khomarlu in the Arax River Basin, is a large mound (77m by 44m, 7m high) with multiple occupation layers from the Bronze Age to the Iron Age. The site was excavated as part of an archeological program related to the construction of the Khoda Afarin Dam in the province of Eastern Azerbaijan. Kohneh Tepesi is located in a forested area limited in the south by the Arax River Basin and in the north by the Arasbaran Mountains. Archeological investigations have revealed cultural interactions and important ties with the Caucasus and Eastern Anatolia.

The Gorgan Wall sites. The recent joint Iranian and British archeological expedition along the Gorgān Wall, a historical wall extending over 195km in the north-east of Iran shed light into poorly understood periods in Iranian archeology including the Sassanian era (AD 235-7th century). The sites of **Qelīch Qōīneq**, **Dasht Qal'eh** and **Qareh Dōyūb** belong to the Achaemenid (the first) and to the Sasanian period (the two last sites). An extensive zooarcheological study revealed insights into the subsistence economies of various type of sites (urban, rural, military) along this wall (Omrani Rekavandi et al. 2007; Mashkour 2012).

The other samples belong Neolithic and Chalcolthic sites along the Zagros Mountains. **Ganj Dareh**, **Tepe Guran**, and **Qaleh Rostam** are the oldest Iranian sites that were analyzed and only the latter has provided a single sequence. **Qaleh Rostam** is located in the Central Zagros and is dated to the mid-Neolithic period. **Chogha Gavaneh** is a Chalcolithic/Bronze Age site in the Central Zagros excavated by Kamyar Abdi (Abdi 1999). The fauna report is being prepared for publication. **Tepe Mehr Ali** is a Chalcolithic site in the Fars Province excavated by A. Sardari as a rescue excavation necessitated by the construction of a dam. The site is located at a high altitude and has a high pastoral component. **Malyan** is also in the Fars Province and belongs to the subsequent Bronze /Iron Age periods. The fauna was studied by Melinda Zeder (Zeder 1985,1991). **Doshan Tepe** is another Bronze Age/ Iron Age site in the northern part of the central plateau near Tehran and was excavated between 2000 and 2002 by Youssef Madjidzadeh within the Ozbaki archeological Zone Project. The abundant animal bones of the site are very well preserved and suitable for genetic studies (Mashkour and Mohaseb Karimlu 2011).

Cypriot sites

Shillourokambos. We attempted to extract DNA from a single bone from this PPNB (pre-pottery Neolithic B) site located six kilometers east of Limassol in southern Cyprus. This site is one of the

largest and most important PPN sites in the region. The site is key for understanding the chronology of animal domestication and human management during the early domestication process (Vigne et al. 2011).

Supplementary Table Descriptions

Supplementary table S1. List of all the ancient specimens from Middle and Near East analysed in the present study. In the AMS indirect dating, upper and lower bounds determined from AMS dating carried out on associated bones from the same stratigraphic layer is reported. The status (wild versus domestic) of some of the specimens was provided following identification based on traditional metrics. Specimens for which status identification was not possible are left blank. Because traditional metrical methods for determining status are not necessarily conclusive (Evin et al. submitted), some of the status calls reported here may be subject to revision.

Supplementary table S2. List of modern wild boars mapped in supplementary figure S2. Each specimen was assigned to a mtDNA clade (Larson et al. 2007a) on the basis of genetic variation in long stretches of the mtDNA control region (380-661bp).

Supplementary table S3. Variable positions in ANC1 clone sequences from 10 ancient pig specimens obtained with Topo-TA Cloning kit (Invitrogen) and Illumina GAII platform. Cloned sequences (in grey) are aligned to the consensus of each specimen. In Topo-TA cloned sequences, direct sequences from PCR products are shown in white and used to create a consensus sequence. A frequent occurrence of some artifacts appeared in the oldest samples (BAD47, BAD52), most likely due to a low initial number of template molecules. Names for the sequenced clones are given as follow: SAMPLE NAME_EXTRACTION_PCR FRAGMENT_# CLONE. Positions are numbered according to Ursing and Arnason (Ursing and Arnason 1998).

Supplementary table S4. Polymorphic sites and associated haplotypes detected in the fragments ANC1 and ANC2 of the mtDNA control region in ancient pig specimens from the Near East. Alignment was done with a reference sequence, in bold (AJ002189, (Ursing and Arnason 1998)). Colors of mtDNA haplotypes and clades (NE2, Near Eastern clade 2; E, European) mimic those in figures 1 and 2. Positions are numbered according to Ursing and Arnason (Ursing and Arnason 1998). Haplotype assignment is based on Larson et al. (Larson et al. 2007a). The status (wild versus domestic) of some of the specimens was provided following identification based on traditional metrics. Specimens for which status identification was not possible are left blank. Because traditional metrical methods for determining status are not necessarily conclusive (Evin et al. submitted), some of the status calls reported here may be subject to revision.

Supplementary table S5. Ancient pig specimens analyzed in the timeframe 6,500-3,000 BC depicted in supplementary figure S3. An asterisk indicated dates based upon direct AMS dating of pig samples unearthed from the same layer.

Supplementary table S6. Differences between pigs with Near Eastern and European lineages in size (Kruskall-Wallis test) and shape (MANOVA) based on geometric and traditional morphometrics. Significant results are in bold for lower M2 and M3 with sample size in parentheses (EU: European lineages, NE: Near East lineages).

Supplementary Figures Legends

Supplementary fig. S1. Panel a) pie charts indicate approximate locations of the archeological sites investigated in the present study. Size of the pie charts is proportional to the number of specimens

analyzed from each site. Colors indicate the fraction of haplotypes encountered (as in figure 1) and the rate of unsuccessful samples (in white). Sites are numbered on the map as follows: 1, Ulucak Höyük; 2, Malkayası; 3, Menteşe; 4, Bademağacı; 5, Sagalassos; 6, Gordion; 7, Çamlıbel Tarlası; 8, Düzen Tepe; 9, Lidar Höyük; 10, Hassek Höyük; 11, Sirkeli Höyük; 12, Göbekli Tepe; 13, Gürcütepe; 14, Nevali Çori; 15, Çayönü Tepesi; 16, Halan Çemi; 17, Tell Leilan; 18, Atij; 19, Mashnaqua; 20, Umm Qseir; 21, Tachti-Perda; 22, Aruchlo; 23, Shengavit; 24, Mokhrablur; 25, Tsakaektsi; 26, Sevkar 4; 27, Lchashen 2; 28, Areni-1; 29, Kohneh Tepesi; 30, Haftavan Tepe; 31, Kalanan Sirlan Bijar; 32, Doshan Tepe; 33, Gohar Tappeh; 34, Dasht Qaleh; 35, Qareh Doyub; 36, Qelīch Qōīneq; 37, Chishko; 38, Choga Gavaneh; 39, Ghar-i-Khar; 40, Ganj Dareh; 41, Guran Tepe; 42, Cham Quleh; 43, Qaleh Rostam; 44, Malyan; 45, Mushki; 46, Mehr Ali; 47, Tol-e-Spid; 48, Shillourokambos. Panel b) yields of successful genetic analyses of the ancient samples plotted against the chronological age of the specimen. The fraction of successful specimens in each chronological period is reported.

Supplementary fig. S2. Panel a) Maximum-Likelihood tree based on 661bp sequences of the mtDNA control region of 267 modern wild boar (including novel sequences and those from previous studies (Larson et al. 2005; Larson et al. 2007a)) from Europe, the western Eurasia and East Asia, the latter of which were used to root the tree . Diagnostic SNPs in the ANC1 fragment that define the main clades are shown on the tree. SNPs are numbered to a reference sequence (AJ002189 (Ursing and Arnason 1998), indicated as 'REF' in the tree and are all transitions unless specified. Recurrent mutations are underlined. It worth noting that mutations 15567.1T and 15592 discriminate halotypes Arm1T and Y1. Statistical support (chi-squared p-values) is indicated in the branches of the main clades. The same topology of the main clades of the tree was obtained in Bayesian trees. Posterior probabilities are indicated in bold in correspondence of the nodes separating the main clades. Panel b) geographic distribution within the west Eurasian continent of modern wild boar mtDNA haplotypes from literature and the present study (supplementary table

S2). In total, 150 specimens are displayed, and colors designate the clade affiliation, as reported in legend. Specifically for the specimens with Near Eastern ancestry, the color designate to one of the two Near Eastern clades (NE1 and NE2, in green and grey) detected by sequencing 661 bp of the D-Loop region. All the ancient pigs possessing one of three Near Eastern mtDNA lineages (Arm1T, Y1 or Y2) (Larson et al. 2007a) belonged to the NE2 clade.

Supplementary fig. S3. Panel a) Geographic distribution and frequency of ancient pig ANC1-haplotypes in the time frame 6,500-3,000 BC analyzed in the present study and in literature (Larson et al. 2007a). A total of 78 pig specimens are included in the map (supplementary table S5). Pie size is proportional to the number of specimens genetically analyzed in each region. Asterisks indicate the fraction of wild specimens. ANC1-haplotypes are represented by different colors as follows (clade assignation is also reported): yellow, Y1 (Near Eastern); light blue, Arm1T (Near Eastern); brown, Y2 (Near Eastern); brown, Arm2T (Near Eastern); red, A and C (European); orange, Italian specific haplotype (Italian). Dates are reported on the map in the areas where Y1 is present. An inset illustrating the geographic distribution of ANC1-haplotype in Europe in the time frame 3,000 BC-1,500 AD, as from Larson et al. (Larson et al. 2007a), has been included on top right. Dots in the inset indicate exclusively the presence of the haplotypes and not the frequency. Panel b) Traditional measurement (maximum tooth lengths and widths) and location of the 2D landmarks (in grey), connected by lines to emphasize their relative positions, and sliding semi-landmarks (in white) along the outlines of the second (left) and third (right) lower molars of *Sus scrofa*.

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