Research Article

Southern Italian wild boar population, hotspot of genetic diversity

Valeria Maselli[1], Daniela Rippa[1], Adriana De Luca[2], Greger Larson[3], Barbara Wilkens[4], Anna Linderholm[3], Marco Masseti[5], Domenico Fulgione[1,*]

[1]*Department of Biology, University of Naples Federico II, Naples, Italy*
[2]*Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Naples, Italy*
[3]*Palaeogenomics & Bio-Archaeology Research Network, Research Laboratory for Archaeology, University of Oxford, Dyson Perrins Building, South Parks Road, Oxford OX1 3QY*
[4]*Department of Science for Nature and Environmental Resources (DipNeT), University of Sassari, Sassari, Italy*
[5]*Department of Biology, University of Florence, Florence, Italy*

Abstract

The wild boar, *Sus scrofa*, is an important game species widely distributed in Eurasia. Whereas the genetic variability of most European wild boar populations is well known, the status of wild boar living in Southern Italy is not as clear. We evaluated the present and past genetic diversity (D-loop, mtDNA) of the South Italian population, comparing it with that observed in other Mediterranean glacial refugia. Italian population showed highest genetic variability, if compared to other two European refugia (Iberian and Balkan). Most of samples from Italy carried sequences belonging to the European E1 haplogroup (80.9%) with a small proportion of the private Italian E2 (10.2%) and of the Asian (8.9%) ones. Italian samples carrying an Asian haplotype were genotyped by MC1R nuclear gene, failing to disclose a recent introgression from domestic pigs. Mismatch distribution analysis of the Italian population was affected by secondary contacts between these different lineages. This genetic melting pot was detected since the Mesolithic and the Neolithic age, during which we found samples belonging to the indigenous Italian and European haplogroups. Further, a Near-Eastern haplotype was found in 1,800 AD samples from Southern and Central Italy. Our results can be in agreement with post-glacial recolonization theories, as well as with the long history of human-mediated translocations of *Sus scrofa* in the Mediterranean basin.

Introduction

The wild boar *Sus scrofa* (Linnaeus, 1758) split from the Asian suid (either in Sundaland or mainland Southeast Asia; Larson et al., 2005) and appeared in Western Eurasia about 1 million of years ago (Rook and Martínez-Navarro, 2010; Frantz et al., 2013a). Quaternary climate change shaped geographical patterns of genetic diversity in this species, resulting in two major Old World clades – one in Asia and the other in Europe (Giuffra et al., 2000; Okumura et al., 2001; Alves et al., 2003; Larson et al., 2005; Alexandri et al., 2012). Within Western Eurasia, three well-supported mitochondrial wild boar clades diversified during glacial Pleistocene period (Larson et al., 2005). One of these clades is present in the Near East (Fertile Crescent), one in continental Europe, and the last one is confined to the Italian peninsula (Larson et al., 2005, 2007b, 2010; Ottoni et al., 2012; Krause-Kyora et al., 2013). In general terms, Quaternary climatic oscillations in Europe provoked species movement along a North-South axis, with the Iberian, Italian and Balkan peninsulas acting as Southern refugia, in the face of climatic deterioration (Bennett et al., 1991; Hewitt, 2004). Phylogeographic studies confirmed that a variety of taxa, including plants (Taberlet et al., 1998), and vertebrates (Hewitt, 1996, 1999, 2000) recolonized Europe from the refugia upon glaciers retreat, and especially so after the Last Glacial Maximum (LGM). It has been proposed that the modern genetic structure of North West European *S. scrofa* was likewise the result of such post-glacial re-colonization of Northern Europe from the Southern European peninsulas (Italy, Iberia and the Balkans), as well as

extra-Mediterranean refugia (Scandura et al., 2008; Alves et al., 2010; Alexandri et al., 2012; Schmitt and Varga, 2012). Like many other species, the wild boar was involved in natural and human-mediated dispersal during the Holocene with the earliest example in the introduction of wild boar to island of Cyprus by at least 11,700-11,400 BP (Vigne et al., 2009, 2011). In fact, *Sus scrofa* is common in both archaeological and paleontological sites (Clutton Brock, 1999; Masseti, 2002; Wilkens and Delussu, 2003; Masseti, 2007) and became economically relevant to humans during the latest Pleistocene (Pushkina and Raia, 2008). This species was particularly important in the Roman economy and its bones were always found in Roman sites, with higher proportion than any other domestic species (MacKinnon, 2001). According to other ancient sources, Cisalpine Gaul to the Northern, and Campania, Samnium, Bruttium, and Basilicata regions in the Southern part of Italy provided a fair share of pig meat for Rome (MacKinnon, 2001).

The medieval history of Southern Italian wild boar is not nearly as clear. It is evident from historical records that during the 18th and beginning of the 19th centuries, wild boar populations underwent extreme demographic decline in several European countries, mainly as a result of hunting (Apollonio et al., 1988; Yalden, 1999), and then became extinct over most of the Italian peninsula (Scandura et al., 2008). The fragmented populations survived after World War II, increasing and spreading again over most of the country. The genetic variation in Southern Italian wild boar population was also possibly affected by artificial long-distance migrations associated with uncontrolled and rarely documented restocking plans over the last 50 years (Apollonio et al., 2010). Sardinia houses a wild boar population and free-living pigs, whose history is similarly intricate and poorly known (Scandura et al., 2011a,b; Canu et al., 2014; Iacolina et al., 2016). Sardinian wild

boars are believed to descend from escaped animals during the Neo-lithic (Albarella et al., 2006; Scandura et al., 2011b). Thence, they experienced a multi-millennial history of independent evolution under geographic isolation (Randi, 1995; Larson et al., 2005; Iacolina et al., 2016) followed by introgressive hybridisation and gene flow with continental wild boars transplanted to the island in recent years (Scandura et al., 2011b). Instead, free-living pigs currently live in some areas of Sardinia under open-air conditions (Maselli et al., 2014b). Although they are domestic pigs, their feral habits exposes them to some similar selective pressures as wild boars.

We designed our study to provide more details on the South Italian wild boar population, in the light of present and past genetic variability, comparing it with that observed in other Mediterranean glacial refugia.

Materials and methods

We collected both modern and ancient specimens from across Italy (Fig. 1). Modern samples were collected in 2008–2012 during legal hunts in accordance with Italian national laws on wild boar management (prot n° 24581 20/07/2014). Ancient samples came from archaeological sites and museum collections.

Modern samples

We analysed a total of 157 modern samples of which 151 wild boars and six free-living pigs collected from six Italian regions (Tuscany, Latium, Campania, Basilicata, Apulia, and Sardinia) (Fig. 1 and Tab. S2).

DNA extraction, PCR amplification and sequencing

Fresh muscle samples were either stored in plastic tubes (15 ml) filled with 96% alcohol or kept frozen at −20 °C temperature. Total genomic DNA was isolated using a commercial DNA isolation kit (QI-Aamp DNA Mini Kit, Qiagen, Hilden, Germany). A fragment (652 bp) of the mtDNA D-loop control region (sites 15435–16088 of the reference mtDNA sequence coded AJ002189, Ursing and Arnason, 1998) was amplified using the primers H16108 and L15387 (Watanobe et al., 2001; Larson et al., 2005). This region was selected in order to maximize the size of possible alignments including already published GenBank sequences. PCR reactions were performed in a total volume of 25 μl, containing 50 ng of DNA template, 1 unit of Fermentas Dream Taq DNA polymerase (Fermentas GmbH, Germany), 2.5 μl of 10x reaction buffer, with MgCl$_2$, 0.25 mM of each dNTP and 12.5 pmol of each primer. The amplifications were carried out in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) and involved one initial denaturation step at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min 30 sec and a single final extension step of 72 °C for 5 min.
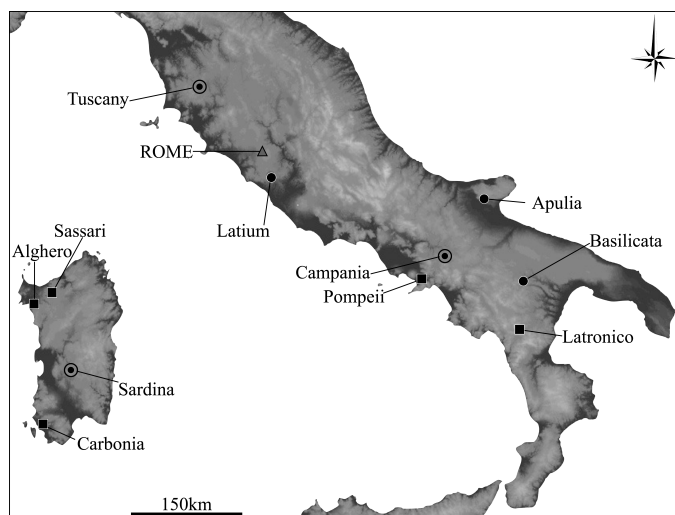

Figure 1 – Sampling locations of modern wild boars and free-living pigs (circles), ancient *Sus scrofa* specimens (square), or both (circle bullet). See Tab. S1 and S2.

PCR products were purified by Exo/SAP (Fermentas) digestion, according to the manufacturer's instructions, and were sequenced using the BigDye©Terminator kit version 3.1 (Applied Biosystems, Foster City, CA). Fragments were finally purified and run in an ABI PRISM 3100 Avant automatic sequencer (Applied Biosystems). All modern samples were sequenced with the forward primer and ambiguous positions were verified by re-sequencing with the reverse primer. Sequences were visually edited using Geneious v. 5.5 software (Biomatters Ltd; Drummond et al., 2011) and multiple sequences from the same sample were compiled into consensus sequence. Multiple alignments of the consensus sequences and published *Sus scrofa* mtDNA reference sequences were finally created for phylogenetic analysis.

Pig introgression analysis

We analysed the polymorphic melanocortin receptor 1 (MC1R) coat colour gene because it is a useful marker for detecting natural hybridisation between pigs and wild boars (Frantz et al., 2012, 2013b; Krause-Kyora et al., 2013). Various studies support the use of this gene as a tool for distinguishing breeds in a range of animal species (Klungland et al., 1995; Crepaldi et al., 2010). In particular, MC1R allelic variants are closely associated to particular breeds and colour morphs in *S. scrofa* (Marklund et al., 1996; Moller et al., 1996; Kijas et al., 1998, 2001; Giuffra et al., 2002; Pielberg et al., 2002; Fang et al., 2009; Koutsogiannouli et al., 2010). For each putative hybrid of modern samples (N=14), we amplified the entire MC1R coding sequence (963 bp) with different pairs of primers to analyse all the polymorphisms in the coding region [MERL1, EPIG1, EPIG3R (Kijas et al., 1998); MF1 and MR1 (Li et al., 2009)]. PCR reactions were performed in a total volume of 25 μl, containing 50 ng of genomic DNA, 1 unit of Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Hudson, NH, USA), 2.5 μl HF-GCrich PCR buffer (10x), 200 mM of each dNTP, 20 pmol of each primer. The amplifications were carried out in a Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) and involved one initial denaturation step at 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 sec, 58 °C for 40 sec, 72 °C for 40 sec and a single final extension step of 72 °C for 5 min. The sequences of MC1R were aligned and edited by Geneious v. 5.5 software (Biomatters Ltd; Drummond et al., 2011) analysing nucleotide composition and variable sites, compared with GenBank sequences and known alleles (Marklund et al., 1996; Moller et al., 1996; Kijas et al., 1998, 2001; Giuffra et al., 2002; Pielberg et al., 2002; Fang et al., 2009; Koutsogiannouli et al., 2010).

Ancient Samples

We collected 19 *Sus scrofa* ancient samples (Tab. S1), from Italian Peninsula (N=9) and Sardinia (N=10). In the former we analysed four specimens dated to about 1,800 AD (one from Tuscany and three from Campania), three specimens dated 79 BC (Pompeii, Campania) and two specimens collected in a Neolithic site dated to about 6,000 BC (Latronico village, Basilicata). In Sardinia we obtained ten samples ranging from 3,500 BC to 1,800 AD. The samples were collected from secure stratigraphic contexts associated with well-defined ceramic assemblages, which provide secure relative chronology (i.e., period and/or sub-period; Wilkens, 1987, 2012).

Prevention of Contamination

Throughout the extraction procedure, we took care to minimize contamination with modern DNA, especially problematic for domestic species (Leonard et al., 2007), and multiple measures were undertaken to exclude contamination by exogenous DNA. Bone powders, DNA extraction and PCR processing were prepared in a facility dedicated to the analysis of ancient specimens, using separate equipment and reagents, following stringent laboratory protocols tailored on ancient DNA (aDNA) processing (Gilbert et al., 2005). Non-disposable equipment (e.g. drill bits) was decontaminated between consecutive treatments when handling the bones. Standard contamination precautions, such as working in a regularly UV-irradiated laboratory exclusively used for pre-PCR work on highly degraded DNA, were employed. Worktops for DNA extraction and PCR setup were separated and cleaned frequently with bleach, distilled water, and 95% eth-

anol. Laboratory personnel wore disposable facemasks, full zip suits, protective shoe covers, and sterile latex gloves. The facility was not entered if PCR products had been handled the same day. All equipment, disposables, and nonorganic buffers, except for the tubes used for the aDNA extracts (Eppendorf®RNA/DNA LoBind microcentrifuge tubes, RNase and DNase free), were autoclaved and/or UV irradiated at 254 nm in a crosslinker for 30'. A separate laboratory was used for preparation and addition of the modern standard DNA to PCR reactions.

DNA Extraction

Ancient samples were surface sterilized by washing with 0.5% sodium hypochlorite and then rinsed with running deionized distilled water (ddH$_2$O) for 5 min, then air-dried and exposed to UV irradiation for 1 h. One-mm diameter drill-bit (VellemanR electric drill and engraving set. VTHD21B-DC 18V) run at 1,000 rpm generated the bone powder. About 5 g of powder were taken from the internal part of each bone, depending on the age and state of preservation. The DNA extraction was performed by using the QIAquick PCR Purification Kit (Qiagen), which is based on a modified procedure of the protocol described by Yang et al. (1998). Bone powders were dissolved in 8 mL extraction buffer (0.5 M EDTA pH 8.0, 0.5% sodium dodecyl sulfate, and 100 μg/mL proteinase K) and incubated in a shaking waterbath at 55 °C overnight. The extraction solution was centrifuged at 2,000 g for 5 minutes, and 1.75 mL aliquots of the supernatant were transferred to 2.0 mL centrifuge tubes and spun in a micro-centrifuge (12,800 g) for 5 minutes. The supernatant was then transferred to a 10-mL tube and mixed with 5 volumes of QIAquick PB buffer. Using a sterile disposable pipette, 750 μl was loaded directly into a QIAquick column and centrifuged at 12,800 g for 1 minute. The flowthrough was discarded and the process was repeated until all of the extract had been passed through the column. The DNA was washed by adding 750 μl of QIAquick PE buffer and centrifuging for 1 min. The flowthrough was discarded and the DNA was then eluted from the column by loading 100 μl TE buffer and centrifuging for 1 min. The isolated DNA was quantified with the NanoDrop 2000 (Thermo Scientific). The duplicate samples were extracted separately, and extraction controls followed each extraction batch with a frequency of one control to every two to five extracts.

PCR amplification

Mitochondrial genes are present in up to a thousand times higher concentration than nuclear genes in bone tissues (Smith et al., 2003) and are, for this reason, extensively used in studies on aDNA (Handt et al., 1996; Krings et al., 1997; Stone and Stoneking, 1998; Caramelli et al., 2003; Gilbert et al., 2003; Larson et al., 2007b). A highly variable 80-bp fragment (that included numerous indels), identified within a 652-bp alignment (Larson et al., 2007a) was amplified using the primers AncV1 (5' TTCGTATGCAAACCAAAAG 3) and AncV2 (5' TGC-ATGGGGACTAGCAATTA 3'). PCR reactions were performed in a total volume of 50 μl, containing 100 ng of DNA template, 2.5–3.5 unit of AmpliTaq Gold LD (Applied Biosystems), 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1.0 mg/mL BSA, and 0.3 μM each primer. The amplifications were carried out in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) and involved one initial denaturation step at 95 °C for 10 min followed by 55 cycles of 94 °C for 20 sec, 60 °C for 30 sec, 72 °C for 50 sec and a single final extension step of 72 °C for 5 min. Each PCR was performed independently twice for each extract. All ancient samples were sequenced with forward and reverse primers as described above.

Authenticity of Ancient DNA Results

The authenticity of the ancient sequences and confidence in haplotype assignments are supported by the precautionary measures implemented in the experimental protocol and the coherence of the results obtained in this study: (i) dedicated ancient DNA facilities were used; (ii) robust decontamination protocol of the bone samples was employed before DNA extraction; (iii) all blank extracts were included in PCRs

and each PCR was performed with negative and positive controls (iv) no PCR amplification was observed in blank extracts and PCR negative controls; (v) multiple haplotypes were obtained as a whole, as well as within most extraction batches; (vi) the same ancient haplotypes were obtained in two separate laboratories, using different extraction and amplification protocols; and (vii) all sequences belong to *Sus scrofa*. Two repeated extractions and amplifications were conducted for all samples. Consistent results were obtained for all replications. Although some differences, likely attributable to post-mortem genetic damage, were detected, these did not influence the designation of the specimens to different haplotypes. Ancient DNA experiments were performed in dedicated facilities in the University of Naples Federico II. In order to verify the authenticity of the data obtained in Naples, seven samples were further analysed for blind replication in the independent laboratory at Durham University. Both laboratories followed the standard criteria for ancient DNA handling (Gilbert et al., 2005).

Data analysis

A first round of analysis was conducted in order to assess the genetic relationship between the identified new haplotypes and additional 132 mtDNA wild boar sequences sampled worldwide, chosen as to represent the current genetic diversity of Western Eurasia (GenBank, Tab. S3) (Watanobe et al., 1999; Giuffra et al., 2000; Okumura et al., 2001; Watanobe et al., 2001; Kim et al., 2002; Randi et al., 2002; Alves et al., 2003; Gongora et al., 2003; Larson et al., 2005, 2007a; Scandura et al., 2008; Lattuada et al., 2009; Alves et al., 2010; Gongora et al., 2011). A second round of analysis was conducted on sequences (n=501) grouped according to specific refugia (Balkan, Italian and Iberian) across eight European and Mediterranean countries (Tab. S4), comparing shorter sequences of 610 bp. Finally we assessed the genetic variability within the Italian refugium (335 sequences) considering populations grouped by regions (Tab. S5). In this dataset the newly generated sequences were trimmed in a shorter segment of 410 bp in order to compare them with the most of Italian sequences deposited in GenBank (Scandura et al., 2008).

In order to deeper analyse the genetic variability of Italian refugium, we aligned the newly 19 ancient sequences (Tab. S1) with sequences of 60 different ancient haplotypes obtained from GenBank, truncated to make them comparable (Larson et al., 2007a) (Tab. S6). All datasets were performed using Geneious v. 5.5 software (Biomatters Ltd; Drummond et al., 2011) and final adjustments were performed by eye. The alignments were collapsed in distinct haplotypes using DnaSP v5 (Librado and Rozas, 2009).

To detect genetic structure within Italy and to reveal the phylogeny and the phylogeography of Italian wild boar within a European framework, Bayesian phylogenetic analyses were carried out, using reference sequences of modern (Tab. S3) and ancient European samples (Tab. S1). The best fit model of nucleotide substitution was estimated using a hierarchical likelihood ratio test (hLRT) as implemented in jMODELTEST 3.7 (Posada and Crandall, 1998; Guindon and Gascuel, 2003). The best model resulted the HKY model with inv-gamma distributed (HKY85+G+I) rate variation across the sites, based on the corrected Akaike Information Criterion (-lnL=3535.78. AICc weight=0.792) The best fit parameters were included as priors for MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and eight Markov Chain Monte Carlo (MCMC) searches were run for 30 million generations. For each MCMC, a tree was sampled every 100 generations. Tracer 1.6 (Rambaut et al., 2014) was used to summarize Bayesian analyses, to inspect the validity of the burn-in fraction applied and to evaluate the convergence. The first 10% of samples were discarded as burn-in (Rambaut et al., 2014) and convergence was assessed by checking the log likelihoods, the average standard deviation of split frequencies (<0.01), and the potential scale reduction factor in MrBayes. We also assessed convergence by visual inspection of the trace and the estimate of the effective sample size (ESS>200). A consensus tree from the retained trees was computed with MrBayes. Sulawesi Warty Pig (*Sus celebensis*) D-loop sequence was included as an outgroup (GenBank accession number: GQ338955). Sometime consistent trees are diffi-

cult to obtain under high rates of homoplasy, this is the reason why we used a median joining (MJ) network (Bandelt et al., 1999). Therefore, in addition to phylogenetic trees, a MJ network of haplotypes was created for the European two alignment sets (modern and ancient, Tab. S3 and Tab. S1), by using the software Network 4.1.0.9 (Fluxus Technology). A maximum parsimony calculation was used to eliminate non-parsimonious links (Bandelt et al., 1999). To obtain better insights into the genetic diversity and demographic history of wild boar among Mediterranean refugia and within Italian refugium we measured the levels of molecular diversity. Number of polymorphic sites (S) and haplotypes (H), average number of pairwise differences (k), Theta (Θ) (Nei, 1987) and nucleotide diversity (π) were computed with DNAsp v5 (Librado and Rozas, 2009). To gain an insight into the possible demographic history over time, a mismatch analysis was carried out (Rogers and Harpending, 1992) for all mtDNA sequences from Italy (Tab. S5) and Mediterranean refugia (Tab. S4). The mismatch analysis does not consider the Asian samples, because attributed to local introgression with domestic pigs. Significance was assessed after 10,000 replicates, using a parametric bootstrap approach, under the null hypothesis that the observed data fit the sudden expansion model (Schneider and Excoffier, 1999). The mismatch analysis and the raggedness index analysis were performed in DnaSp v5 (Librado and Rozas, 2009).

Results

Modern samples

In modern samples, we identified 39 different haplotypes (named from VM_H1 to VM_H39; [Genbank: HM-051193-201, HM448432, KF873473-501]), of which 32 had been never described before. We identified 17 distinct haplotypes exclusive to Sardinia, 20 exclusive to the Italian peninsula and only two haplotypes were shared by Southern Italy and Sardinia. Bayesian phylogenetic analysis (using the HKY85+G+I model) from living specimens pointed out three distinct clades: European E1, Italian E2, and East Asian A (Larson et al., 2005; Scandura et al., 2008) (Fig. S7). The European E1 clade accounted for 80.9% of sequences, 10.2% for the Italian E2 clade, and 8.9% for the Asian A clade. Private Italian haplotypes (clade E2) were found both in Sardinia and in Italian peninsula, including the most common BG_Italy described in Larson et al. (2005). We observed also some differentiation among haplotypes assigned to the Italian clade. Sardinian samples carried two distinct haplotypes, basal to other E2 haplotypes detected in peninsular samples, possibly reflecting the long geographic isolation experienced (Fig. 2).

Inside the European clade only 29% of the 157 sequences were ascribed to the most common European haplotypes (W, BK, AF, E and M in Larson et al., 2005). Among Southern Italian samples (Fig. 2a), one free-living domestic pig and 13 wild boars, belonged to the East Asian haplogroup (D2 in Larson et al., 2005). These samples were genotyped at MC1R gene, revealing that just the free-living pig (from Castelnuovo Cilento) showed a typical domestic pig sequence (accountable to genotype 0501), whereas all wild boars were wild-type (genotype 0101; Fang et al., 2009). In addition to the phylogenetic tree, we performed a network analysis (Fig. 2a) in which we identified three distinct clades (European, Italian, and Asian) corroborating the Bayesian analyses results. The Italian haplotypes showed a scattered pattern highly dispersed according to the private mutations and new haplotypes. Common haplotypes were surrounded by a starlike pattern, suggesting a population expansion (Fig. 2a). In the map showing the frequency of the three haplogroups (E1, E2, and A) in each of the eight Italian populations (Fig. 2b) we observed a high degree of variability in the Southern populations, sampled in Campania and Apulia. Resolving the European clade (Fig. 3) in consideration of C-side and A-side assignment (Larson et al., 2005) we observed that the Southern Italy haplotypes occurred within both European core lineages, with similar proportions (46.6% A-side and 53.4% C-side). Genetic variability in Italian wild boar populations can be inferred also comparing nucleotide characteristics of sequences among three refugia.

Southern Italian samples thus had higher nucleotide diversity overall than other refugia (Tab. 1; π=0.00391 for Balkans, π=0.00396 for
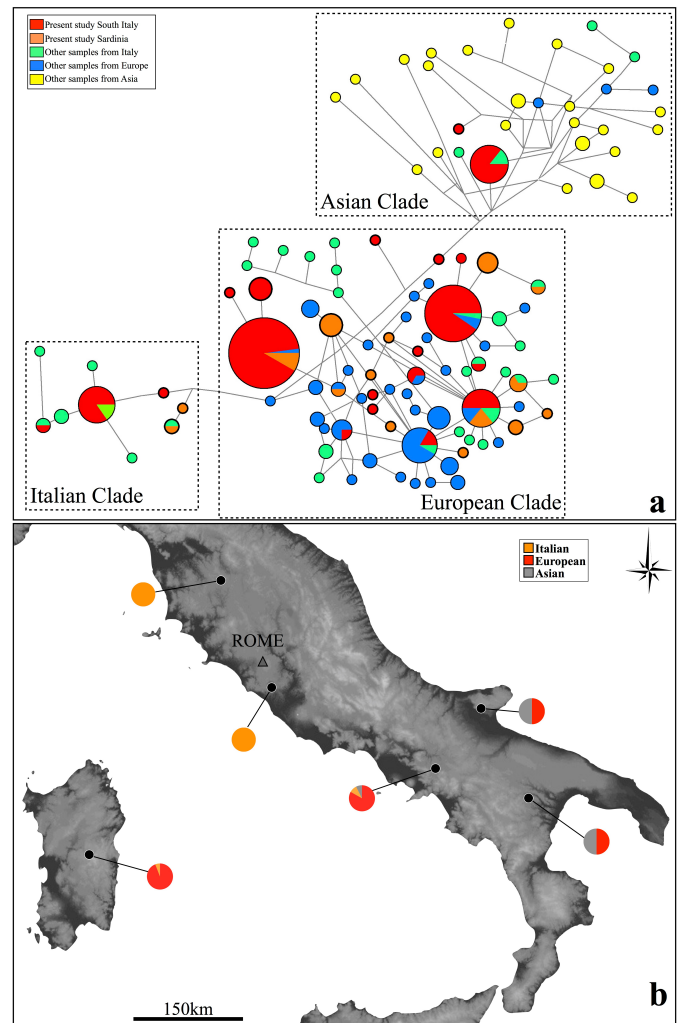

Figure 2 – (a) Median-joining network based on the joint (original and published sequences) data set from modern mtDNA of wild boar and pig breed. Each circle represents a haplotype, size is proportional to the frequency of collapsed sequences. Solid branches connecting circles represent nucleotide changes among haplotypes. The branch length is proportional to the number of mutations. Colours within circles illustrate the relative frequency of sequences from different geographic areas. Bold circles indicate haplotypes found for the first time in this study. (b) Geographical distribution of the sampled populations, and distribution among them of main mtDNA lineages, shown as pie diagrams.

Iberia, and π=0.00994 for South Italy). We observed a similar trend for the average number of nucleotide differences (k) (Tab. 1; k=2.3017 for Balkans, k=2.3888 for Iberia, and k=5.9668 for South Italy). The number of haplotypes was quite similar for South Italy and Balkans (38 and 40 respectively), but they showed a high frequency of private haplotypes (89.5% and 90.0% respectively) and only two haplotypes were shared between these two refugia. The Iberian refugium showed a lower number of haplotypes (16) and percentage of private haplotypes (68.8%). Focusing on samples from Italy, owing to the reduction in the total length of sequences used for the analyses (410 bp), several Italian haplotypes collapsed together (Tab. 2). Among the different Italian populations, it was clear that Campania and Sardinia showed the highest diversity in the peninsula, with higher number of haplotypes (18 and 15 respectively) and private haplotypes (66.7% and 77.8% respectively). Moreover mtDNA allowed us to make further inference on the demographic characteristic of population. The pattern of mismatch distribution suggested that the Iberian and Balkan populations experienced a sudden expansion, which was absent in Italian populations (Fig. 4). High peaks in pairwise difference were in accordance with a demographic history including different haplogroups (secondary contacts), especially in Southern Italy and Sardinia (Fig. 4).

Table 1 – Genetic diversity observed in modern wild boar populations of Mediterranean refugia (D-loop, 610 bp). *Pop*, population; *n*, sample size of each population; *S*, number of polymorphic sites; *H*, number of haplotypes; *AU*, number of private haplotypes; *U%*, percentage of private haplotypes; Θ, Theta; π, nucleotide diversity; *k*, average number of pairwise differences.

Pop	*n*	*S*	*H*	*AU*	*U‰*	Θ	π	*k*
Balkans	176	38	38	34	89.5	0.01155	0.00391	2.3017
Iberia	159	15	16	11	68.8	0.00441	0.00396	2.3888
South Italy	166	30	40	36	90.0	0.00881	0.00994	5.9668

Table 2 – Genetic diversity observed in modern Italian wild boar populations (D-loop, 410bp). *Pop*, population; *n*, sample size of each population; *S*, number of polymorphic sites; *H*, number of haplotypes; *AU*, number of private haplotypes; *U%*, percentage of private haplotypes; Θ, Theta; π, nucleotide diversity; *k*, average number of pairwise differences.

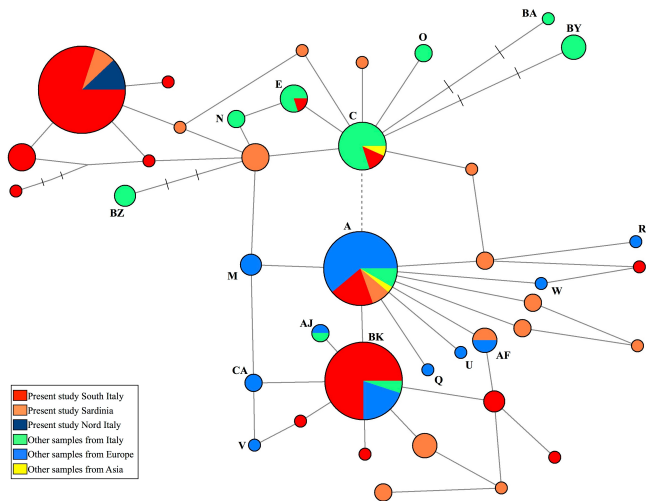| *Pop*	*n*	*S*	*H*	*AU*	*U‰*	Θ	π	*k*
Piedmont	19	12	7	1	14.3	0.00846	0.01089	4.4211
Emilia Romagna	10	5	3	0	0.0	0.00435	0.00657	2.6667
Tuscany	57	13	6	1	16.7	0.00694	0.01248	5.0664
Latium	13	1	2	1	50.0	0.00079	0.00126	0.5128
Campania	107	19	15	10	66.7	0.00897	0.01153	4.6563
Basilicata	10	14	5	3	60.0	0.01219	0.01806	7.3333
Apulia	2	8	2	1	50.0	0.01970	0.01970	8.0000
Sardinia	117	20	18	14	77.8	0.00926	0.00867	3.5111

Figure 3 – Median-joining network of European clade (EI). Detail of the main European clade depicting the relationships among haplotypes detected in the different Italian populations. The A and C haplotypes correspond to the two core lineages. Size of circles is proportional to the number of sampled individuals; if more than one mutation occurs between two haplotypes, they will be connected by dashed line, each dash representing one mutational step.

Figure 4 – Mismatch distributions based on pairwise site differences between sequences, performed in DNAsp (Librado and Rozas, 2009) for different populations: (a) Balkans; (b) Iberia; (c) South Italy; (d) Sardinia. The expected data were computed under the model of demographic expansion. On the horizontal axis there is the number of nucleotide differences between pairs of sequences and on the vertical axis there is the relative frequencies of pairwise comparisons. Grey and black bars represent expected and observed frequencies, respectively.

Ancient samples

We successfully extracted and sequenced DNA from 19 ancient *Sus scrofa* bones sampled from archaeological sites or museum. Nucleotide sequences from each replicate were identical for 18 samples. Just in one sequence from Pompeii (PR_VM03) we found a misincorporation (A instead of T, at position 20), but this did not influence the designation of the haplotypes. In ancient samples we identified five different haplotypes (named from VM_AH3 to VM_AH7; Additional file 1 [GenBank: KF873503-06, HM051202]). Phylogenetic Bayesian consensus tree (Fig. S7b) drawn using only the 80-bp partial sequence of mtDNA D-loop was useful to identify four distinct clades (European, Italian, Asian, and Near East Asian). Surprisingly, we found a Near Eastern haplotype (NE2-Y1) in three museum samples from Campania, (about 1,800 AD) and one museum sample from Tuscany (about 1,800 AD), suggesting that *Sus scrofa* with this genetic trait was present in Italy at least until 200 BP, before disappearing from modern populations (Fig. 2, Fig. 5, Tab. S1, Tab. S6, Fig. S7) (Zinser et al., 2003).

Two haplotypes belonging to the endemic Italian clade came from ancient samples from Sardinia (Alghero, Neolithic) and Basilicata (Mesol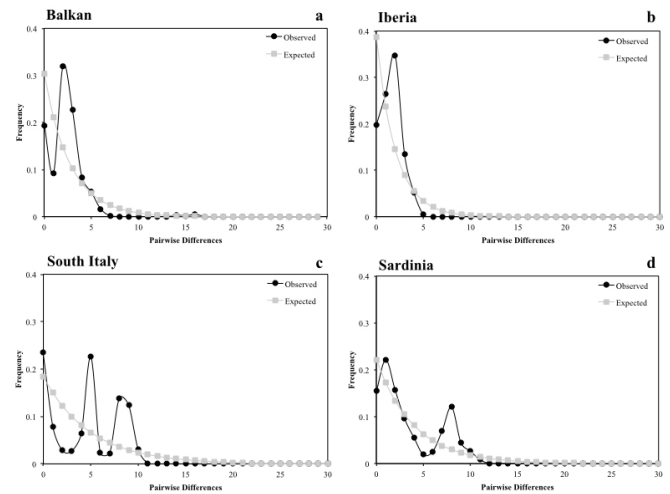ithic). The Sardinian haplotype was consistent with those observed in modern samples. All other haplotypes from Sardinia (Carbonia, Sassari, Alghero) and all samples from Pompeii (79 AD) belonged to the European clade (ANC-C side), indicating the presence of this clade on Sardinia during Neolithic age. The alignment with ancient samples was used to draw a median-joining network (Fig. 5a) to further elucidate the differences between the major clades (European, Italian, Asian, and Near East Asian). The time series of maps (Fig. 5b) depicting the shifting geographic positions for the Italian, European and Near Eastern haplotypes over the past, is consistent with those previously reported Larson et al. (2007a) and expanded the range and timing of the Italian clade, observed in Sardinia.

Discussion

The role of Italy as a refugium in Europe for a lot of species during the LGM is already well known (Hewitt, 1999, 2004) and there is evidence that the wild boar distribution followed the general pattern exhibited by the majority of mammal species (Scandura et al., 2008; Alexandri et al., 2012). We analysed the genetic structure and variability of wild boar populations from Sardinia and South Italy, previously suggested as
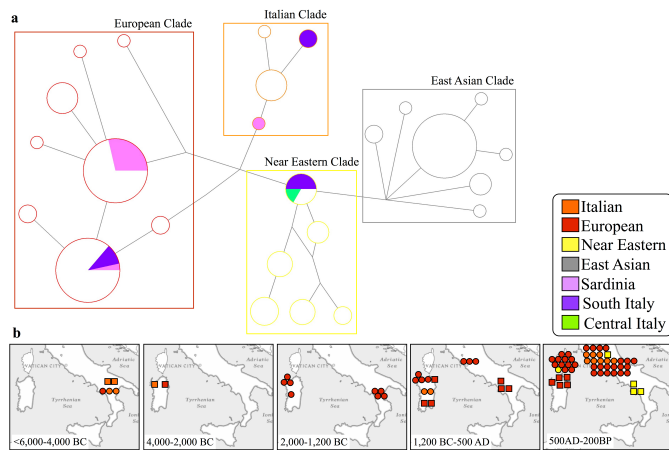
Figure 5 – Relationships and distribution of mtDNA haplotypes from ancient samples (80 bp). (a) Median-joining network created from sequences deriving from ancient and museum samples to further elucidate the principal differences between the major clades. Orange, Red, grey and yellow represent haplotypes from Italy, Europe, East Asia and the Near East, respectively. The colours in the circles indicate the geographic position of our haplotypes. (b) Time series of maps depicting the geographic positions of Italian, European and Near Eastern haplotypes over the past. Each symbol corresponds to a single sample, and the shape indicates both to the Larson (2007) and our haplotypes (circle and square, respectively). The colour of each symbol indicates the belonging haplogroup as in the frame of the network.

one of the most important glacial refugium (Scandura et al., 2008; Vilaça et al., 2014). We provided further evidence supporting the role of this refugium as hotspot of genetic variability for wild boar. Our findings were based on data from Mesolithic age to recent times, elucidating how both glaciation and human movement affected the wild boar variability pattern. Data on mtDNA from Southern Italian wild boar populations, principally due to the presence of the three major mtDNA lineages (E1, E2, and A) and private haplotypes, corroborated the general pattern of decreasing northward genetic diversity (Vilaça et al., 2014). The most frequent wild boar haplotypes in Italy belonged to E1 haplogroup, which was also shared among the different refugia (Italian, Balkans and Iberian) as well as by all other European populations analysed so far (Kusza et al., 2014; Vilaça et al., 2014). The existence of a large number of private haplotypes in South Italy suggested that a large fraction of the pre-glacial wild boar diversity was maintained in this area during the last glacial period. Similar cases were found in other species (Sommer et al., 2005). These findings supported the scenario of a post-glacial northward expansion from Italy to Central Europe and beyond, with the gene pool from North Italy contributing more to the recolonization process than the gene pool from Southern parts. The genetic variability observed in Central Italy (Tuscany) was mainly due to the presence of a high proportion of samples belonging to the private Italian clade (E2). Finally, only one private Italian haplotype was found in North Italy (Piedmont and Emilia Romagna).

A minority of Southern Italian haplotypes clustered with East Asian wild boar and pigs. Indeed, the Asian A haplotypes made up as much as 29% of European domestic pig breeds (Fang and Andersson, 2006), due to historical introgression with Chinese pigs (18th–19th century) (Giuffra et al., 2000). The presence of A haplotypes in wild individuals thus proved the occurrence of a domestic pig ancestor in their maternal line. The occurrence of free-ranging pigs in our sampled areas and to the practice of restocking in southern regions, possibly using captive hybrids, can explain this presence. In the Southern Italy wild boar population the contact between wild and domestic pigs is well known and it is due to local farming practices still existing in few regions of the Mediterranean basin (Randi, 2005; Maselli et al., 2014a,b). When pigs escape from farms or are deliberately allowed to graze freely, their chance to get into contact with wild boars is high. In Italy, another source of genetic variability came from the Sardinian population. It shared the major mtDNA haplotypes with the Italian Peninsula, mostly European E1, but a lot of haplotypes were nonetheless endemic. In Sardinia, the Italian lineage (E2=6%) confined on the island was char-

acterised by a nucleotide divergence generating a sub-clade within the Italian one. High level of genetic variability in wild boar of Southern regions (Latium, Campania, Basilicata, Apulia and Sardinia) can be explained by the long-term persistence of favourable environmental conditions within the refugium. They allowed for the maintenance of stable and genetically variable populations (Canestrelli et al., 2010), and probably, a constantly high effective population size. This condition was probably maintained by a series of secondary contacts as suggested by mismathch analysis. In fact in Italy multiple peaks can be explained by the presence of E1 and E2 haplotypes as well as to wild boar introductions occurred in the past. Whereas Balkan and Iberian sequences showed an unimodal distribution, that likely reflected a long-term demographic expansion (Fig. 4).

Ancient DNA from *Sus* contributed to provide information about the complex temporal and geographical pattern. In South Italy, the private Italian haplotype (Anc-Italy) was originally present, as shown in the remains of Latronico (Basilicata). Similarly, Sardinian samples, dating back to the Neolithic age, belonged to the Italian clade, reflecting contacts between Peninsula and island cultures (Fig. 5). With our contribution we expanded the range and timing of some of the haplotypes (Larson et al., 2007a), e.g. samples belonging to the Italian clade observed in Sardinia during Neolithic age, and samples assignable to the Near Eastern lineage occurring in Tuscany and Campania in 1,800 AD (Fig. 5b). As plainly indicated also by our ancient mtDNA data (see Pompeii [79 AD], Sassari [27 BC - 476 AD] and Carbonia [VII–VI sec. BC] samples), the European E1 haplotypes were well represented in the past in South Italy, either as autochthonous populations previously settled or as a consequence of repeated, ancient introductions. In Italy the Near Eastern haplotype NE2-Y1 was found in museum samples 1,800 AD from Tuscany and Campania, previously recorded in archaeological remains from Romania to France and in modern populations on Corsica, where it still exists (Larson et al., 2007a; Larson, 2012). The first domestic pigs introduced into Europe possessed mitochondrial signatures acquired from Anatolian wild boar, but this signature was replaced by mtDNA haplotypes possessed by European wild boar about 4,000 years ago (Larson et al., 2007a). However recent studies typed the presence of the Near Eastern haplotype NE2-Y2 in North Italy in some pre-Neolithic *Sus* remains (Vai et al., 2015), so our samples can descend from early domesticated pigs arrived into Europe during the Neolithization of the continent or from a legacy of previous contacts with some northern populations. The difference between the two haplotypes observed in North and Central-South Italy (NE2-Y2 and NE2-Y1, respectively) is in a single mutation (an indel), and together with the large temporal difference from those remains, made this interpretation of results very difficult. Both hypotheses should be verified, considering more samples from Mesolithic to recent times. Finally, considering data on modern and ancient samples altogether, we can speculate about a high genetic diversity in Italian wild boar as a result of several additive factors including glaciation cycles, natural expansion and human translocation of animals, since Neolithic. ✍

References

Albarella U., Manconi F., Rowley-Conwy P., Vigne J.-D., 2006. Pigs of Sardinia and Corsica: a biometrical re-evaluation of their status and history. In: Tecchiati U., Sala B. (Eds.). Archaeozoological Studies in Honour of Alfredo Riedel. Province of Bolzano, Bolzano. 285–302.

Alexandri P., Triantafyllidis A., Papakostas S., Chatzinikos E., Platis P., Papageorgiou N., Larson G., Abatzopoulos T., Triantaphyllidis C., 2012. The Balkans and the colonization of Europe: the post-glacial range expansion of the wild boar, *Sus scrofa*. J. Biogeogr. 39(4): 713–723.

Alves C.P., Pinheiro I., Godinho R., Vicente J., Gortàzar C., Scandura M., 2010. Genetic diversity of wild boar populations and domestic pig breeds (*Sus scrofa*) in South-western Europe. Biol. J. Linn. Soc. 101(4): 797–822.

Alves E., Óvilo C., Rodríguez M.C., Silió L., 2003. Mitochondrial DNA sequence variation and phylogenetic relationships among Iberian pigs and other domestic and wild pig populations. Anim. Genet. 34(5): 319–324.

Apollonio M., Randi E., Toso S., 1988. The systematics of the wild boar (*Sus scrofa* L.) in Italy. Boll. Zool. 55(1-4): 213–221.

Apollonio M., Andersen R., Putman R., 2010. European Ungulates and their Management in the 21st st Century. Cambridge University Press, Cambridge.

Bandelt H.J., Forster P., Röhl A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16(1): 37–48.

Bennett K.D., Tzedakis P.C., Willis K.J., 1991. Quaternary refugia of north European trees. J. Biogeogr. 18(1): 103–115.

Canestrelli D., Aloise G., Cecchetti S., Nascetti G., 2010. Birth of a hotspot of intraspecific genetic diversity: notes from the underground. Mol. Ecol. 19(24): 5432–5451.

Canu A., Costa S., Iacolina L., Piatti P., Apollonio M., Scandura M., 2014. Are captive wild boar more introgressed than free-ranging wild boar? Two case studies in Italy. Eur. J. Wildl. Res. 60(3): 459–467.

Caramelli D., Lalueza-Fox C., Vernesi C., Lari M., Casoli A., Mallegni F., Chiarelli B., Dupanloup I., Bertranpetit J., Barbujani G., Bertorelle G., 2003. Evidence for a genetic discontinuity between Neandertals and 24,000-year-old anatomically modern Europeans. PNAS. 100(11): 6593–6597.

Clutton Brock J., 1999. A Natural History of Domesticated Mammals, 2nd ed., Cambridge, UK.

Crepaldi P., Fornarelli F., Marilli M., 2010. MC1R gene: comparison between different farm animal species. J. Anim. Sci. 4(Supp. 2): 43–45.

Drummond A., Ashton B., Buxton S., Cheung M., Cooper A., Duran C., Field M., Heled J., Kearse M., Markowitz S., Moir R., Stones-Havas S., Sturrock S., Thierer T., Wilson A., 2011. Geneious v 5.5. Available from: http://www.geneious.com [accessed 30 August 2011].

Fang M., Andersson L., 2006. Mitochondrial diversity in European and Chinese pigs is consistent with population expansions that occurred prior to domestication. Proc. R. Soc. Lond. B. Biol. Sci. 273(1595): 1803–1810.

Fang M., Larson G., Soares Ribeiro H., Li N., Andersson L., 2009. Contrasting Mode of Evolution at a Coat Color Locus in Wild and Domestic Pigs. PLoS. Genet. 5(1): e1000341. doi:10.137l/journal.pgen.1000341

Frantz A.C., Bertouille S., Eloy M.C., Licoppe A., Chaumont F., Flamand M.C., 2012. Comparative landscape genetic analyses show a Belgian motorway to be a gene flow barrier for red deer (*Cervus elaphus*), but not wild boars (*Sus scrofa*). Mol. Ecol. 21(14): 3445–3457.

Frantz A.C., Zachos F.E., Kirschning J., Cellina S., Bertouille S., Mamuris Z., Koutsogiannouli E.A., Burke T., 2013a. Genetic evidence for introgression between domestic pigs and wild boars (*Sus scrofa*) in Belgium and Luxembourg: a comparative approach with multiple marker systems. Biol. J. Linn. Soc. 110(1): 104–115.

Frantz L., Schraiber J., Madsen O., Megens H., Bosse M., Paudel Y., Semiadi G., Meijaard E., Li N., Crooijmans R., Archibald A., Slatkin M., Schook L., Larson G., Groenen M., 2013b. Genome sequencing reveals fine scale diversification and reticulation history during speciation in Sus. Genome. Biol. 14(9): R107.

Gilbert M.T.P., Bandelt H.-J., Hofreiter M., Barnes I., 2005. Assessing ancient DNA studies. Trends Ecol. Evol. 20(10): 541–544.

Gilbert M.T.P., Willerslev E., Hansen A.J., Barnes I., Rudbeck L., Lynnerup N., Cooper A., 2003. Distribution Patterns of Postmortem Damage in Human Mitochondrial DNA. Am. J. Hum. Genet. 72(1): 32–47.

Giuffra E., Kijas J., Amarger V., Carlborg Ö., Jeon J., Andersson L., 2000. The Origin of the Domestic Pig: Independent Domestication and Subsequent Introgression. Genetics 154(4): 1785–1791.

Giuffra E., Törnsten A., Marklund S., Bongcam-Rudloff E., Chardon P., Kijas J.M.H., Anderson S.I., Archibald A.L., Andersson L., 2002. A large duplication associated with dominant white color in pigs originated by homologous recombination between LINE elements flanking KIT. Mamm. Genome. 13(10): 569–577.

Gongora J., Peltoniemi O.A.T., Tammen I., Raadsma H., Moran C., 2003. Analyses of Possible Domestic Pig Contribution in Two Populations of Finnish Farmed Wild Boar. Acta Agric. Scand. Sect. A. Anim. Sci. 53: 161–165.

Gongora J., Cuddahee R., Nascimento F., Palgrave C., Lowden S., Ho S., Simond D., Damayanti C., White D., Tay W., Randi E., Klingel H., Rodrigues-Zarate C., Allen K., Moran C., Larson G., 2011. Rethinking the evolution of extant sub-Saharan African suids (Suidae, Artiodactyla). Zool. Scripta. 40: 327–335.

Guindon S., Gascuel O., 2003. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. Syst. Biol. 52(5): 696–704.

Handt O., Krings M., Ward R.H., Pääbo S., 1996. The retrieval of ancient human DNA sequences. Am. J. Hum. Genet. 59(2): 368–376.

Hewitt G., 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biol. J. Linn. Soc. 58: 247–276.

Hewitt G., 2000. The genetic legacy of the Quaternary ice ages. Nature. 405(6789): 907–913.

Hewitt G.M., 1999. Post-glacial re-colonization of European biota. Biol. J. Linn. Soc. 68(1–2): 87–112.

Hewitt G.M., 2004. Genetic consequences of climatic oscillations in the Quaternary. Phil. Trans. R. Soc. Lond. B. 359: 183–195.

Iacolina L., Scandura M., Goedbloed D.J., Alexandri P., Crooijmans R.P.M.A., Larson G., Archibald A., Apollonio M., Schook L.B., Groenen M.A.M., Megens H.J., 2016. Genomic diversity and differentiation of a managed island wild boar population. Heredity. 116(1): 60–67.

Kijas J.M.H., Moller M., Plastow G., Andersson L., 2001. A Frameshift Mutation in MC1R and a High Frequency of Somatic Reversions Cause Black Spotting in Pigs. Genetics. 158(2): 779–785.

Kijas J.M.H., Wales R., Törnsten A., Chardon P., Moller M., Andersson L., 1998. Melanocortin Receptor 1 (MC1R) Mutations and Coat Color in Pigs. Genetics. 150(3): 1177–1185.

Kim K.I., Lee J.H., Li K., Zhang Y.P., Lee S.S., Gongora J., Moran C., 2002. Phylogenetic relationships of Asian and European pig breeds determined by mitochondrial DNA D-loop sequence polymorphism. Anim. Genet. 33(1): 19–25.

Klungland H., Vage D.I., Gomez-Raya L., Adalsteinsson S., Lien S., 1995. The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat color determination. Mamm. Genome. 6(9): 636–639.

Koutsogiannouli E.A., Moutou K.A., Sarafidou T., Stamatis C., Mamuris Z., 2010. Detection of hybrids between wild boars (*Sus scrofa scrofa*) and domestic pigs (*Sus scrofa* f. domestica) in Greece, using the PCR-RFLP method on melanocortin-1 receptor (MC1R) mutations. Mamm. Biol. 75(1): 69–73.

Krause-Kyora B., Makarewicz C., Evin A., Flink L.G., Dobney K., Larson G., Hartz S., Schreiber S., von Carnap-Bornheim C., von Wurmb-Schwark N., Nebel A., 2013. Use of domesticated pigs by Mesolithic hunter-gatherers in northwestern Europe. Nat. Commun. 4: 2348.

Krings M., Stone A., Schmitz R.W., Krainitzki H., Stoneking M., Pääbo S., 1997. Neandertal DNA Sequences and the Origin of Modern Humans. Cell. 90(1): 19–30.

Kusza S., Podgórski T., Scandura M., Borowik T., Jávor A., Sidorovich V.E., Bunevich A.N., Kolesnikov M., Jędrzejewska B., 2014. Contemporary Genetic Structure, Phylo-

geography and Past Demographic Processes of Wild Boar Sus scrofa Population in Central and Eastern Europe. PLoS. ONE. 9(3): e91401. doi:10.137l/journal.pone.0091401

Larson G., 2012. Using pigs as a proxy to reconstruct patterns of human migration. Population Dynamics in Prehistory and Early History. In: Kaiser E., Burger J., Schier W. (Eds.) New Approaches by Using Stable Isotopes and Genetics. De Gruyter, Berlin, Boston. 31–40.

Larson G., Burger J. 2013. A population genetics view of animal domestication. Trends Genet. 29(4): 197–205.

Larson G., Dobney K., Albarella U., Fang M., Matisoo-Smith E., Robins J., Lowden S., Finlayson H., Brand T., Willerslev E., Rowley-Conwy P., Andersson L., Cooper A., 2005. Worldwide phylogeography of wild boar reveals multiple centers of pig domestication. Science. 307: 1618–1621.

Larson G., Albarella U., Dobney K., Rowley-Conwy P., Schibler J., Tresset A., Vigne J.-D., Edwards C.J., Schlumbaum A., Dinu A., Bălăçsescu A., Dolman G., Tagliacozzo A., Manaseryan N., Miracle P., Van Wijngaarden-Bakker L., Masseti M., Bradley D.G., Cooper A., 2007a. Ancient DNA, pig domestication, and the spread of the Neolithic into Europe. PNAS. 104(39): 15276–15281.

Larson G., Cucchi T., Fujita M., Matisoo-Smith E., Robins J., Anderson A., Rolett B., Spriggs M., Dolman G., Kim T.-H., Thuy N., Randi E., Doherty M., Due R., Bollt R., Djubiantono T., Griffin B., Intoh M., Keane E., Kirch P., Li K.-T., Morwood M., Pedrina L., Piper P., Rabett R., Shooter P., Van den Bergh G., West E., Wickler S., Yuan J., 2007b. Phylogeny and ancient DNA of *Sus* provides insights into neolithic expansion in Island Southeast Asia and Oceania. PNAS. 104(12): 4834–4839.

Larson G., Liu R., Zhao X., Yuan J., Fuller D., Barton L., Dobney K., Fan Q., Gu Z., Liu X.-H., Luo Y., Lv P., Andersson L., Li N., 2010. Patterns of East Asian pig domestication, migration, and turnover revealed by modern and ancient DNA. PNAS. 107(17): 7686–7691.

Lattuada L., Quaglia F., Iannelli F., Gissi C., Mantecca P., Bacchetta R., Polli M., 2009. Mitochondrial DNA sequence variations in some Italian wild boar populations. J. Anim. Breed. Genet. 126(2): 154–163.

Leonard J.A., Shanks O., Hofreiter M., Kreuz E., Hodges L., Ream W., Wayne R.K., Fleischer R.C., 2007. Animal DNA in PCR reagents plagues ancient DNA research. J. Archaeol. Sci. 34(9): 1361–1366.

Li H., Handsaker B., Wysoker A., Fennell T., Ruan J., Homer N., Marth G., Abecasis G., Durbin R., 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 25: 2078–2079.

Librado P., Rozas J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 25(11): 1451–1452.

MacKinnon M., 2001. High on the hog: linking the zooarchaeological, literary and artistic evidence for pig breeds in Roman Italy. Am. J. Archaeol. 105: 1–25.

Marklund L., Moller M.J., Sandberg K., Andersson L., 1996. A missense mutation in the gene for melanocyte-stimulating hormone receptor (MC1R) is associated with the chestnut coat color in horses. Mamm. Genome. 7(12): 895–899.

Maselli V., Rippa D., Russo G., Ligrone R., Soppelsa O., D'Aniello B., Raia P., Fulgione D., 2014a. Wild boars' social structure in the Mediterranean habitat. Ital. J. Zool. 81(4): 610–617.

Maselli V., Polese G., Larson G., Raia P., Forte N., Rippa D., Ligrone R., Vicidomini R., Fulgione D., 2014b. A Dysfunctional Sense of Smell: The Irreversibility of Olfactory Evolution in Free-Living Pigs. Evol. Biol. 41(2): 229–239.

Masseti M., 2002. Uomini e (non solo) topi. Gli animali domestici e la fauna antropocora. Università degli Studi di Firenze. [in Italian]

Masseti M., 2007. The economic role of *Sus* in early human fishing communities. In: Albarella U., Dobney K., Ervynck A., Rowley-Conwey P. (Eds.) Pigs and humans 10,000 years of interaction. Oxford University Press, Oxford, New York. 156–170.

Moller M.J., Chaudhary R., Hellmén E., Höyheim B., Chowdhary B., Andersson L., 1996. Pigs with the dominant white coat color phenotype carry a duplication of the KIT gene encoding the mast/stem cell growth factor receptor. Mamm. Genome. 7(11): 822–830.

Nei M., 1987. Molecular Evolutionary Genetics,. Columbia University Press, New York.

Okumura N., Kurosawa Y., Kobayashi E., Watanobe T., Ishiguro N., Yasue H., Mitsuhashi T., 2001. Genetic relationship amongst the major non-coding regions of mitochondrial DNAs in wild boars and several breeds of domesticated pigs. Anim Genet. 32(3): 139–147.

Ottoni C., Flink L.G., Evin A., Geörg C., De Cupere B., Van Neer W., Bartosiewicz L., Linderholm A., Barnett R., Peters J., Decorte R., Waelkens M., Vanderheyden N., Ricaut F.-X., Rus Hoelzel A., Mashkour M., Karimlu A.F.M., Seno S.S., Daujat J., Brock F., Pinhasi R., Hongo H., Perez-Enciso M., Rasmussen M., Frantz L., Megens H.-J., Crooijmans R., Groenen M., Arbuckle B., Benecke N., Vidarsdottir U.S., Burger J., Cucchi T., Dobney K., Larson G., 2012. Pig domestication and human-mediated dispersal in western Eurasia revealed through ancient DNA and geometric morphometrics. Mol. Biol. Evol. 30(4): 824–832.

Pielberg G., Olsson C., Syvänen A.-C., Andersson L., 2002. Unexpectedly High Allelic Diversity at the KIT Locus Causing Dominant White Color in the Domestic Pig. Genetics. 160(1): 305–311.

Posada D., Crandall K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics. 14(9): 817–818.

Pushkina D., Raia P., 2008. Human influence on distribution and extinctions of the late Pleistocene Eurasian megafauna. J. Hum. Evol. 54(6): 769–782.

Rambaut A., Suchard M., Xie D., Drummond A., 2014. Tracer v1.4. Available from: http://beast.bio.ed.ac.uk/Tracer [accessed 1 February 2014].

Randi E., 1995. Conservation genetics of the genus *Sus*. Ibex. J. Mt. Ecol. 36:-12.

Randi E., 2005. Management of Wild Ungulate Populations in Italy: Captive-Breeding, Hybridisation and Genetic Consequences of Translocations. Vet. Res. Commun. 29(2): 71–75.

Randi E., D'Huart J.P., Lucchini V., Aman R. 2002. Evidence of two genetically deeply divergent species of warthog, *Phacochoerus africanus* and *P. aethiopicus* (Artiodactyla: Suiformes) in East Africa. Mamm. Biol. 67(2): 91–96.

Rogers A.R., Harpending H., 1992. Population growth makes waves in the distribution of pairwise genetic differences. Mol. Biol. Evol. 9(3): 552–569.

Ronquist F., Huelsenbeck J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19(12): 1572–1574.

Rook L., Martínez-Navarro B., 2010. Villafranchian: The long story of a Plio-Pleistocene European large mammal biochronologic unit. Quatern. Int. 219(1): 134–144.

Scandura M., Iacolina L., Apollonio M., 2011a. Genetic diversity in the European wild boar *Sus scrofa*: phylogeography, population structure and wild x domestic hybridization. Mammal Rev. 41(2): 125–137.

Scandura M., Iacolina L., Cossu A., Apollonio M., 2011b. Effects of human perturbation on the genetic make-up of an island population: the case of the Sardinian wild boar. Heredity. 106(6): 1012–1020.

Scandura M., Iacolina L., Crestanello B., Pecchioli E., Di Benedetto M.F., Russo V., Davoli R., Apollonio M., Bertorelle G., 2008. Ancient vs. recent processes as factors shaping the genetic variation of the European wild boar: are the effects of the last glaciation still detectable? Mol. Ecol. 17(7): 1745–1762.

Schmitt T., Varga Z., 2012. Extra-Mediterranean refugia: The rule and not the exception? Front. Zool. 9(1): 22.

Schneider S., Excoffier L., 1999. Estimation of Past Demographic Parameters From the Distribution of Pairwise Differences When the Mutation Rates Vary Among Sites: Application to Human Mitochondrial DNA. Genetics. 152(3): 1079–1089.

Smith C.I., Chamberlain A.T., Riley M.S., Stringer C., Collins M.J., 2003. The thermal history of human fossils and the likelihood of successful DNA amplification. J. Hum. Evol. 45(3): 203–217.

Sommer R., Zoller H., Kock D., Bohme W., Griesau A., 2005. Feeding of the barn owl, *Tyto alba* with first record of the European free-tailed bat, Tadarida teniotis on the island of Ibiza (Spain, Balearics). Folia. Zool. 54(4): 364–370.

Stone A.C., Stoneking M., 1998. mtDNA Analysis of a Prehistoric Oneota Population: Implications for the Peopling of the New World. Am. J. Hum. Genet. 62(5): 1153–1170.

Taberlet P., Fumagalli L., Wust-Saucy A.-G., Cosson J.-F., 1998. Comparative phylogeography and postglacial colonization routes in Europe. Mol. Ecol. 7(4): 453–464.

Ursing B.M., Arnason U., 1998. The Complete Mitochondrial DNA Sequence of the Pig (*Sus scrofa*). J. Mol. Evol. 47(3): 302–306.

Vai S., Vilaça S.T., Romandini M., Benazzo A., Visentini P., Modolo M., Bertolini M., MacQueen P., Austin J., Cooper A., Caramelli D., Lari M., Bertorelle G., 2015. The Biarzo case in northern Italy: is the temporal dynamic of swine mitochondrial DNA lineages in Europe related to domestication? Sci. Rep. 5: 16514.

Vigne J.-D., Carrère I., Briois F., Guilaine J., 2011. The Early Process of Mammal Domestication in the Near East: New Evidence from the Pre-Neolithic and Pre-Pottery Neolithic in Cyprus. Curr. Anthropol. 52(S4): S255–S271.

Vigne J.-D., Zazzo A., Saliège J.-F., Poplin F., Guilaine J., Simmons A., 2009. Pre-Neolithic wild boar management and introduction to Cyprus more than 11,400 years ago. PNAS. 106(38): 16135–16138.

Vilaça S.T., Biosa D., Zachos F., Iacolina L., Kirschning J., Alves P.C., Paule L., Gortazar C., Mamuris Z., Jędrzejewska B., Borowik T., Sidorovich V.E., Kusak J., Costa S., Schley L., Hartl G.B., Apollonio M., Bertorelle G., Scandura M., 2014. Mitochondrial phylogeography of the European wild boar: the effect of climate on genetic diversity and spatial lineage sorting across Europe. J. Biogeogr. 41(5): 987–998.

Watanobe T., Okumura N., Ishiguro N., Nakano M., Matsui A., Sahara M., Komatsu M., 1999. Genetic relationship and distribution of the Japanese wild boar (*Sus scrofa leucomystax*) and Ryukyu wild boar (*Sus scrofa riukiuanus*) analysed by mitochondrial DNA. Mol. Ecol. 8(9): 1509–1512.

Watanobe T., Ishiguro N., Okumura N., Nakano M., Matsui A., Hongo H., Ushiro H., 2001. Ancient Mitochondrial DNA Reveals the Origin of *Sus scrofa* from Rebun Island, Japan. J. Mol. Evol. 52(3): 281–289.

Wilkens B., 1987. Il passaggio tra Mesolitico e Neolitico attraverso lo studio delle faune di alcuni siti dell'Italia centro-meridionale. University of Pisa, Pisa. [in Italian]

Wilkens B., 2012. Archeozoologia. Il Mediterraneo, la storia, la Sardegna. Edes edizioni, Sassari [in Italian]

Wilkens B., Delussu F., 2003. Wild and domestic mammals in holocenic Sardinia. In: Legakis A. (Ed.) The new panorama of the animal evolution. Proceedings XVIII International Congress of Zoology, Athens. 303–308.

Yalden D., 1999. The History of British Mammals. Poyser/Academic Press, London.

Zinser E.R., Schneider D., Blott M., Kolter R., 2003. Bacterial evolution through the selective loss of beneficial genes: trade-offs in expression involving two loci. Genetics. 164: 1271–1277.

Associate Editor: M. Scandura

Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 A list of ancient *Sus scrofa* specimens obtained for this study, extracted, and sequenced and deposited on GenBank.

Table S2 A list of modern *Sus scrofa* specimens analyzed in this study. Corresponding sequences were deposited on Genbank.

Table S3 A list of modern *Sus scrofa* sequences deposited on GenBank used to build Bayesian tree.

Table S4 Sequences used to define the genetic variability among different populations each assigned to specific refugia (Balkans, Iberia, and Italy).

Table S5 Sequences used to define the genetic variability within the Italian refugium, considering populations divided by regions (Piedmont, Emilia Romagna, Tuscany, Latium, Campania, Basilicata, Apulia, Sardinia).

Table S6 A list of ancient *Sus scrofa* specimens available on Genbank and used in this study.

Figure S7 Bayesian trees.