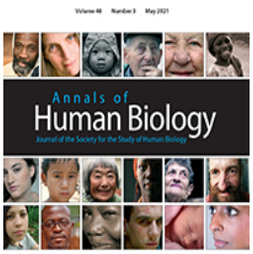

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RESEARCH PAPER

More data on ancient human mitogenome variability in Italy: new mitochondrial genome sequences from three Upper Palaeolithic burials

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ABSTRACT

Background: Recently, the study of mitochondrial variability in ancient humans has allowed the definition of population dynamics that characterised Europe in the Late Pleistocene and Early Holocene. Despite the abundance of sites and skeletal remains few data are available for Italy.

Aim: We reconstructed the mitochondrial genomes of three Upper Palaeolithic individuals for some of the most important Italian archaeological contexts: Paglicci (South-Eastern Italy), San Teodoro (South-Western Italy) and Arene Candide (North-Western Italy) caves.

Subjects and methods: We explored the phylogenetic relationships of the three mitogenomes in the context of Western Eurasian ancient and modern variability.

Results: Paglicci 12 belongs to sub-haplogroup U8c, described in only two other Gravettian individuals; San Teodoro 2 harbours a U2'3'4'7'8'9 sequence, the only lineage found in Sicily during the Late Pleistocene and Early Holocene; Arene Candide 16 displays an ancestral U5b1 haplotype already detected in other Late Pleistocene hunter-gatherers from Central Europe.

Conclusion: Regional genetic continuity is highlighted in the Gravettian groups that succeeded in Paglicci. Data from one of the oldest human remains from Sicily reinforce the hypothesis that Epigravettian groups carrying U2'3'4'7'8'9 could be the first inhabitants of the island. The first pre-Neolithic mitogenome from North-Western Italy, sequenced here, shows more affinity with continental Europe than with the Italian peninsula.

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Mitochondrial DNA; ancient DNA; Upper Palaeolithic; Italian hunter-gatherers; LGM

Introduction

According to mitochondrial DNA (mtDNA) data, peopling of Europe by Early Modern Humans occurred less than 55,000 years ago (ya) through a single, late and rapid dispersal that brought about expansion of haplogroups (hg) M and N (Fu et al. 2016; Posth et al. 2016). The estimated most recent common ancestor (TMRCA) for these basal non-African clades are about 49,000 ya for hg M and 51,000 ya for hg N (Posth et al. 2016). After this spread, the founder population experienced a first slow increase in size, accompanied by a differentiation of the mtDNA variability. Between the most ancient available individuals (45,000–24,000 ya), haplotypes close to the root of hg R, N, M and U are present, as well as hg U5, U2 and U8. This period was followed by a genetic bottleneck during the Last Glacial Maximum (LGM, between 25,000 and 19,500 ya) (Posth et al. 2016). Small groups survived in climatic *refugia* and re-expanded after the LGM as the ice sheets retracted (Fu et al. 2016; Posth et al. 2016). Hg M seems to disappear in Europe in the post-LGM, probably as a consequence of the genetic bottleneck. The available mitogenomes for the post-LGM period (19,500–14,500 ya) are represented by the already mentioned hg U sub-haplogroups. A period of climatic instability characterised the Late Glacial period (14,500–11,500 ya) and it reflected its consequences on the hunter-gatherer population dynamics. A major population turnover occurred around 14,500 ya in correspondence with the warm Late Glacial interstadial Bølling-Allerød (Posth et al. 2016). The post-LGM mtDNA variability was partially replaced by lineages coming from a still undefined glacial *refugium*; as a consequence of this shift, the Holocene mtDNA genomes available so far mainly belong to derived sub-lineages of hg U5, with the

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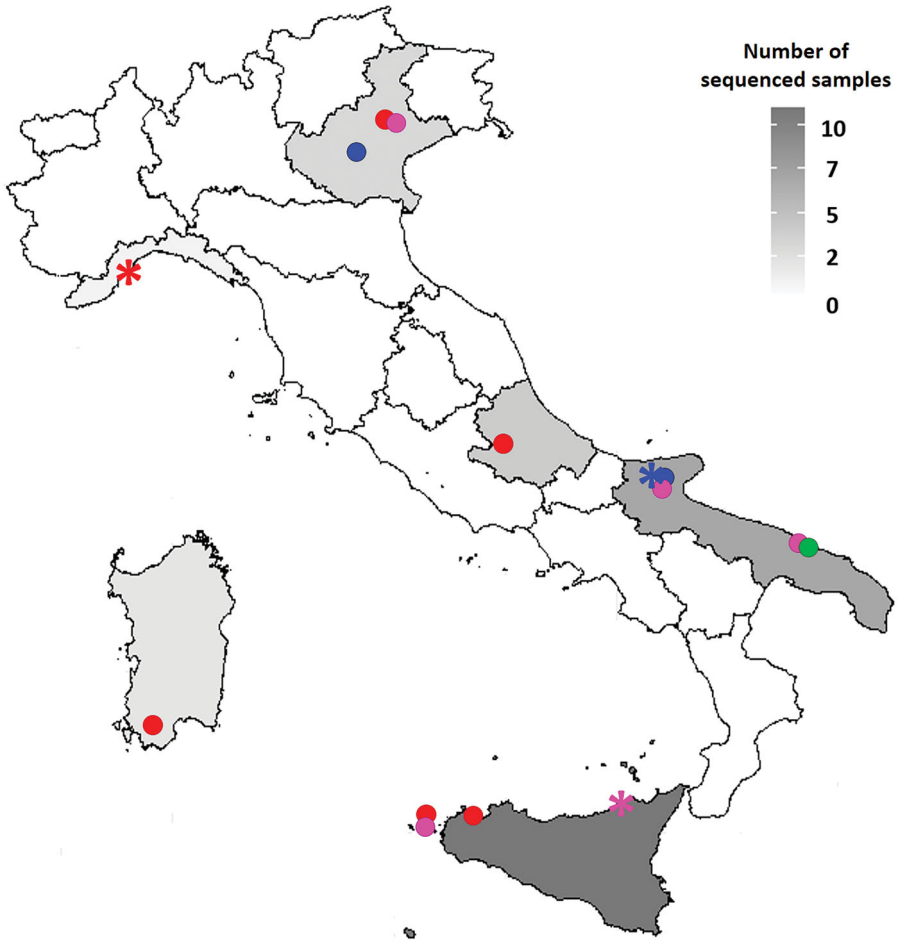

Figure 1. Overview of the Italian Upper Palaeolithic sites characterised for mtDNA genomes. Sites are marked according to the sample chronological assignment: samples dated to the Pre-LGM are coloured in blue, Post-LGM in green, Late Glacial in magenta and Holocene hunter-gatherers in red. Samples analysed in this study are marked with an asterisk. The map was generated with Rstudio.

appearance of U4 lineages and the persistence in southern regions of U2'3'4'7'8'9 subclade, sporadically present in the previous periods.

A large number of archaeological sites and abundance of skeletal remains characterise the region of present-day Italy. Twenty-one complete mtDNA genomes from 9 Italian Upper Palaeolithic sites have been sequenced to date (Fu et al. 2013; Benazzi et al. 2015; Fu et al. 2016; Posth et al. 2016; Modi et al. 2017; Antonio et al. 2019; Modi et al. 2020; van de Loosdrecht et al. 2020; Bortolini et al. 2020) (Figure 1). It is of particular interest to increase the number of specimens with genetic information for this country, considering its role as a glacial refugium (Blockley et al. 2018) and harbour between the Mediterranean and continental Europe, as well as its internal geographical boundaries that could have shaped the genetic variability in a different way compared to the rest of Europe. It has been proposed that Italy could form a regionally distinct part of the Western European metapopulation, as the material culture record seems to suggest, with the transition to Epigravettian during the LGM distinguishing Italy from the other regions (Wren and Burke 2019).

Here we present the mtDNA genomes of individuals recovered from three key Palaeolithic sites in Italy (Figure 1): one Gravettian juvenile skeleton from Paglicci cave (Puglia, South-Eastern Italy), one Late Epigravettian individual from San Teodoro cave (Sicily, South-Western Italy), and one Late Epigravettian adolescent from Arene Candide cave (Liguria, North-Western Italy). We then explored the genetic relationships and geographic affinities of the three newly characterised sequences in the context of Western Eurasian ancient and modern variability.

Subjects and methods

Sample description

To improve the recovering of ancient DNA (aDNA), we analysed two already disarticulated petrous bones and one auditory ossicle from three Italian hunter-gatherer individuals. A description of the samples and archaeological sites is provided in the next paragraphs.

Paglicci 12

Paglicci Cave is located on the western side of the Gargano promontory in Apulia (Rignano Garganico, Foggia, Italy). The site attests a human frequentation between the Middle Palaeolithic and the Final Epigravettian, and the Upper Palaeolithic sequence is one of the most complete in southern Europe. Since 1961, the numerous field excavations allowed the discovery of abundant lithic and large mammal

assemblages (Boschin et al. 2018; Boschin et al. 2020 and references therein), and art objects (Mezzena and Palma di Cesnola 2004; Arrighi et al. 2008; Arrighi et al. 2012). Paglicci also yielded the only Upper Palaeolithic wall paintings known in Italy so far (Zorzi 1963; Arrighi et al. 2012), and additionally, the site provided the most ancient evidence of plant-food manipulation and flour consumption (Mariotti Lippi et al. 2015). Regarding human fossil records, the cave yielded more than 100 isolated remains (Condemi et al. 2014) as well as one partial Late Epigravettian and two complete Evolved Gravettian burials belonging to one female adult individual (Paglicci 25 from the burial Paglicci III, found in layer 21B) and one adolescent (Paglicci 12, PA12, from the burial Paglicci II, found at the base of layer 21 D) (Ronchitelli et al. 2015; Fu et al. 2016; Posth et al. 2016). aDNA analysis was performed on the right petrous bone collected from individual PA12. Radiocarbon dating performed on 21 D archaeological level, locates the sample between 29,750 and 27,872 cal. BP (24,720 ± 420 uncal. BP, F-55) (Ronchitelli et al. 2015).

San Teodoro 2. The San Teodoro Cave is located in northern Sicily (San Fratello Acquedolci, Messina, Italy) and represents one of the most important prehistoric archaeological sites to understand the island's peopling dynamics (Sineo et al. 2015). The site has been known since the 19th century (Bonfiglio et al. 2001) and yielded a rich Pleistocene vertebrate fossil assemblage (Catalano et al. 2020; Garilli et al. 2020 and references therein) as well as late Upper Palaeolithic human burials and artefacts (Graziosi and Maviglia 1946; D'Amore et al. 2009 and references therein). The Upper Palaeolithic remains from San Teodoro Cave represent the oldest human skeletal sample yet found in Sicily. Seven variously preserved *Homo sapiens* individuals (ST1-7) were excavated from different stratigraphic settings in the outer part of the cave (Graziosi and Maviglia 1946; Graziosi 1947); they could possibly be considered the direct descendants of the earliest Epigravettian settlers who arrived in Sicily crossing the Messinian strait (D'Amore et al. 2009). The sample analysed here is one of the three auditory ossicles (left incus) collected from the San Teodoro 2 (ST2) individual during a restoration activity (Carotenuto et al. 2013). ST2 is represented by an almost complete cranium housed at the Geological Museum "G. G. Gemmellaro" of the University of Palermo. It was discovered by Maviglia in 1938 in a disturbed burial and only the cranium was preserved to be unearthed in the subsequent year. Morphological study attributed the specimen to a 40–50 years-old individual according to dental wear (Graziosi 1947; D'Amore et al. 2009). It was found in the same stratum of San Teodoro 1 (ST1). Morphological features suggest the skeleton belongs to an Epigravettian individual. The skeleton ST1, found in close proximity to ST2, gave a calibrated age of 14,700 years BP (15,232–14,126 cal BP) (Incarbona et al. 2010; Mannino et al. 2011).

Arene Candide 16. Arene Candide Cave is located at an altitude of about 90 m above sea level, on the west upper margin of Monte Caprazoppa in Liguria (Finale Ligure, Savona, Italy). The archaeological site provides several findings, burials and human remains between the Palaeolithic and historical times. In addition to the famous burial of the "Prince," a Gravettian adolescent with a rich grave, goods and shell ornaments (Sergi et al. 1974; Pettitt et al. 2003), more than 20 individuals dating back to the final Epigravettian (10,500–10,000 BCE) were found in a large necropolis (Sparacello et al. 2018). Thanks to the well-preserved funerary complexes and objects, Arene Candide is considered one of the most important sites in Europe for understanding funerary behaviour in the Upper Palaeolithic. Genetic analysis was performed on an adolescent individual, Arene Candide 16 (AC16), discovered by Cardini in 1970. This finding represents early evidence of the funerary use of the cave during the Late Epigravettian (Formicola and Toscani 2014). AC16 was directly radiocarbon dated to 10,810 uncal. BP (12,820 cal BP) (Formicola and Toscani 2014; Sparacello et al. 2018). DNA was extracted from disarticulated left petrous bone.

Methods

Sample preparation and DNA extraction

Experimental steps of DNA isolation and library preparation were carried out at the Laboratory of Anthropology and Palaeogenetics at University of Florence, exclusively dedicated to aDNA analysis, and appropriate criteria to prevent contamination with present-day DNA were followed. DNA extraction and library preparation reactions included negative controls. The petrous portion of the temporal bone was collected for the samples AC16 and PA12, while one of the three auditory ossicles (left incus) was available for ST2. To remove potential contamination, the outer layer of the petrous bone samples was brushed with disposable tools and irradiated by ultraviolet light (254 nm) for 45 min in a Biolink DNA Crosslinker (Biometra). The petrous pyramids were sectioned using a disc saw, and the densest part of inner ear was selected to collect bone powder using a dentist microdrill with disposable tips (Pinhasi et al. 2015). The incus was processed as described in Sirak et al. 2020. 50–65 mg of petrous bone powder and the whole auditory ossicle were used for DNA extraction using a silica-based protocol that allows aDNA molecules to be efficiently recovered even if highly fragmented (Dabney et al. 2013). DNA was eluted twice in 50 µl of TET buffer (10 nM Tris, 1 mM EDTA, 0.05% Tween-20).

NGS library preparation and sequencing

NGS libraries were prepared starting from 20 µl of DNA extract for each specimen following a double-stranded DNA protocol optimised for ancient samples, in order to make the DNA immortalised, barcoded and available for the Next Generation Sequencing (NGS) on Illumina platforms. A partial uracil-DNA-glycosylase treatment (Rohland et al. 2015) was performed for ST2 sample. This treatment removes uracile residues and abasic sites in the internal portion of the molecules, but partially preserves the characteristic deamination pattern associated with aDNA damage at the ends of the fragments, which can be used to discriminate endogenous DNA from possible modern contaminants (Rohland et al.

2015). No uracil-DNA glycosylase treatment was performed for AC16 and PA12 samples (Meyer and Kircher 2010). A unique combination of two indexes per specimen was used for barcoding. Libraries were enriched for mtDNA following a multiplexed capture protocol (Maricic et al. 2010) and sequenced on an Illumina MiSeq v3 $2 \times 75 + 8 + 8$ bp chemistry. For ST2, the enriched library was sequenced on Illumina NovaSeq 6000, setting $1 \times 100 + 8 + 8$ run parameters.

Bioinformatics analysis on sequence data

Sequences were demultiplexed and sorted according to the sample, then raw sequence data were analysed using the pipeline described in Peltzer et al. (2016).

Adapters were clipped-off and reads with a minimum overlap of 10 bp were merged in a single sequence using Clip&Merge version 1.7.4 (read merging was skipped for ST2). Merged reads were then mapped on the revised Cambridge Reference Sequence, rCRS (GenBank Accession Number NC_012920) using CircularMapper and BWA v.0.6.2. Reads with mapping quality below 30 were discarded. Duplicates were removed using DeDup v0.12.1, a tool that considers both ends of the fragments to recognise them as clonal. Length and deamination patterns were estimated using MapDamage 2.0 (Jónsson et al. 2013). Deamination rates were estimated by schmutzi (Renaud et al. 2015), with default parameters in order to consider 2 bases to be deaminated on each end for ST2, and setting –lengthDeam 5 for PA12 and AC16. Endogenous consensus sequences were called using endoCaller, a program integrated in schmutzi. The program takes into account deaminations at the ends of the molecules for consensus calling. Bases with individual likelihood < 20 were considered as missing positions (Ns). Present-day human contamination was estimated using a database of 256 Eurasian mitochondrial genomes distributed with schmutzi.

Mitochondrial haplogroup definition and phylogenetic analysis

The mitochondrial haplogroups were assigned according to PhyloTree Build 17 by Haplogrep2 (van Oven 2015; Weissensteiner et al. 2016).

A phylogenetic tree was constructed to infer the phylogenetic position of the new mtDNA genomes. The three assembled mitogenomes were aligned to previously published sequences from 53 worldwide modern humans (Ingman et al. 2000) and 74 Upper Palaeolithic and Mesolithic Eurasian specimens (Table S1), using the MUSCLE software (Edgar 2004). Feldhofer 2 mtDNA (FM865408, Briggs et al. 2009) was included as a phylogenetic out-group, for a total of 128 sequences. Nucleotide position 16519, as well as the poly-C stretches and AC-indels at positions 16180–16193, 303–315, 515–524 and 573–576, were excluded from the phylogenetic analysis (Derenko et al. 2014). A Maximum Parsimony tree was built using MEGA X (Kumar et al. 2018) with SPR algorithm and 99% partial deletion. The phylogeny was tested with the bootstrap method and 1000 replications.

Then, the dataset used for the construction of Maximum Parsimony tree was filtered to retain only sequences from samples with an associated radiocarbon date and enriched with further ancient and modern mitogenomes selected for representing specific sub-haplogroups of interest. The new dataset included the three newly assembled mitogenomes, sequences from 65 modern individuals and 85 ancient samples ranging from Upper Palaeolithic to Bronze Age, as well as Feldhofer 2 mtDNA (FM865408, Briggs et al. 2009) as outgroup, for a total of 154 sequences (Table S2). The alignment obtained through MUSCLE software (Edgar 2004), excluding positions with gaps, was then analysed with jModelTest v2.1.7 (Guindon and Gascuel 2003; Darriba et al. 2012) to identify the best-fit model of evolution according to the AIC and BIC. A Bayesian phylogenetic analysis was performed to explore phylogenetic relationships between samples and estimate the time to the most recent common ancestor (TMRCA) for specific mitochondrial lineages using BEAST v.2.6.2 (Bouckaert et al. 2019) on a total of 16535 nucleotide positions. Hasegawa-Kishino-Yano with invariant sites and gamma distributed rates (HKY + G + I) was selected as a substitution model, according to the model testing result. The mutation rate was set at 2.74×10^{-8} (95% HPD: $2.44 \times 10^{-8} - 3.01 \times 10^{-8}$) mutations/site/year, and a Bayesian Skyline coalescent with 20 as group number with a strict molecular clock model were used as priors as this combination was estimated to be the best supported in Posth et al. (2016). Tip dates were indicated for the ancient samples, according to their radiocarbon calibrated BP ages, considering their confidence intervals as uniform priors. Tip dates of zero were selected for present-day mtDNA sequences. Markov chain Monte Carlo (MCMC) was performed with 50 000 000 iterations and trees were sampled every 1,000 generations. Effective sample size (ESS) values and the adequate convergence of the MCMC chains were checked using Tracer v1.7.1 (Rambaut et al. 2018). After discarding the first 10% of iterations as burn-in, TreeAnnotator v.2.6.2 (Bouckaert et al. 2019) was used to produce a Maximum Clade Credibility tree, visualised and edited by FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

The genetic relationships and geographic affinities within Western Eurasia of the three newly characterised mitogenomes were explored in more detail with haplotype networks. An additional database including 71 ancient and 12 modern full-length mitochondrial sequences attributed to haplogroups U2'3'4'7'8'9 and U8 and U5b1 (including 3 sequences belonging to ancestral U5 haplogroup), was assembled from the published literature (Table S3 and S4). Sequences were grouped according to their mitochondrial lineage and aligned with MUSCLE software (Edgar 2004). After removing gaps and missing sites, 12494 positions were retained for U5b1 database and 15696 for U2'3'4'7'8'9/U8. Nexus files were generated with MEGA X (Kumar et al. 2018) and used to build two Median-Joining networks in PopART ($\varepsilon = 0$) (Leigh and Bryant, 2015). The networks nodes are colour-coded according to the "Traits block" of the Nexus-format alignments.

Results

We reconstructed nearly complete mtDNA genomes for the three pre-Neolithic individuals analysed, with a sequencing coverage between 83x and 301x. Only 1 missing position is present in Arene Candide 16 (AC16) and San Teodoro 2 (ST2) consensus sequences and 3 in Paglicci 12 (PA12). The samples show typical features of aDNA: short molecules, with average length of ~53 base pairs (bp), and high rate of cytosine deamination at the 5′ end of the molecules (Table 1). Contamination was estimated at 2% for all the samples, allowing us to assess the authenticity of the reconstructed consensus sequences for all the three individuals (Table 2). We defined three different lineages all belonging to haplogroup U: U8c for PA12, U2′3′4′7′8′9 for ST2 and U5b1 for AC16 samples (Table S5). The three new mitogenomes were placed into a Bayesian phylogenetic tree together with a dataset of modern and ancient sequences. The three new sequenced mitogenomes fall into three different branches of the phylogenetic tree (Figure 2, Figure S1), in agreement with the general frame of replacement between different hunter-gatherer populations through time in Europe, as already described (Posth et al. 2016). The time to the most recent common ancestor (TMRCA) for U8, U2′3′4′7′8′9 and U5b1 clades was estimated at 41,387 years BP (95% HPD: 36,706–45,987), 42,771 years BP (95% HPD: 37,434–46,945) and 21,434 years BP (95% HPD: 17,565–26,295), respectively. Coalescent times obtained here are in agreement with independent estimates from modern mitogenomes (Behar et al. 2012), with the exception of U5b1 TMRCA that is earlier in our estimate. We also estimated the TMRCA of U8 sub-lineages and of different nodes within the U2′3′4′7′8′9 clade, that represent the only lineage in our analysis encompassing LGM (Table S6).

Additionally, the new mtDNA sequences were depicted in two Median-Joining Networks, including also post-Neolithic and modern individuals (Figure 3). When compared to other sequences belonging to U8 and U2′3′4′7′8′9 sub-haplogroups, PA12 falls in a separate arm branching from one of the ancestral nodes and including only two other pre-LGM individuals, Paglicci133 from the same site and Dolni Vestonice 13 from Eastern Europe (Fu et al. 2013; Fu et al. 2016; Posth et al. 2016) (Figure 3(a)). According to the younger age, PA12 shows a slightly more derived haplotype than the other samples in the branch. ST2 seems related to the other individuals from Sicily (two samples from Grotta d'Oriente and two samples from Grotta dell'Uzzo) as well as to coeval and later samples from Southern Italy (Grotta Paglicci), France (Rigney) and Iberia (Balma Guilanyà), the latter showing the most diversified haplotype (Fu et al. 2013; Posth et al. 2016; Catalano et al. 2020; Modi et al. 2020; Villalba-Mouco et al. 2019; van de Loosdrecht et al. 2020) (Figure 3(a)). The clade branches from an ancestral node after the separation of the only pre-LGM sample assigned to the same U2′3′4′7′8′9 lineage, a 28,000 year-old individual from Southern Italy (Paglicci 108) (Posth et al. 2016). In the context of U5b1 haplotypic variation, AC16 brings an ancestral haplotype highly similar to the sequences found in other Late Pleistocene and Early Holocene hunter-gatherers from Central and Northern Europe (Figure 3(b)) (Fu et al 2013; Posth et al 2016; Mathieson et al. 2020; Table S4). This haplotype appears ancestral to all the more derived U5b1 sub-lineages found in Europe in later ages up to modern time (Figure 3(b)).

Discussion

The new mitogenomes presented here were obtained from three Upper Palaeolithic Italian samples coming from different geographical contexts: Puglia (South-Eastern Italy), Sicily (South-Western Italy) and Liguria (North-Western Italy). They are also representative of different chronological periods: Paglicci 12 (PA12) dated back to 29,000 years BP, during the pre-Last Glacial Maximum, while San Teodoro 2 (ST2) (14,700 years BP) and Arene Candide 16 (AC16) (12,820 years BP) dated back to the Late Glacial Period. Their phylogenetic position fits well with the already known distribution of mtDNA variability in space and time during the Late Pleistocene and Early Holocene in Europe.

The lineage U8c found in PA12 sample is shared with another slightly older individual from the same archaeological site, Paglicci 133, dated to 33,000 years BP, bringing a less derived haplotype (Fu et al. 2016; Posth et al. 2016). In the context of the pre-Last Glacial Maximum samples, only

Table 1. Bioinformatics analysis results.

Sample ID	# of Raw Reads prior to Clip & Merge	# Reads after C&M prior to mapping	# Mapped Reads prior to RMDup	# of Duplicates removed	Mapped Reads after RMDup	Cluster Factor	mtDNA Average Coverage Depth	5′-end deamination pattern (%)	3′-end deamination pattern (%)	average fragment length (bp)
AC16	1164678	578612	132150	39650	92500	1,429	301.2	35.11	34.98	53.95
PA12	770952	374068	112691	54082	58609	1,923	184.28	50.69	49.11	52.1
ST2*	1051346	970137	255954	229100	26854	9,531	83.33	20.45	19.88	51.41

*library with partial-UDG treatment

Number of raw reads, number of mapped reads prior to and after PCR duplicates removal, mtDNA average depth of coverage, deamination patterns at molecule termini and average fragment length are reported.

Table 2. Schmutzi results.

Sample ID	First iteration (low-high)	Final iteration (low-high)	Number of missing positions	mtDNA haplogroup
AC16	0 (0–0.05)	0.02 (0.01–0.03)	1	U5b1
PA12	0 (0–0.05)	0.02 (0.01–0.03)	3	U8c
ST2	0.05 (0.025–0.075)	0.02 (0.01–0.03)	1	U2′3′4′7′8′9

Contamination estimate, number of unassigned positions and mitochondrial haplogroup are reported for each sample.

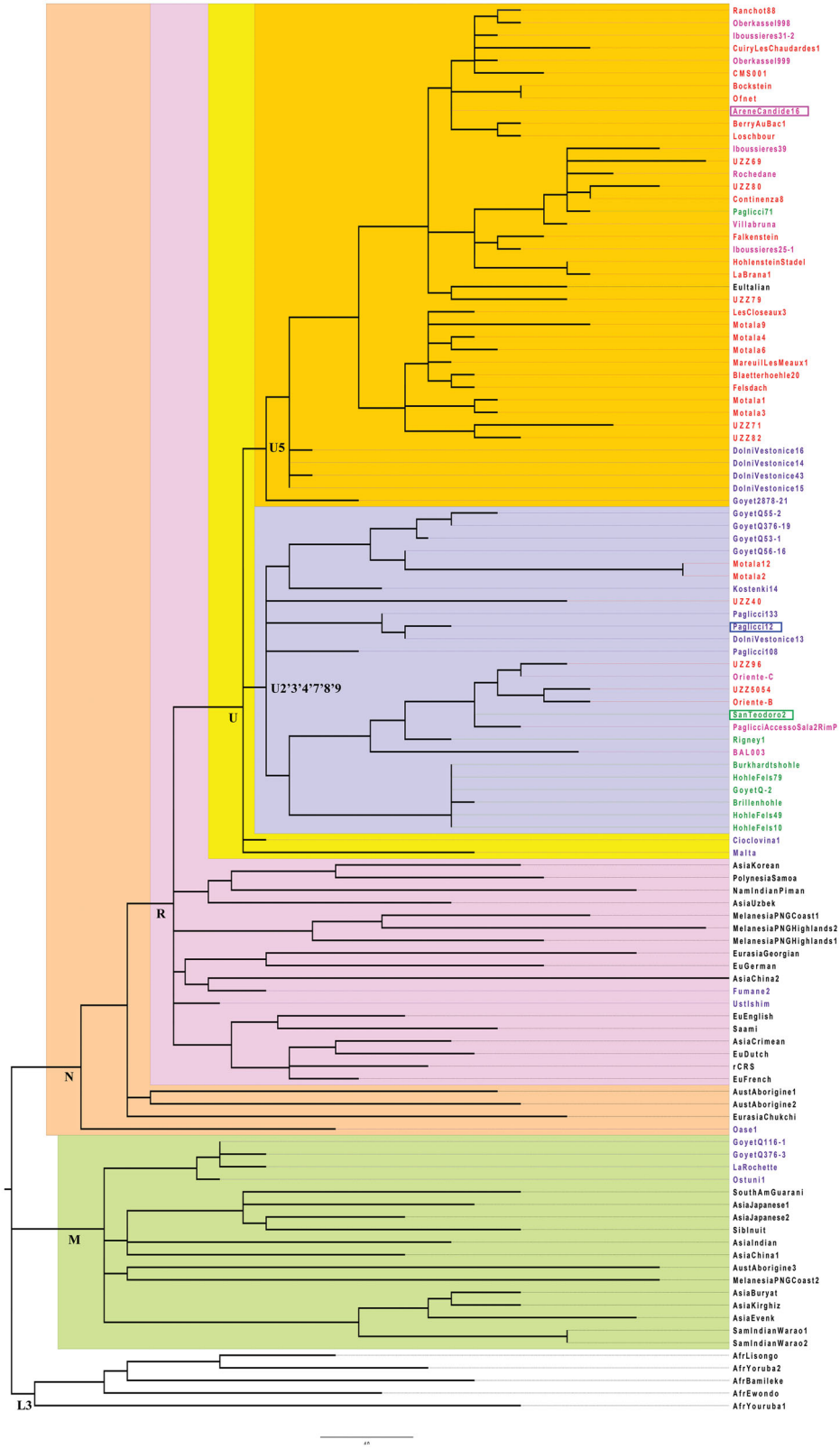

Figure 2. Maximum Parsimony Tree of 53 Present-Day and 77 Pre-Neolithic mitogenomes. The tree root (Feldhofer Neandertal) and 16 deeply divergent African genomes are not shown. Samples dated to the Pre-LGM are coloured in blue, Post-LGM in green, Late Glacial in magenta and Holocene hunter-gatherers in red. Haplogroups are highlighted in different colours and indicated at their root.

another individual from Dolni Vestonice in Czech Republic, dated around 31,000 years BP and previously generally assigned to U8 (Fu et al. 2013; Fu et al. 2016) belongs to sub-haplogroup U8c. We estimated a TMRCA of 32,078 years BP (95% HPD: 31,077–34,615) for the two U8c samples associated with a direct date (PA12 and Dolni Vestonice 13),

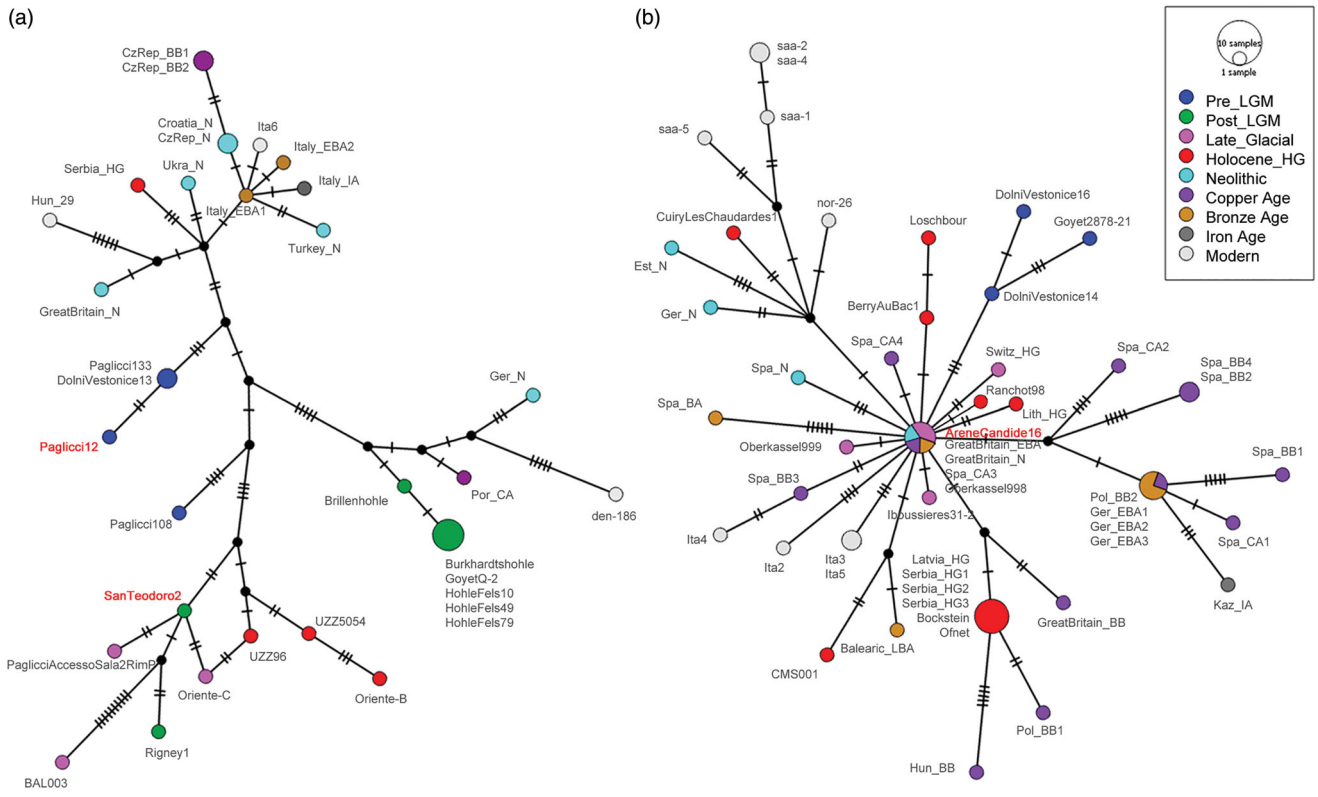

Figure 3. Median joining (MJ) network obtained from complete mtDNA genomes. a) MJ network for haplogroup U8 and U2'3'4'7'8'9; b) MJ network for haplogroup U5b1. The sizes of the circles are proportional to the frequency of each haplotype and the cross lines in the branches between adjacent nodes denote the mutations. The branch length is not proportional to the number of mutations occurred as they were adjusted to fit the page. The samples used in the analysis are labelled with their geographical origin and period/culture with the exception of the Palaeolithic individuals (Table S3 and S4).

highlighting a close phylogenetic relationship between the clade despite the geographic distance. Overall, the results from PA12 confirm the mtDNA heterogeneity of the European pre-Last Glacial Maximum samples and support the connection between East European and South European groups already inferred through analysis at the genomic level (Fu et al. 2016). Moreover, the new sequence of PA12 highlights a possible regional genetic continuity, from 33,000 to at least 29,000 years BP, in the pre-LGM Gravettian groups that succeeded in Paglicci cave (Figures 2 and 3). U8c is the only sub-lineage of U8 hg found in Europe before LGM (Figure 4(a)). As previously reported, U8c haplogroup is no more detected between the available genotyped samples from later periods, mainly due to the contraction of human populations during LGM and the subsequent bottleneck (Posth et al 2016; Villalba-Mouco et al. 2019; van de Loosdrecht et al. 2020) (Figure 4(a)); U8a, a different sub-lineage most likely diversified in an unidentified glacial *refugium*, is the most frequent lineage in the few post-LGM individuals typed so far (TMRCA of 20,444 years BP (95% HPD: 16,776–25,825)) (Figure 4(a)), while U5 and U2 sub-lineages become the most represented mtDNA genomes in Europe starting from Late Glacial times.

Unsurprisingly, ST2 carries the U2'3'4'7'8'9 haplogroup that is exclusively present in samples from Southern Italy, Spain and France, almost all living after the LGM and up to Early Mesolithic (Posth et al. 2016; Villalba-Mouco et al. 2019; Catalano et al. 2020; Modi et al. 2020; van de Loosdrecht et al. 2020) (Figure 4(b)). The ST2 sequence is shown to be phylogenetically closer to other later Sicilian samples from Grotta d'Oriente and Grotta dell'Uzzo and to an individual from Grotta Paglicci (PagliccAccessoSala2Rim) dated around 13,000 BP (Figures 3 and 4(b)). Interestingly, the data from ST2 confirmed that U2'3'4'7'8'9 is the only mitochondrial lineage found in Sicily during the Late Pleistocene and Early Holocene (Catalano et al. 2020; Modi et al. 2020) (Figure 4(b)). The earliest evidence of this lineage in Europe is represented by a 28,000 years-old individual from Grotta Paglicci in Southern Europe (Paglicci 108) that is phylogenetically more distant from ST2 and all other post-LGM samples, and brings a different and less derived haplotype (Figures 2 and 3). San Teodoro Cave represents the oldest human attestation yet found in Sicily; therefore, the data from ST2 strengthen the hypothesis that Epigravettian groups carrying U2'3'4'7'8'9 haplogroup could be the first inhabitants of Sicily arriving from Southern Italy around the Last Glacial Maximum, as supported also by the estimated divergence times of U2'3'4'7'8'9 sequences (18,492 years BP (95% HPD: 15,641–21,673) for the five samples from Sicily). In mainland Europe, only two additional samples display U2'3'4'7'8'9 sequences: a 15,000 years-old individual from French Jura (Rigney1) (Posth et al., 2016) and a Late Upper Palaeolithic sample from Balma Guilanyà in Iberian peninsula (Villalba-Mouco et al., 2019) (Figure 4(b)). In the phylogenetic tree, the Balma Guilanyà sequence falls in a separate branch of the post-LGM U2'3'4'7'8'9 cluster (Figure 2), as depicted also in the haplotype network (Figure 3(a)), while Rigne1 seems more related to ST2 and the other samples from Southern

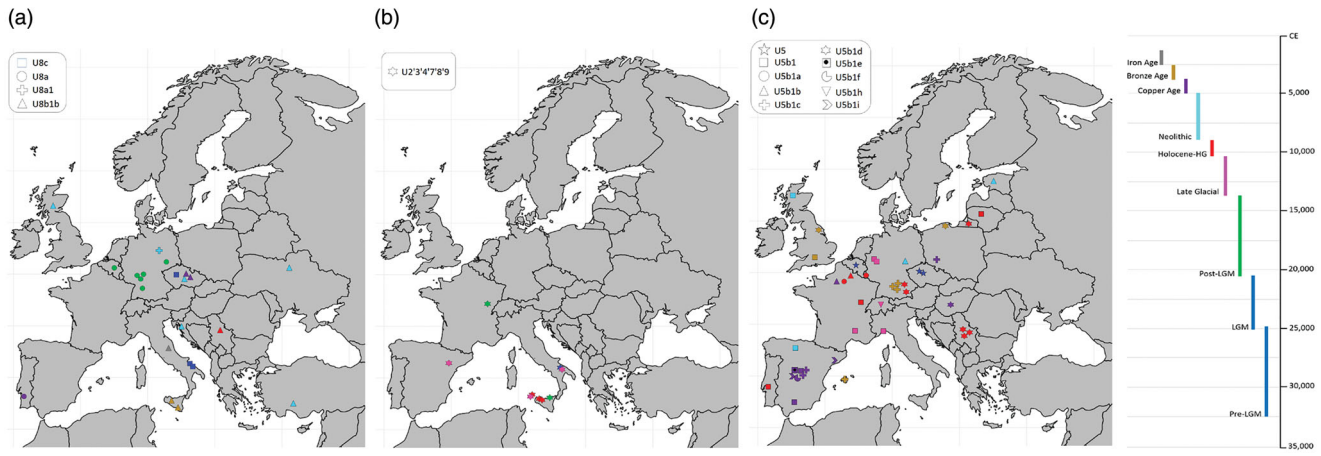

Figure 4. Geographic/temporal maps of ancient mitogenomes belonging to the three haplogroups identified in our samples. See Table S3 and S4 for more details. a) Map for haplogroup U8; b) Map for haplogroup U2'3'4'7'8'9; c) Map for haplogroup U5b1. Maps were generated with Rstudio.

Italy. As previously reported (Modi et al. 2020), we estimated that the "Sicilian clade" diverged from mainland U2'3'4'7'8'9 individuals 23,669 years BP (95% HPD: 18,926–29,111); when also including the Late Upper Palaeolithic sample from Balma Guilanyà, the TMRCA of the post-LGM U2'3'4'7'8'9 cluster is pushed back to 30,704 years BP (95% HPD: 24,355–37,366). Overall, the observed temporal and geographic distribution of the different haplotypes of U2'3'4'7'8'9 points to possible early connections between different LGM *refugia* in Europe, as previously suggested by genome-wide analysis (Villalba-Mouco et al. 2019; van de Loosdrecht et al. 2020); more pre- and post-LGM mitogenome data from France and Spain would be necessary to better address the phylogeographic structure of this haplogroup. U2'3'4'7'8'9 haplogroup seems to disappear later in Mesolithic samples that are characterised by different lineages belonging to U5 haplogroup.

Haplogroup U5b1 characterises the AC16 sample that represents the first pre-Neolithic mitogenome from North-Western Italy. The sequence falls in the same cluster with both slightly earlier and younger samples from North and Central Europe (Figures 2 and 3(a)). The U5b1 sub-lineage is spread across continental Europe since late glacial times (Figure 4(c)) and the estimated TMRCA (21,434 years BP (95% HPD: 17,565–26,295)) is consistent with its diversification during the LGM. Interestingly, the novel data from AC16 shows that its mtDNA genome is different from the U5 sub-lineages that characterise the other samples from peninsular or insular Italy since post-LGM times (Paglicci 71, Villabruna, Continenza, and some Mesolithic individuals from Grotta dell'Uzzo) (Figure 2), suggesting a greater genetic affinity of this sample with individuals from continental Europe than with the Italian groups. More derived U5b1 genomes are widespread in both Northern and Central Europe as well in the Iberian Peninsula starting from the Neolithic age (Figures 3(a) and 4(c)), suggesting a direct mtDNA legacy to the later European populations from the hunter-gatherer group which AC16 belonged to.

The new data reported here contribute to better describe the mtDNA variability in Italy during the Upper Palaeolithic. Thanks to the new mtDNA genome from the Paglicci 12 burial, we highlighted a regional genetic continuity in the Gravettian groups that succeeded in Paglicci cave before LGM, and confirmed the connection between East European and South European groups associated with the same material culture. The data from San Teodoro, the site with the oldest human attestation yet found in Sicily, strengthen the hypothesis that Epigravettian groups carrying U2'3'4'7'8'9 haplogroup could be the first inhabitants of Sicily arriving from Southern Italy around the Last Glacial Maximum and support the scenario of genetic continuity between Palaeo-Mesolithic hunter-gatherers in Sicily (Modi et al. 2020). Finally, we generated the first genetic data from the Arene Candide cave in North-Western Italy, a geographic region of Italy not investigated in previous studies, showing that the mtDNA genome of AC16 is more related with the sequences found in individuals from continental Europe than with the Italian groups.

Moreover, thanks to the accessibility of petrous bone or inner ear bone samples, we confirmed the feasibility of obtaining good quality genetic data for samples from southern latitudes and/or older chronologies that can show high levels of degradation of the genetic material. Further samplings of selected Italian specimens associated with direct radiocarbon dates and an accurate definition of the archaeological context will allow a deeper description of the regional dynamics that characterised hunter-gatherer populations with their possible contacts and cultural/genetic interactions.

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Disclosure statement

The authors report no conflict of interest.

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Data availability statement

Sequence data are available in GenBank under Accession numbers MW209008-MW209010

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