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Research article

Analysis of ancient mtDNA from the medieval archeological site of *Amiternum* (L'Aquila), central Italy

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ABSTRACT

Study of ancient DNA makes it possible to analyze genetic relationships between individuals and populations of past and present. In this paper we have analyzed remains of human bones, dating back to the 8th-10th century AD, from the burials found in the Cathedral of *Santa Maria in Civitate*, archaeological site of *Amiternum*, L'Aquila, Italy. As a genetic marker, the hypervariable region 1 of mitochondrial DNA (HVR1) was selected. To obtain reliable sequences from the hypervariable region 1 of mtDNA (HVR1) were performed: multiple extractions, template quantification and cloning of PCR products. The sequences obtained were compared with Anderson's sequence for the identification of polymorphisms (SNP) and haplogroups. The data obtained were analyzed with various software and phylogenetic methods. For the comparison between populations, ancient and modern sequences found in databases and literature have been used. This work provides preliminary information on the correlation between the population of Amiternum, the migrant populations transited and/or established in the territory of Amiternum such as Byzantines, Longobards (Lombards), which dominated the Italian peninsula between 568 and 774 AD, and the current populations of Italy.

The study of haplogroups, the analysis of genetic variability and phylogenesis studies on the sequences considered show a genetic closeness between the individuals of Amiternum, the current population of central-northern Italy and the Germanic tribe of Longobards, however, also highlights genetic traits of Byzantines in some samples of Amiternum. Using the analysis of amelogenin gene fragments, we successfully determined the sex of the bone remains on all samples.

1. Introduction

Amiternum was an ancient italic city, founded by the Sabines, whose ruins are located near san Vittorino, a town 11 km north of L'Aquila. Lombard presence there has been confirmed by historical sources (Antonelli and Tornese, 2012).

In a recent research (Vai et al., 2019), in order to investigate the degree of genetic affinity between the various cultures and to clarify the migratory dynamics of the early Middle Ages in Europe, medieval tombs located in Italy (Collegno), Czech Republic and Hungary were examined, some of which can be attributed to the Lombard culture and others not. The Lombard arrival in Italy began in 568, passing through Veneto and expanding throughout Northern Italy (Christie, 1998). They also reached southern Italy, where they founded two important poles: the ducats of Spoleto (570) and of Benevento (576).

The stages of the Lombard conquest in Abruzzo were gradual and spread out over a period of several decades. It is plausible to suppose that between 580 and 595, the Lombard arrived in the *Amiternum* area along the route of via Claudia-Valeria. Their expansion went through the Marsica area and along the main routes leading to the Adriatic coast.

During that period, the Lombard stabilized their power, also within the establishment of the gastaldato of *Amiternum*, and they resumed activities and interventions of restoration, expansion, enrichment of the cult buildings and funerary spaces for the Lombard elites (Redi and Savini, 2016).

The Lombard invasion reduced the Byzantine presence, which had been quite consistent until then along the Aterno valley, where it controlled the routes of communication between the Adriatic coast and Rome (Paroli, 1997; Redi et al., 2012).

The Byzantine presence in the territory is confirmed by various

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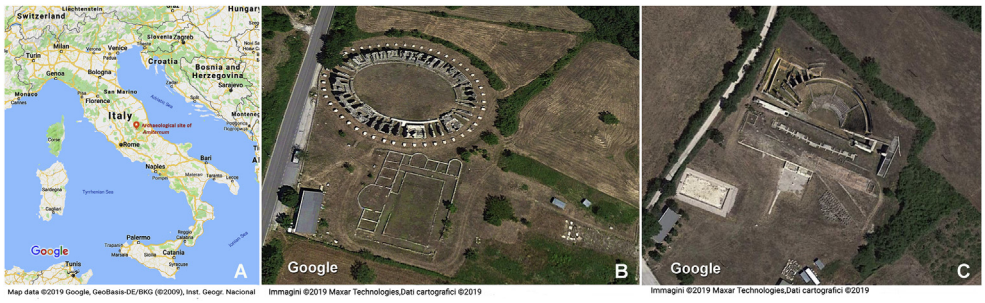

Fig. 1. The ancient city of *Amiternum* (L'Aquila). Fig. 1A geographical location of the archaeological area of *Amiternum*; Fig. 1B the theatre (I century B.C.) city of *Amiternum*; Fig. 2C The amphitheate built in the I century A.D. city of *Amiternum*.

indicators such as toponymy, the dedication of churches to cult imported from the Orient and by excavation finds (Redi et al., 2012).

Considering the strategic position of *Amiternum* along the Via Claudia Nova, which connected the agro Amiternino to the Val Pescara, it seems plausible to assume the defensive importance of the ancient town of Amiternum for the Byzantines on the Adriatic coast of the region starting from the sixth century (Staffa, 1995). The archaeological and historical data (seem to) attest with certainty that a progressive militarization of the Byzantine presence on the Abruzzo coast counterbalanced a strengthening of the Lombard presence in the internal areas.

Amiternum was an episcopal seat at least until the seventh century (Giuntella, 1999) and the presence of a cathedral dedicated to Holy Mary has been attested indirectly between the V and VI century from literal and archival sources (*Auctorum Antiquissimorum*, 1894; Redi, 2010).

The Lombard raid destroyed these places (Staffa, 1992) and led the Episcopal seat into a crisis that soon became irreversible: in 970 *Amiternum* was a pile of ruins (Gemblacensi, 1841; Sereni, 2001).

Nevertheless, archaeological evidences showed that this area continued to be populated throughout the early Middle Ages.

In the city of *Amiternum*, in an area still known today as *Campo Santa Maria*, recent archaeological excavations have brought back residential facilities including a cathedral dedicated to *Santa Maria*. The cathedral stands almost in the center of the city, next to the decumanusmaximus and the temples that overlook it, not far from the amphitheater and it seems to have had no relationship with pagan or early Christian places of worship of martyrs (Fig. 1). It is a large building with three naves divided by columns and with a single semicircular apse probably belonging to the period of the episcopate of Ceteo, that is during the Lombard conquest or a little later. Various tombs dating back to the 8th and 9th century AD have been found inside the cathedral.

In this study, we want to integrate the historical knowledge deriving from writings and archaeological finds, with biological and genetic data coming from the burials, in order to understand the Byzantine and Lombard influence in the territory and their genetic contribution on the current population.

In recent years the interest in point mutations (single nucleotide polymorphism SNP) has increased not only for the numerous applications in medical genetics (Lai, 2001), but also for those in evolutionary (developmental) genetics (Morin et al., 2004) and in the forensic field (Sobrino and Carracedo, 2005).

SNPs located within the non-recombinant part of the mitochondrial genome are particularly valuable because they have a uniparental (maternal) inheritance.

The analysis of mutations in the sequence of the hypervariable region (HVR1, about 400 sequential bases) of mitochondrial DNA (mtDNA) are a formidable tool for understanding evolutionary history and human migration at a global and regional level (Vigilant et al., 1991; Ward et al., 1991; Mountain et al., 1995; Fareed and Afzal, 2013).

Studies on human mtDNA have shown that Italy shows molecular variation models similar to other European countries: the regional differences found have arisen over time and show both continuity and genealogical discontinuity in the various Italian regions (Brisighelli et al., 2012).

In this study we extracted DNA from the remains of 4 medieval skeletons found in the Cathedral of *Santa Marie in Civitate* of *Amiternum*, remains which date back to the VIII and X century. The purpose of this research was sequencing the mtDNA hypervariable region (HVR-I), performing comparative analyses of genetic diversity and determining sex.

The phylogenetic comparison was carried out among modern populations from different geographical districts (central, southern and northern Italy) and Lombard and Byzantine finds, in order to analyze the genetic contribution left by the ancient populations to modern individuals and to correlate, if possible, the available historical and archaeological data with genetic data.

2. Materials and methods

2.1. Sample

The samples analyzed were remains of human bones dating back to 8th and 9th centuries AD, recovered from the cathedral of *Santa Maria in Civitate* of *Amiternum* L'Aquila, Italy (Redi et al., 2013, 2014, 2015), the position of the burials in the cathedral is shown in Fig. 2. The recovery of skeletal remains occurred during the 2016/17 archaeological expeditions carried out by researchers from the Archeology Laboratory of the Department of Human Sciences of the University of L'Aquila and in the presence of a molecular biologist (OP1Z_AQ) from the Genetics and Mutagenesis laboratory of the Department of Life, Health and Environmental Sciences of the University of L'Aquila, which drafted the protocol so that the excavation and recovery of the samples were carried out following all the necessary precautions to avoid contamination of the remains from modern DNA (Pilli et al., 2013; Llamas et al., 2017). The bone remains for genetic analysis, after a light brushing with a dry brush pretreated with 10% bleach, were packed separately in plastic bags, pretreated with 10% bleach, and transported in airtight containers in the Genetics and Mutagenesis laboratory where they were stored in a freezer at –20 °C until they were used.

The number of skeletal remains to be analyzed was conditioned primarily by the discovery during the archeologia excavation of only 9 burials, of which:1 does not correspond to the original context (S. 41); 6 are reworked (S. 40, 42, 43, 44, 45 and 47) and 2 are intact (S.46 and 48). For the biomolecular analyzes we have chosen: two intact burials S.46 and S. 48 and two burials reworked, but well preserved, S.42 and S.45. The skeletons 42, 45 and 46, dated to 9th-10th century, have all been found in area 3, while the skeleton 48, dated to 8th century, was located in area 6 (Fig. 2), they are inhumations, laid in graves with wooden elements, preserved in good condition, even if not completely intact; these archaeological finds at the first molecular analysis tests presented useful and interesting characteristics for molecular experimentation.

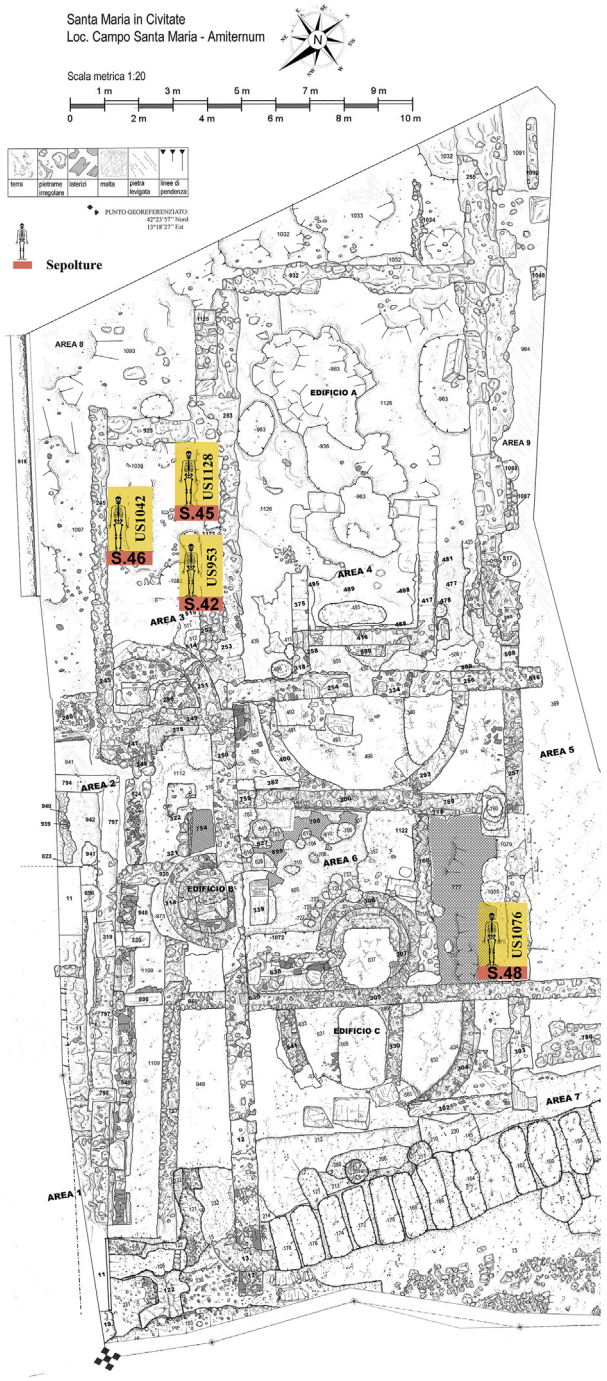

Fig. 2. The plan of the cathedral of Santa Maria in Civitate di Amiternum with the burial sites indicated.

Table 1
Skeletal remains analyzed.

Burial	Bones	Age (years)	Period
S.42	Femore dx	Child (4–6)	IX–X sec
S.45	Femore dx	Adult (45–50)	IX–X sec
S.46	Femore sx	Teenager (16–18)	IX–X sec
S.48	Femore sx	Adult (45–50)	VIII sec

The other skeletal remains were not analyzed because there was not a certain dating and also because, due to physical and chemical factors related to the nature of the site, they were profoundly contaminated and gave negative results in the preliminary DNA extraction tests.

The anthropological analysis on bone remains allowed to estimate the age at death and in some cases also determine the sex of the individuals (Redi et al., 2018) (Table 1). For the skeleton 42 it was possible to estimate the age at death between 4 and 6 years, but the incomplete skeletal maturity did not allow to determine the sex of the child. The skeleton S. 45 is referable to a robust adult individual for whom it was not possible to determine sex due to the absence of diagnostic districts. The skeleton S. 46, deposited in dorsal decubitus in a sub-rectangular pit of modest size, allowed to estimate the age at death between 16 and 18 years and that it is a subject presumably male. The skeleton of S. 48 was found in the probably best preserved tomb. According to the characters of the skull and the pelvis it is possible to state that it is an adult, 45–50 years old, male.

2.2. DNA extraction and PCR amplifications

The study was conducted, following the appropriate guidelines for the analysis of human skeletal remains, according the recommendations to control DNA contamination, two separate laboratories were used to perform the experimental procedures (Llamas et al., 2017; Fulton and Shapiro, 2019; Pilli et al., 2013). In the pre-PCR laboratory we perform: cleaning and pulverization of bone remains, DNA extraction, preparation of the PCR reaction mixture; in the post-PCR laboratory the work phases are carried out with DNA already amplified and no longer at risk of contamination by modern DNA; dedicated equipment is used in the two laboratories. Access to the pre-PCR laboratory is limited to three molecular biologists (OP1Z_AQ, OP2P_AQ, OP3C_AQ) who carry out all the work phases using the appropriate devices. Workstations are carefully cleaned with 10% bleach and irradiated with UV rays at 254 nm for one night before use, so sample extraction takes place on different days. All the materials are extensively washed with 6% bleach and, if necessary, with ethanol and then irradiated for 4 hours with UV rays at 254 nm before each use (Adler et al., 2013; Korlević et al., 2015; Salamon et al., 2005).

The surface of the bone fragments was carefully washed with a cloth soaked in a 6% solution of bleach in sterile water, followed by ultraviolet exposure at 254 nm for 20-20 minutes on each side in a Hoefer UVC 500 Ultraviolet. An area of the bone fragments was milled with a mini DEXTER drill, at a speed of 800 rpm, to remove about 2 mm thick, the powder samples, 200 mg, were collected in a 40 ml sterile falcon tube placed under the impact area of the DEXTER diamond tip. The powder thus collected was stored at -20 °C until its use. The DNA was extracted through silica-based spin columns with the GeneAll® ExgeneTM DNA genomic micro kit following the manufacturer's instructions, three DNA extractions were made for each sample. The Molecular Biologists who had contact with ancient bone samples provided blood samples, in order to obtain the sequences of the HVR1 region of mtDNA, which were deposited in GenBank database (Accession numbers: MG972629.1; MG972630.1; MG972631.1). Peripheral blood sampling (100 μl) was performed on the operators who worked on the experiment and the total DNA was extracted using the same kit, following the instructions for the blood samples.

PCR amplification was carried out using Hybaid PCR Express Thermo Cycler using the kit KAPA2G Fast HotStart ReadyMix 2X (Kbiosystems). Table 2 shows the primers used, some of them have been found in the literature (Caramelli, 2009; Plantinga et al., 2012; Kim et al., 2008; Del Gaudio et al., 2013), others constructed on gene sequences with Primer Express 3.0 software software (Applied Biosystems, USA).

The reaction mixture contained in a 25 μl reaction system: 50–150 copies of template, 12.5 μl of ReadyMix 2X; 1.25 μl of forward and reverse primers 5 μM; water up to a final volume of 25 μl. All the PCRs were performed under following conditions: 3 min at 95 °C; 40 cycles of 20 s at 95 °C, 20 s annealing temperature primers (Table 2), 20 s at 72 °C; and 5 min final extension at 72 °C.

A negative control was introduced to monitor contaminants that can

Table 2
PCR primers used for DNA amplification.

Names primers	Primers Sequences 5′–3′	Temperatura di annelling	Amplicon size (bp)
F-15995	5′-CCACCATTAGCACCCAAAG-3′	55,2 °C	180
R-16132	5′-CTACAGGTGGTCAAGTATTTATGGT-3′	54,5 °C	
F-16131	5′-CACCATGAATATTGTACGGT-3′	49,5 °C	126
R-16218	5′TGTGTGATAGTTGAGGGTTG-3′	46,8 °C	
F-16196	5′-CCCCTCCCCATGCTTACAA-3′	59,3 °C	180
R-16345	5′-GGGCGAGAAGGGATTTGACT-3′	59,1 °C	
F-16009	5′-GCACCCAAAGCTAAGATTCTAATT-3′	56,2 °C	472
R-16439	5′-CCCGGAGCGAGGAGAGTAG-3′	58,6 °C	
F_amel R_amel	5′-GGGCACCCTGGTTATATCAAC-3′	56,9 °C	Y_201/X_ 195
	5′-AGGCCAACCATCAGAGCTT-3′	56,0 °C	
Famel_A Ramel_B	5′-CCCTGGGCTCTGTAAAGAATAGTG-3′	59,2 °C	X_106/Y_112
	5′-ATCAGAGCTTAAACTGGGAAGCTG-3′	59,1 °C	
Fam_XY	5′-GGTTATATCAACTTCAGCTATGAG-3′	51,0 °C	Y_190/X_184
R-AMXY	5′-GCCAACCATCAGAGCTTAAAC-3′	55,3 °C	
F_AMY2	5′-TGGATTCTTCATCCCAAATAAAGTG-3′	59,3 °C	Y_85/Y it does not amplify
Ramel_B	5′-ATCAGAGCTTAAACTGGGAAGCTG-3′	59,1 °C	

Some primers were found in the literature others constructed on gene sequences with Primer Express 3.0 software software (Applied Biosystems, USA).

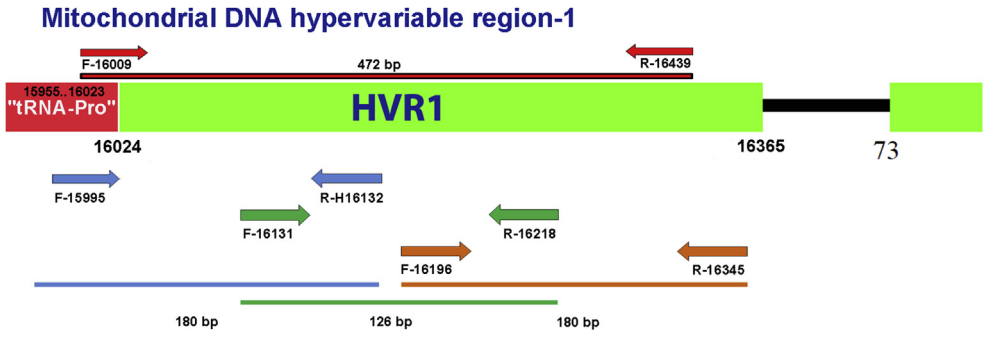

Fig. 3. Strategy used to produce the fragments of the HVR 1 region to be cloned.

Table 3
Quantification of DNA extracted from skeletal remains by qPCR.

Samples	ExtractionI Copies/µl ± S.E.		ExtractionII Copies/µl ± S.E.		ExtractionIII Copies/µl ± S.E.		Average value of the three extractions
Amiternum S. 42 IX-X sec years 4–6	15 30 21	22 ± 4,3	35 66 53	51,3 ± 8,9	32 22 35	29,6 ± 3,9	34,33 ± 5,37
Amiternum S. 45 IX-X sec years 45–50	80 95 100	92 ± 8,6	78 101 100	93 ± 7,5	115 132 118	121,6 ± 5,2	102,11 ± 5,82
Amiternum S. 46 IX-X sec years 16–18	35 55 31	40,3 ± 7,4	54 67 39	53,3 ± 8,0	31 33 45	36,3 ± 4,3	43,33 ± 4,26
Amiternum S. 48 VIII sec years 45–50	33 22 21	25,3 ± 3,8	13 13 12	12,8 ± 3,1	32 22 18	24 ± 4,16	20,66 ± 2,59

Determination of the number of copies/µl extracted DNA, carried out on three independent extractions. The table shows the number of copies of the "target" gene of the amelogenin/µl for each sample extracted ± the Standard Error (E.S.).

enter any phase of the entire process.

2.3. PCR amplification of the amelogenin gene for sex determination

The amelogenin is a gene that codes for a protein, found in tooth enamel. The Y-chromosome and the X-chromosome both contain this gene, but the X-chromosome has a deletion of 6 bp, with the resulting product 106 bp, compared to the Y-chromosome product length of 112 bp (Sullivan et al., 1993). The extracted DNA was amplified for the amelogenin gene (Sullivan et al., 1993; Kim et al., 2008), obtaining two fragments of 112 bp (chromosome Y) and 106 bp (chromosome X) in male individuals and only a fragment of 106 bp in female individuals.

In order to control the results obtained and to make the determination of the sex more robust, we performed a nested PCR with specific primers F_AMY/R_amel_B (Kim et al., 2008) designed to amplify only the amplicone produced on the Y allele. The PCR product obtained, using the Famel/Ramel primers, with 10 amplification cycles, is used as template (first-PCR) for a second amplification (nested-PCR) with the internal primers "nested" F_AMY/R_amel_B (85 bp Its Y, does not amplify his X). The reaction mixture contained in a 25 µl reaction system: 2 µl reaction system first-PCR; 12,5 µl ReadyMix 2x; 1,25 µl of F_AMY/Ramel_B primers 10 µM; water up to a final volume of 25 µl.The PCR reaction was carried under the following conditions: 3 min at 95 °C; 32 cycles of 20 s at 95 °C, 20 s at 60 °C, 20 s at 72 °C; and 5 min final extension at 72 °C. The allelic variants were highlighted with an electrophoretic run in low melting point agarose gel at 2.8% colored with ethidium bromide.

A negative control was introduced to monitor contaminants that can enter any phase of the entire process.

2.4. qPCR to evaluate the concentration of DNA in the samples

The remains of ancient bones contain little DNA, so quantization in a sample is essential for most PCR-based analyzes (Zoppis et al., 2012), so we built a standard curve.

The X and Y alleles of the operators were amplified in PCR with the Famel/Ramel primers, the amplicons obtained were cloned in the pCRII-TOPO vector, the recombinant vectors were linearized with the restriction enzyme SacI-HF® (NEB) in a reaction volume of 50 µl, according to

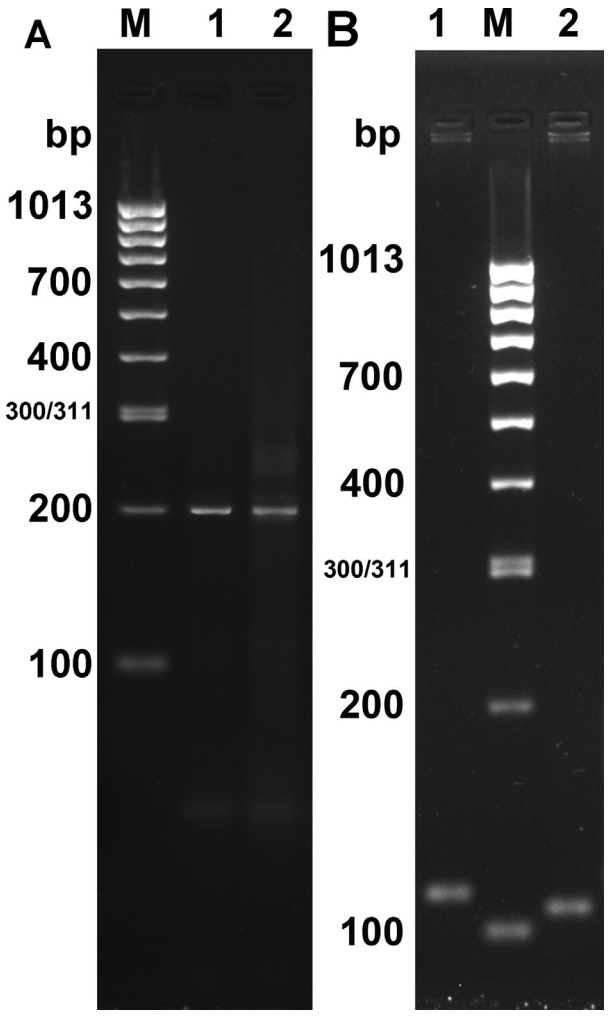

Fig. 4. Cloned fragments in the pCR ™ 2.1-TOPO® vector of the amelogenin gene. A) Electrophoretic run in 1.8% agarose gel colored with ethidium bromide 0.5 μg/ml: M, Marker HyperLadder 100 bp (Bioline); lanes 1 and 2, cloned PCR products, obtained with the F_amel/R_amel primers (Y_ 201 - X_195) from modern control DNA, respectively from the male and female phenotype. B) Electrophoretic run in agarose gel with low melting point 2.8% colored with ethidium bromide 0.5 μg/ml: lane 1 product of PCR on the linearized recombinant plasmid with insert (Y_201 bp) obtained with specific primers FamelA/RamelB (112 bp); M, Marker HyperLadder 100 bp (Bioline); lane 2 product of PCR on the linearized recombinant plasmid with insert (X_195 bp) obtained with the specific FamelA/RamelB primers (106 bp).

the manufacturer's instructions, the reaction product was purified with the NucleoSpin® Extract II (Clontech) kits according to the manufacturer's instructions.

The absorbance at 260 and 280 nm obtained with the NanoDrop spectrophotometer (Thermo Scientific) and the electrophoretic run in agarose gel at 1.2% colored with ethidium bromide (0.5 micrograms/ml) allowed to determine the concentration and the purity of linearized plasmids. Serial dilutions have been made of the linearized recombinant plasmid. Subsequently, 1 μl of each dilution was subjected to qPCR amplification with the Famel_A/Ramel_B primers.

The DNA concentration was converted into number of copies with the DNA Copy Number and Dilution Calculator software (Thermo Scientific). The qPCR tests were performed in triple, with three technical replicates. The respective equations were used to determine the efficiency of the reaction (E) and the concentration of the extracted samples. The experimental conditions, the standard curves and the dissociation curves are reported in the supplementary information (Note-S1, Fig.-S1, Fig.-S2).

A negative control was introduced to monitor contaminants that can enter any phase of the entire process.

2.5. Production of PCR fragments of the hypervariable region of mtDNA (HVR1)

He hypervariable region-1 of mtDNA (HVR1), from position 15995 to 16345, was amplified in three overlapping fragments, the primers used are shown in the Table 2. Three primer pairs (F15995/R16132, F16131/R16218, F16196/R16345) were used to target a subdivided 350 bp of the HVR-I via three overlapping fragments (Fig. 3). These samples were PCR-amplified with the F-15995/R-16345 primers and the purified amplicons were sent to MWG Eurofins (Germany) and sequenced with the F-16009/R-16439 primers.

PCR amplification was carried out using Hybaid PCR Express Thermo Cycler in a 25 μl reaction volume containing:10–150 copies of template, 12,5 μl di 2X KAPA2G Fast HotStart ReadyMix 2X (Kbiosystems), 1,25 μl di forward e reverse primers 10 μM, water up to a final volume of 25μl.The PCR reaction was carried under the following conditions: 3 min at 95 °C; 40 cycles of 20 s at 95 °C, 20 s annealing temperature primers (Tab.2), 20 s at 72 °C; and 5 min final extension at 72 °C.

Contaminations like primers, dNTPs, salts and soluble macromolecular components are removed by NucleoSpin® Extract II Kits (Clontech) following the manufacturer's instructions. The purity and size of PCR fragments they were verified in electrophoresis on 1.8% agarose gels, in TAE (Tris-acetate-EDTA) buffer containing ethidium bromide (EtBr) 0.5 μg/ml. Markers were used as molecular weight markers:E-Gel® 1 kb Plus DNA Ladder (Invitrogen); HyperLadder™ I 1 kb (Bioline). The gels were visualized and the concentration of amplification products was estimated by comparative analysis with Gel Doc 2000 (Biorad). When necessary the amplicons of the HVR1 region were extracted and purified from the agarose gel used in the kit PureLink™ Quick Gel Extraction (Invitrogen), following the manufacturer's instructions. All PCR products were cloned using TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Screening of white recombinant colonies was accomplished by PCR (Guimaraes et al., 2009) and agarose gel electrophoresis. Plasmids from the positive colonies were purified with commercial kits PureLink™ Quick Plasmid Miniprep (Invitrogen) according to the manufacturer's instructions. After purification of plasmid extracts containing the cloned inserts of the samples and the operators were sent to the sequencing service DNA of MWG Eurofins (Germany). The amplicons of the operators and skeletal remains were analyzed with the web application mtDNAprofiler: mithochondrial DNA sequence analysis (http://mtprofiler.yonsei.ac.kr/index.php?cat=5) (Yang et al., 2013).

A negative control was introduced to monitor contaminants that can enter any phase of the entire process.

2.6. Software used for the analysis of the sequences

The ancient sequences HVR-1 obtained were compared to GenBank sequences through the BLAST application (http://www.ncbi.nlm.nih.gov/BLAST/), to determine their closest match and to ensure that they did not match any other unexpected species or sequences. The multiple alignment, relative to particular traits of sequences belonging to different individuals, was carried out with the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/EMBL-EBI) (Sievers and Higgins, 2014) e Multalin (http://multalin.toulouse.inra.fr/multalin/) (Corpet, 1988). The consensus sequence was compared to the Cambridge reference sequence (CRS) (Anderson et al., 1981; Andrews et al., 1999)to define the mutational motif HVR-I, on the basis of the HVR-I haplotype and the SNPs in the mtDNA coding region, each individual was assigned

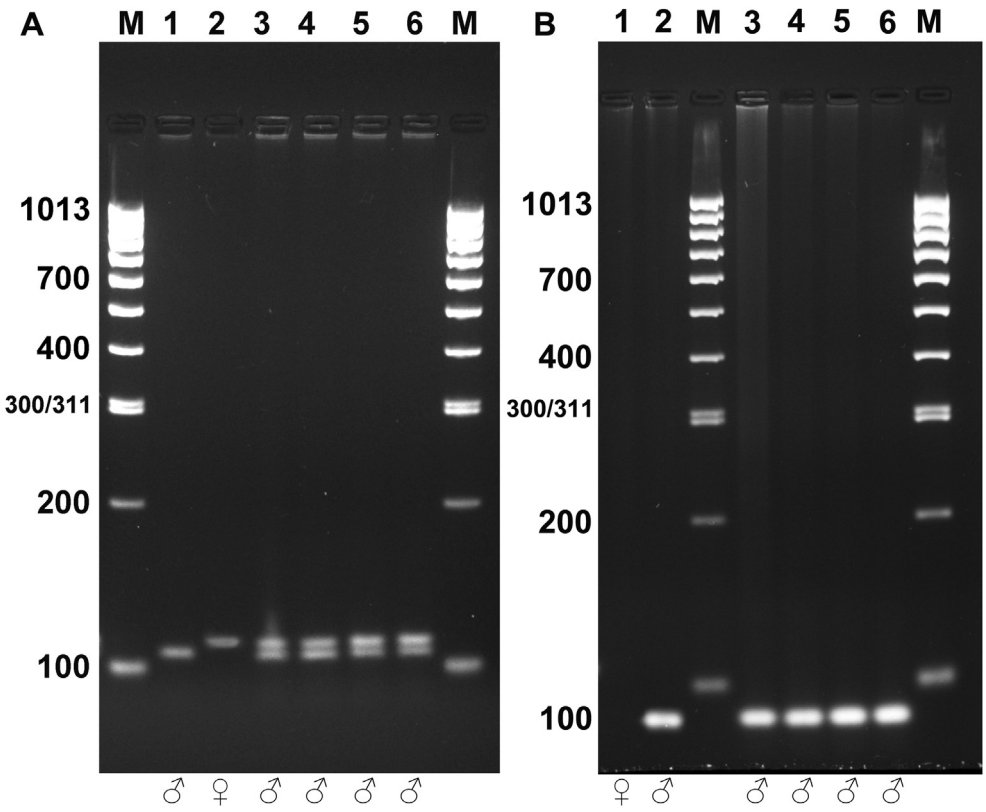

Fig. 5. Determination of the sex of *Amiternum* samples, amplification products obtained for PCR and nested-PCR. Electrophoretic run in agarose gel with low melting point 2.8% colored with ethidium bromide 0.5 μg/ml. Fig. 5 A: M marker HyperLadder 100 bp (Bioline); lane 1, a PCR product obtained from the recombinant allele X plasmid with the F_amelA/R_amelB primers (106 bp); lane 2, a PCR product obtained from the recombinant Y allele plasmid with the F_amelA/R_amelB primers (112 bp); lanes 3,4,5 and 6, amplicons obtained with the F_amelA/R_amelB primers respectively on the DNA extracted from samples S.42, S.45, S.46, S.48 of *Amiternum*. Fig. 5 B nested-PCR products obtained with the primers F_AMY/R_amel_B: M marker HyperLadder 100 bp (Bioline); lanes 1 and 2 control amplicons on modern DNA, respectively, of X and Y phenotype (85 bp); lanes 3,4,5 and 6 amplicons obtained for nested-PCR with the primers F_AMY/R_amel_B on the genomic DNA extracted from the skeletal remains of *Amiternum* respectively, samples S.42, S.45, S.46, S.48.

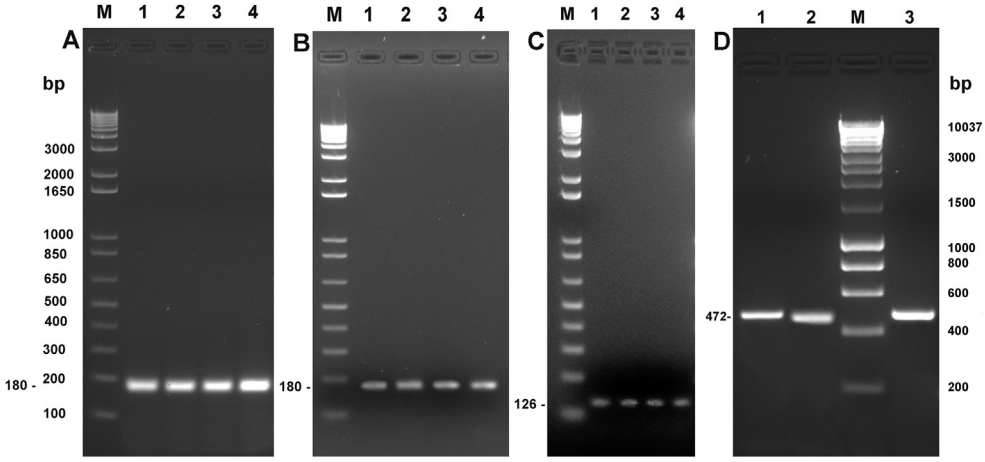

Fig. 6. Electrophoretic runs in agarose gel 1.8% of the cloned amplicons. Fig. 6 A, B, C purified fragments, obtained respectively from the amplification of DNA extracted from *Amiternum* samples (S. 42, S. 45, S. 46, S.48), with the primers: A) F-15995/R-16132 (180 bp), B) F-16196/R-16345 (180 bp); C) F-16131/R-16218 (126 bp) respectively. Fig. 6 D amplicons obtained with primers F-16009/R-16439 (472 bp) on modern DNA, extracted from operators (OP1Z_AQ, OP2P_AQ, OP3C_AQ). Fig. 6 A, B and C, (M) E-Gel® Marker 1 kb Plus DNA Ladder. Fig. 6 D, (M) Marker HyperLadder ™ I 1kb (M).

Table 4
mtDNA sequences of the HVR-1 region of of the *Amiternum* samples and the operators, deposited in GenBank.

Source organism	bp	Accession numbers GenBank database
Homo sapiens isolate *Amiternum*_S.42_IX-X D-loop	388 bp	MG972625.1
Homo sapiens isolate *Amiternum*_S.45_IX-X D-loop	388 bp	MG972626.1
Homo sapiens isolate *Amiternum*_S.46_IX-X D-loop	388 bp	MG972627.1
Homo sapiens isolate *Amiternum*_S.48_VIII D-loop	388 bp	MG972628.1
Homo sapiens isolate OP1Z_AQ D-loop	472 bp	MG972629.1
Homo sapiens isolate OP2P_AQ D-loop	472 bp	MG972630.1
Homo sapiens isolate OP3C_AQ D-loop	472 bp	MG972631.1

to a haplogroup according to the latest mtDNA phylogeny (http://www.phylotree.org/) (van Oven and Kayser, 2009).

Haplogroup assignment was carried out using the web application: HaploGrep, automatic mtDNA haplogroup classification using PhyloTree 17, (http://haplogrep.uibk.ac.at) (Kloss-Brandstätter et al., 2011); a human mitochondrial genome database MITOMAP-MITOMASTER (https://www.mitomap.org/foswiki/bin/view/MITOMASTER/WebHome) (Brandon et al., 2005; Lott et al., 2013); EMPOP mtDNA database, v4/R11 (https://empop.online/) (Parson and Dür, 2007).

All sequences, which were compared with the sequences of the skeletal remains of *Amiternum*, were obtained from the mtDB - Human Mitochondrial Genome Database (www.genpat.uu.se/mtDB) (Ingman and Gyllensten, 2006) and the literature.

Phylogenetic analyses and evolutionary relationships have been studied through software Molecular Evoluzionary Genetics Analisys

Table 5
Polymorphisms and Haplogroup/Subhaplogroup identified in the samples studied.

Samples *Amiternum*	HVR-I motif (16024–16384) Mutations/rCRS	Haplogroup/Subhaplogroup HAPLOGREEP	Haplogroup/Subhaplogroup MITOMASTER	Haplogroup/Subhaplogroup EMPOP
S. 42 IX-X	A16183C, C16234T	H13a1d	H13a (H13a1d)	H13a1d
S. 45 IX-X sec	T16126C, C16234T 16362C	R0a	H14b (H14b1)	H14b1
S. 46 IX-X sec	C16069T, T16126C, C16256T	J1c2a2	J1c (J1c2a2)	J1c2a2
S. 48 VIII sec	G16129T, C16234T	H13a1d	H13a (H13a1d)	H5n

(MEGA7) (http://www.megasoftware.net/docs) (Tamura et al., 2013)e SplitsTree4 V4.14.6 (http://www.splitstree.org/) (Huson and Bryant, 2006).

3. Results and discussion

3.1. Determination of DNA concentration for qPCR

Table 3 shows the number of initial copies of the amelogenin gene in the studied samples, obtained through the equation of the standard curve of qPCR (Fig.-S1, Fig.-S2). The lowest concentration of DNA, expressed as the number of copies of the amelogenin gene, results in the sample "*Amiternum* S.42" attributable to a child of IX-X century and in the sample "*Amiternum* S.48" attributable to an adult of eighth century. This diversity in extraction can be attributed: for sample S.42, to the fact that the bones of a child are more fragile with more pronounced diagenetic phenomena; for sample S.48 to the fact that it is presumably the most degraded, being the oldest sample dating back to the eighth century (Allentoft et al., 2012).

3.2. Cloned fragments in vector PCR ™ 2.1-TOPO ® gene Amelogenin

The agarose gel electrophoretic run of Fig. 4A shows the fragments, used in the cloning, produced by PCR with the F_amel/R_amel primers on the amelogenin gene of the modern DNA of male (lane 1) and female (lane 2) phenotypes. Fig. 4B shows the PCR amplicons with the FamelA/RamelB primers on the recombinant plasmids with the insert Y_201 bp (lane 1–112 bp) and X_195 bp (lane 2–106 bp).

Fig. 5 shows the amplifications obtained for PCR and nested-PCR with the primers F_amelA/R_amelB (Fig. 5A) and F_AMY/R_amelB (Fig. 5B) respectively, using as a template the DNA extracted from the skeletal remains of *Amiternum*, samples S.42, S.45, S.46, S.48 lanes 3–6 respectively.

The PCR amplicons in all samples (Fig. 5A), indicate that the skeletal remains of *Amiternum* derive from male individuals, as they show two distinct amplicons (106 and 112 bp). The amplicons produced for nested-PCR on the samples (Fig. 5B) validate that the skeletal remains of *Amiternum* derive from male individuals, as they show only one amplicon of (85 bp).

The results obtained from the technical triplicates of the three extractions for each sample were coherent and Figs. 4 and 5 are representative of them, negative controls without a DNA template always gave a negative result.

In PCR tests, the initial "target" DNA quantity, to obtain a good amplification, must contain from 100 to 150 copies of the gene of interest and the amplification must be pushed up to 42 cycles with the F_amel/R_amel primers. In nested PCR experiments performed on our samples, the first amplification requires only 25–30 initial copies to obtain a well detectable amount of product in the second amplification with nested primers (F_AMY/R_amelB). Nested PCR associated with classical PCR makes the determination method more reliable and robust.

3.3. Fragments of the HVR region 1 products for cloning

The agarose gel electrophoretic runs of Fig. 6 show the PCR fragments produced by the mtDNA HVR1 region, used for cloning and subsequent sequencing.

Fig. 6A, B, C shows the PCR amplifiers on the HVR1 region of mtDNA of samples S.42, S.45, S.46, S.48 of *Amiternum* obtained with various pairs of primers (F15995/R16132, F16131/R16218, F16196/R16345), it can be seen that amplification is achieved in all samples and the lengths of the amplicons are approximately equal to 180 bp (Fig. 5A), 180 bp (Fig. 6B), 126 bp (Fig. 6C). Fig. 6D shows PCR amplifies of 472 bp on the mtDNA HVR1 region of the operators samples with primers F-16009/R-16439. The dimensions of the amplicons correspond to those expected. The same primer pair was used to amplify the DNA of the bone remains; as expected, as the ancient DNA is fragmented (Del Gaudio et al., 2013), there was no amplification (data not shown).

The cloned inserts in the pCRII-TOPO vector of the *Amiternum* samples and the operators were sequenced by MWG Eurofins (Germany). The sequences obtained were aligned and compared between the clones in order to define the consensus sequence and deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) (Table 4). The results of alignment of the ancient DNA sequences, for each extraction and for each amplification, are consistent and in agreement. (Fig.-S3 A,B,C)

3.4. Haplogroup assignment

Before the determination of the haplotypes and the phylogenetic analyzes, the DNA sequence profiles of the operators and of the skeletal remains were studied, the analysis of the SNIPs allow to exclude the contamination between the two (Fig.-S3 A,B,C).

Table 5 shows the polymorphic variations identified for each sample of skeletal remains of *Amiternum* and the haplogroups and subhaplogroup identified with the softwares indicated in materials and methods section.

Although the size of the sample studied is numerically small, haplogroup H is the most represented and is consistent with the frequency of haplogroup in the Italian population of central and northern Italy, which is about 55%, compared to southern Italy where the the percentage is around 33%; haplogroups J and R0a have a greater percentage in southern Italy (Brisighelli et al., 2012). Further investigations were conducted on haplogroups identified with the mtDNA database, v4/R12 - EMPOP, Fig. 7 shows the geographical distribution of haplogroups, in Europe and the Middle East, performed with Haplogroup Browser (www.empop.org), from which we can deduce the high frequency of some haplogroups in Northern Europe and the Middle East, geographical areas that they could have genetically contaminated the populations we studied.

Fig. 8 shows the analysis with the MEGA 7 software (Kumar et al., 2016) of 44 mtDNA nucleotide sequences (HVR1 region): 4 sequences obtained from the skeletal remains of *Amiternum*; 40 sequences of modern individuals: from the north (Vai et al., 2015), center and south (Ingman and Gyllensten, 2006) of Italy. The phylogenetic tree obtained shows the evolutionary relationship of our samples with the populations of northern Italy.

In Fig. 9 shows the evolutionary relationships studied through SplitsTree4 V4.14.6 (Huson and Bryant, 2006), which is a leading application for the calculation of non-branched phylogenetic networks. The sequences were aligned with Clustal W (Larkin et al., 2007). The analysis involved 56 mtDNA nucleotide sequences (HVR1 region 388 bp): 4

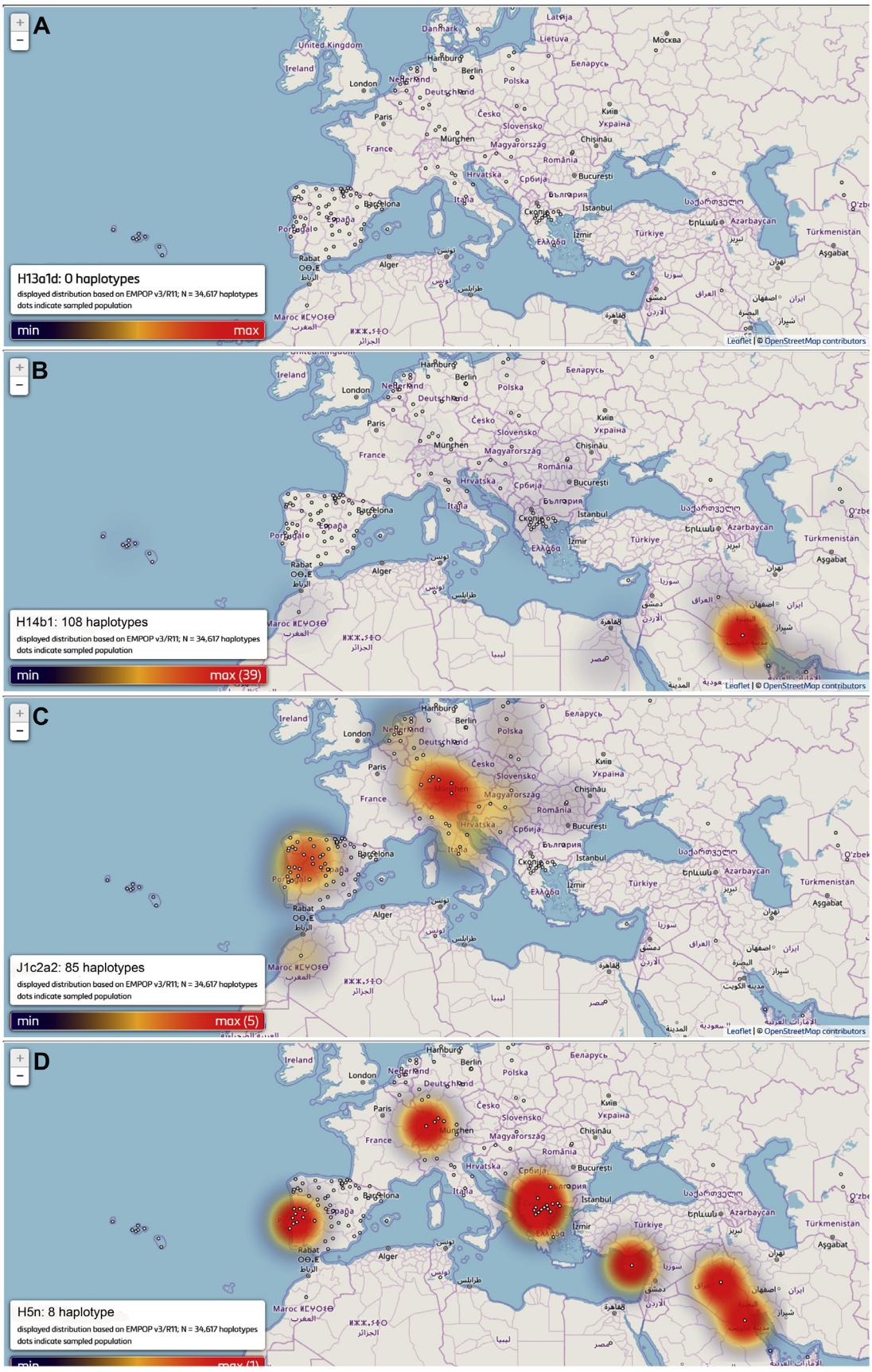

Fig. 7. Distributions of haplogroups performed with Haplogroup Browser. The database contains mtDNA sequences from all over the world and generates a geographical representation of the frequency of the haplogroup identified. The distribution depends on the coverage of that particular haplogroup in EMPOP. A: sample *Amiternum* S.42, Haplogroup H13a1d; B: sample *Amiternum* S. 45, Haplogroup H14b1; C: sample *Amiternum* S. 46, Haplogroup J1c2a2; D: sample *Amiternum* S. 48, Haplogroup H5n.

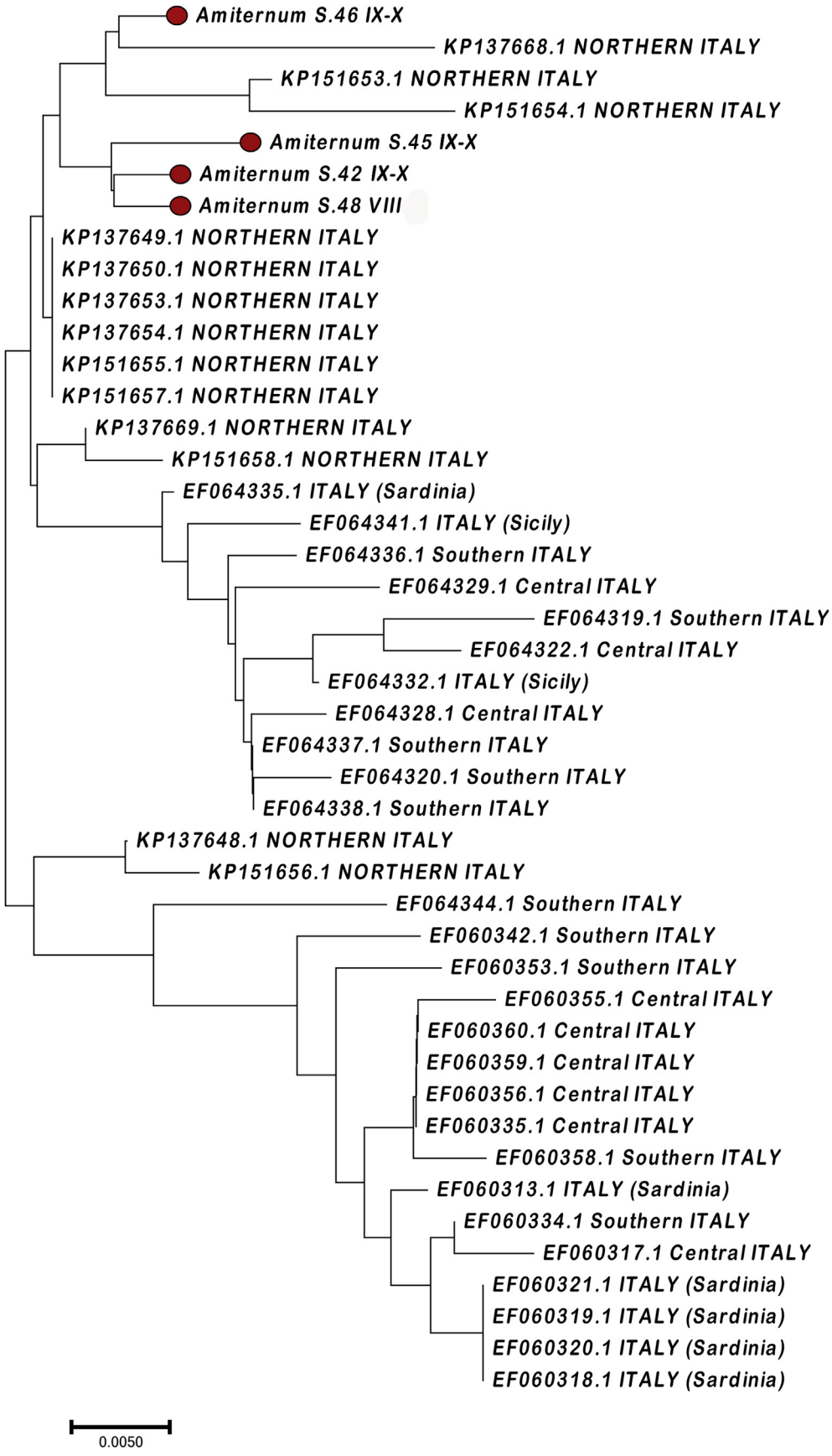

Fig. 8. Evolutionary analysis conducted with the MEGA7 software. Evolutionary history has been deduced using the Neighbor-Joining method. The optimal tree is shown with the sum of the branch length = 0,19757947. The phylogenetic tree is drawn in scale, with lengths of the branches of the same units as those of the evolutionary distances. The analysis involved 44 mtDNA nucleotide sequences (HVR1 region): 4 sequences obtained from the skeletal remains of *Amiternum*;40 sequences belonging to current Italians in the north, central and southern Italy and to the regions of Sicily and Sardinia (Vai et al., 2015; Ingman and Gyllensten, 2006). All GenBank access numbers are shown for all analyzed sequences.

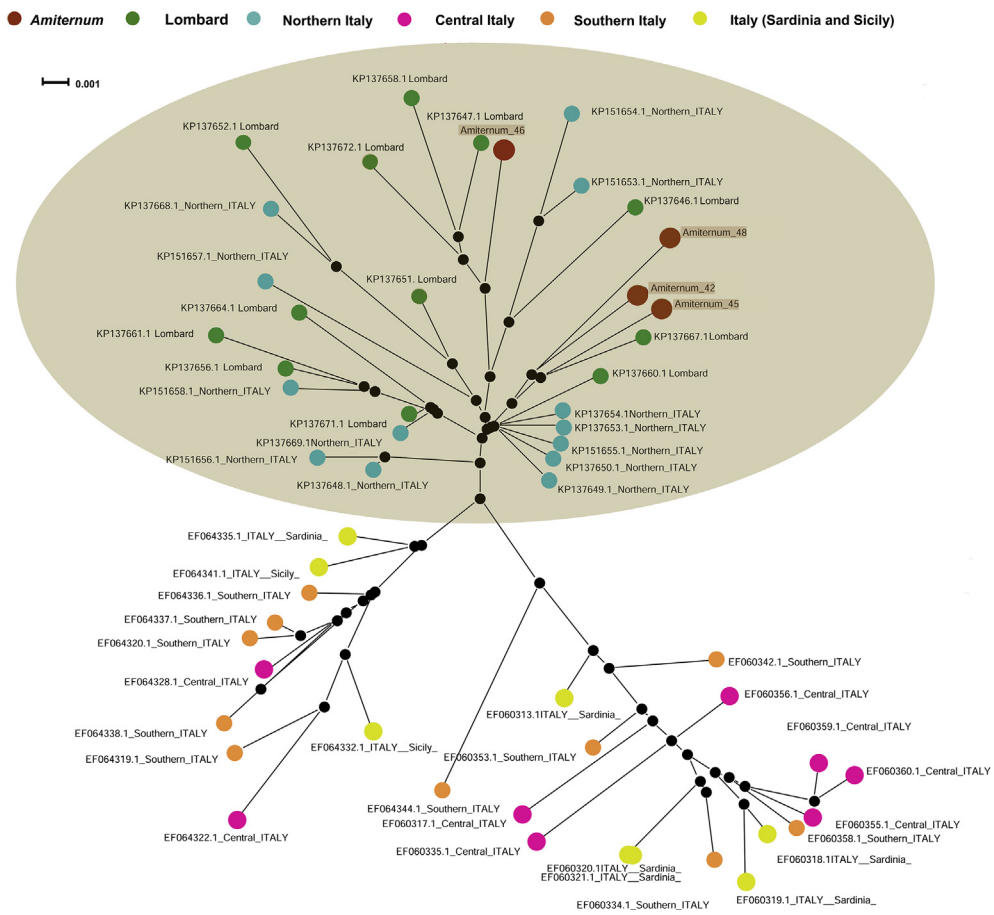

Fig. 9. Phylogenetic network for hypervariable segment (HVR1) of the mtDNA generated using SplitsTree4 with default settings. The analysis involved 56 mtDNA nucleotide sequences: 4 sequences obtained from the skeletal remains of *Amiternum*; 12 sequences of Lombard individuals (Vai et al., 2015); 40 sequences belonging to current Italians in the north, central and southern Italy and to the regions of Sicily and Sardinia (Vai et al., 2015; Ingman and Gyllensten, 2006). All GenBank access numbers are shown for all analyzed sequences.

sequences obtained from the skeletal remains of *Amiternum*; 12 sequences of Lombard individuals (Vai et al., 2015); 40 sequences of modern individuals: from the north (Vai et al., 2015), center and south (Ingman and Gyllensten, 2006) of Italy. From this analysis it is evident the genetic contamination of our populations by the Lombard population.

Fig. 10 shows the phylogenetic tree, obtained with the MEGA 7 software (Kumar et al., 2016), of 67 mtDNA nucleotide sequences (HVR1 388 bp region): 4 sequences obtained from the skeletal remains of *Amiternum*; 12 sequences of Lombard individuals (Vai et al., 2015), 12 sequences of Byzantine individuals (Ottoni et al., 2011), 39 sequences of modern individuals: from the north (Vai et al., 2015), center and south (Ingman and Gyllensten, 2006) of Italy, where it is evident the genetic contamination of the populations studied by us both by the Byzantines and by the Lombards.

4. Conclusion

The possible contamination with modern DNA is one of the difficulties to be faced in the analysis of ancient DNA, to avoid it, in our work all the precautions suggested in the literature have been adopted (Llamas et al., 2017; Fulton and Shapiro, 2019; Pilli et al., 2013; Adler et al., 2013; Korlević et al., 2015; Salamon et al., 2005). In addition, controls were carried out such as: repeated extractions and amplifications, cloning, SNPs analysis, use of primers that produce amplicons greater than 400bp, which as a whole suggest non-contamination.

In this work we successfully sequenced the HVR1 region (388 bp) of mtDNA of 4 skeletal remains found in the cathedral of *Santa Maria in Civitate* of *Amiternum*, dating back to the 8th and 9th century AD and we have identified the haplotypes (Table 4). The most representative haplogroup in the *Amiternum* samples is the predominantly European haplogroup H, which accounts for about 40% of all mitochondrial lineages in Europe and is fairly evenly distributed, suggesting its important role in the European population from around 20,000 years to date (Achilli et al., 2007).

The haplogroup R0a identified in the sample *Amiternum* S.45 is an ancestral clade of the haplogroup HV and therefore antecedent to H and V, it seems to originate from the Arabian peninsula and it spread about 15,000 years ago in the late glacial period (Gandini et al., 2016).

The haplogroup J identified in the sample of *Amiternum* S.46 is present in about 12% of the native European populations (Costa et al., 2013), the subhaplogroup, J1 occupies four-fifths of the total and extends widely around the Mediterranean, Greece, Italy and Spain, the subhaplogroup J1c2a was found previously in the British Isles and in Scandinavia. The subhaplogroup identified in the *Amiternum* samples (S.42, S. 45, S. 48) are H13, H14, non-common branches, linked to the expansion of Neolithic farmers in the European area some 7000 years ago and which are found at low frequencies in Europe, in the Near East and in the Caucasus but also in the Mediterranean coasts (Roostalu et al., 2007). The work provides preliminary information on the genetic correlation between the individuals of *Amiternum* and the current individuals of northern, southern and central Italy and of the Lombard and Byzantine peoples. The limited number of samples and the analysis of a single mtDNA locus, allow us to make only very broad generalizations and obtain information for future studies.

In particular, phylogenetic analyzes (Figs. 8 and 9) show a genealogical continuity between the medieval individuals of *Amiternum* and modern individuals of northern Italy and the Lombard individuals, a Germanic population that dominated a vast area of the Italian peninsula between 568 and 774 AD. The Lombards settled mainly in the North-East and in Lombardy but also in central Italy, including Abruzzo and *Amiternum*, certainly leaving their genetic imprinting on the populations of these regions.

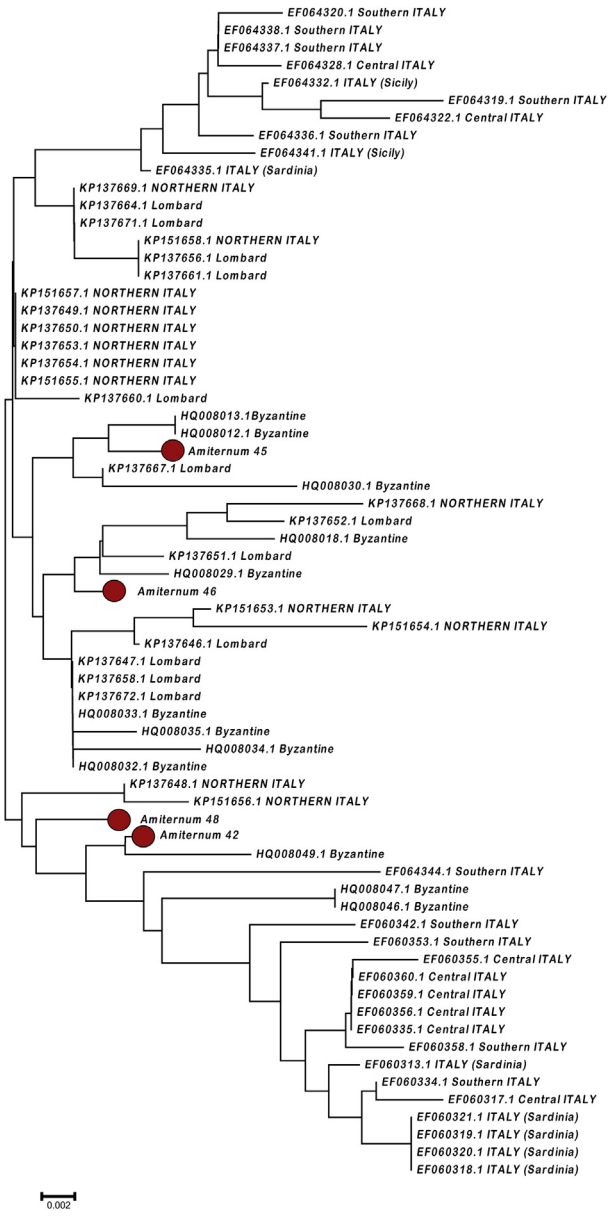

Fig. 10. Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.25357387 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 67 mtDNA nucleotide sequences (HVR1 region 388 bp): 4 sequences obtained from the skeletal remains of *Amiternum*; 12 sequences of Lombard individuals (Vai et al., 2015); 12 Byzantine individuals (Ottoni et al., 2011); 39 sequences belonging to current Italians in the north, central and southern Italy and to the regions of Sicily and Sardinia (Vai et al., 2015; Ingman and Gyllensten, 2006). For all sequences the GenBank access numbers are shown.

However, the evolutionary analyzes conducted by introducing Byzantine sequences (Ottoni et al., 2011) reveal their influence on *Amiternum* samples (Fig. 10).

Thus, these preliminary data indicate that the individuals of *Amiternum* have a genetic similarity with both ancient populations, this datum could agree with the historical movements of the populations occurred in the territory of *Amiternum*, which saw both the Byzantine and Lombard presence.

These molecular anthropology studies do not give a simple and unambiguous response to the interaction of *Amiternum* individuals with migrant populations, but provide indications that will help to plan a broader and more precise genetic research to identify aspects of historical and demographic changes of the populations involved.

Our goal, in the light of the results of this research, will be to introduce some loci of the y chromosome into the study, which will allow us to have a more robust and reliable response on genetic relationships. Moreover, to understand the genetic contribution of the Lombard and Byzantine migration on modern populations, the number of medieval samples of a wider geographical area will be increased, as suggested by historians.

From literature (Chavarria Arnau and Giacomello, 2015) it is clear that the use of cathedrals as a funerary space is a late phenomenon that starts from 7 and spreads mainly from the 8th to the 9th century, so the burials studied are consistent with the funeral ritual of the period. The test of amelogenin on *Amiternum* samples showed that individuals are all male (Fig. 3); characteristic that is often found in similar archaeological contexts; however, anthropological analysis only, without written texts and the presence of equipment, does not allow us to identify whether the buried S.45 and S.48 were ecclesiastical figures.

Declarations

Author contribution statement

Anna Maria Giuseppina Poma, Fabio Redi: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Patrizia Cesare, Antonella Bonfigli: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Giulia Vecchiotti, Sabrina Colafarina: Performed the experiments.

Francesca Savini, Osvaldo Zarivi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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