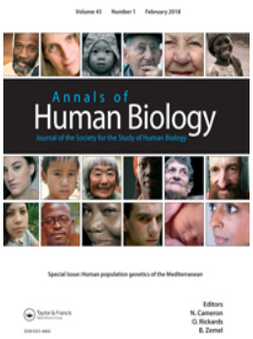

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RESEARCH PAPER

Iron Age Italic population genetics: the Piceni from Novilara (8th–7th century BC)

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ABSTRACT

Background: Archaeological data provide evidence that Italy, during the Iron Age, witnessed the appearance of the first communities with well defined cultural identities. To date, only a few studies report genetic data about these populations and, in particular, the Piceni have never been analysed.
Aims: To provide new data about mitochondrial DNA (mtDNA) variability of an Iron Age Italic population, to understand the contribution of the Piceni in shaping the modern Italian gene pool and to ascertain the kinship between some individuals buried in the same grave within the Novilara necropolis.
Subjects and methods: In a first set of 10 individuals from Novilara, we performed deep sequencing of the HVS-I region of the mtDNA, combined with the genotyping of 22 SNPs in the coding region and the analysis of several autosomal markers.
Results: The results show a low nucleotide diversity for the inhabitants of Novilara and highlight a genetic affinity of this ancient population with the current inhabitants of central Italy. No family relationship was observed between the individuals analysed here.
Conclusions: This study provides a preliminary characterisation of the mtDNA variability of the Piceni of Novilara, as well as a kinship assessment of two peculiar burials.

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Ancient DNA; Mitochondrial DNA; autosomal markers; Italian population genetic; Piceni

Background

According to recent studies, the Italian population is characterised by a higher degree of internal genomic variability than other European people (Boattini et al., 2013; Brisighelli et al., 2012; Di Gaetano et al., 2012; Fiorito et al., 2016; Sarno et al., 2014). This scenario is the result of complex demographic dynamics, dating back mainly from the Late Palaeolithic and Neolithic, but also dating to Metal Ages (Boattini et al., 2013; Capocasa et al., 2014; Pesando, 2005; Piazza et al., 1988; Sazzini et al., 2016), the Middle Ages and the Early Modern Period, which have influenced in a more or less marked way the present-day Italian genetic pool.

In particular, an appreciable population structure for Y-chromosome lineages and a more homogeneous mitochondrial DNA background were highlighted in Italy (Boattini et al., 2013; Brisighelli et al., 2012; Capelli et al., 2007). The population dynamics that shaped the Italian gene pool are not completely clear and, among the several studies carried out on the current Italian genetic variability (Barbujani et al., 1995; Boattini et al., 2013; Capocasa et al., 2014; Piazza et al., 1988; Sarno et al., 2014, 2017; Sazzini et al., 2016), only a few of them are based on ancient populations. These studies mainly focussed on specimens recovered from mainland Italy, in particular Etruscans and Lombards (Ghirotto et al., 2013; Guimaraes et al., 2009; Vai et al., 2015; Vernesi et al., 2004), or from Sardinia, which discloses a particular genetic history and is a well-known outlier in the general European genetic landscape (Caramelli et al., 2007; Modi et al., 2017; Olivieri et al., 2017).

Before its political, juridical, linguistic and cultural unification under the Roman Empire, the Italian Peninsula was a mixture of regional groups characterised by different cultural identities, languages and dialects. At present, insufficient information is available about the origin and possible events of admixture of these populations, and our knowledge is still almost incomplete from a genetic point of view. In this respect, research based on the study of ancient DNA would

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Supplemental data for this article can be accessed here.

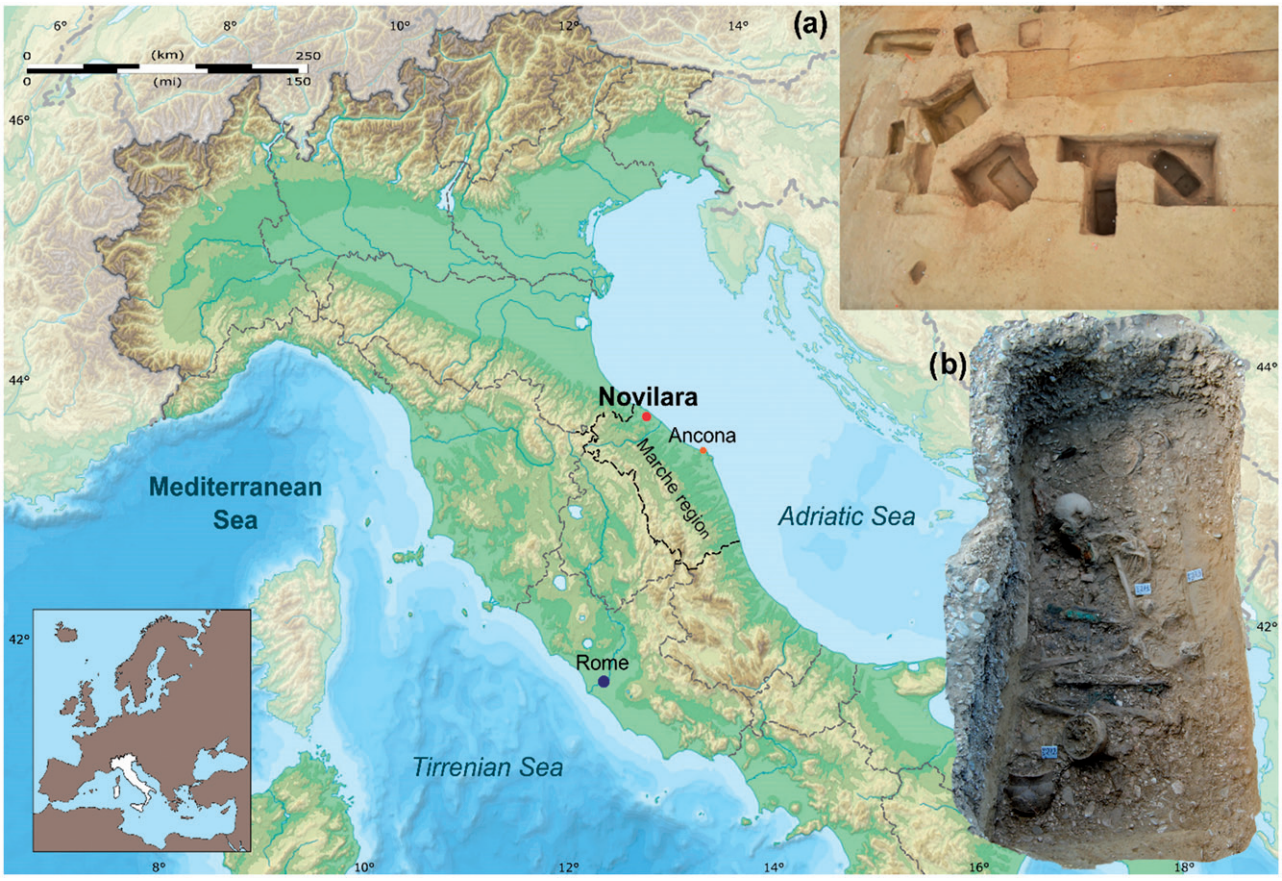

Figure 1. Geographic location of Novilara site: (a) archaeological fieldwork area during the campaign of 2012, (b) example of burial found during 2012–2013 (woman's grave n. 10, 7th century BC).

allow a better understanding of the past events that have led to the current genetic pattern.

In traditional archaeological terms the Italian Early Iron Age covers the 9th and 8th centuries BC and it is during this period that the first communities with strong and well defined cultural identities appeared (e.g. Etruscans, Piceni, Umbrians, Samnites). It was a period of great changes characterised by the adoption of new productive technologies, important social transformations and more extended trade-routes with a full interaction with both continental Europe and Eastern-Mediterranean regions. The economy, previously based on small-scale manufacturing, began to accommodate new classes of specialised craftsmen, thereby encouraging the growth of trades and production on a larger scale (Bietti Sestrieri, 2010).

In the wide panorama of Iron Age populations, we focussed our attention on the Piceni, a civilisation of the central Adriatic coast who lived in an area that roughly corresponds to the present-day region of Marche (Figure 1). Based on archaeological data, this civilisation developed from the 9th century BC to the beginning of the 3rd century BC, when Romans conquered this territory. The Piceni, in their whole distribution area, shared a common culture with some distinctive local features, favoured by the peculiar landscape of Marche, mostly consisting of hills with a linear pattern and narrow river valleys extending from the Apennines to the Adriatic coast. The lack of well-defined single urban centres in this territory maintained this Adriatic culture at a proto-urban stage and, more importantly, facilitated the permanence of local cultural diversities until the dominance of Rome. Knowledge of this civilisation is mostly based on archaeological findings, largely coming from necropolises (Naso, 2000).

The Novilara archaeological site (Figure 1) was recently excavated with a rigorous stratigraphic methodology (Delpino et al., 2016) and produced numerous data and samples for an Italian Iron Age necropolis. A preliminary analysis, already carried out on the osteological finds from Novilara, can help us to determine the contribution of the genetic approach to the interpretation of past and/or recent population dynamics, as well as to the palethnological interpretation of archaeological contexts. In fact, the Novilara site provides outstanding evidence of an Iron Age necropolis in the Marche region: it is characterised by an abundance of grave goods, systematically excavated starting from the end of the 19th century, good conservation status of the skeletal remains and, lastly, by a considerable amount of scholarly literature (see Supplementary Appendix S1). Moreover, within the necropolis, some areas clearly appear to have been more densely used and others show small groups of graves (Supplementary Figure 1). From an archaeological perspective, the burials repartition potentially shows that the funerary space of the necropolis was structured following family or clan-related groups (see Supplementary Appendix S1). Understanding the genealogical relationships among individuals buried together could clarify the social structure of this

ancient population and could identify the presence of specific social behaviours, such as exogamous or endogamous marriages.

The aims of this study are (i) to provide new data about mitochondrial DNA (mtDNA) variability of an Iron Age Italic population and to expand the knowledge of the populations of this period, for which few genetic data are available; (ii) to evaluate the genetic continuity of the population of Novilara with current Italian populations, in particular with respect to areas geographically or historically related to Piceni; and (iii) to study the possible kinship between some individuals buried together inside the Novilara necropolis.

To achieve these objectives, we performed a deep sequencing of the first hypervariable segment (HVS-I) of the mitochondrial DNA in 27 ancient samples from the Novilara necropolis followed by the genotyping of a set of 22 SNPs in the mtDNA coding region of the same individuals. To test kinship relationships, we also performed a forensic analysis on autosomal STRs and insertion/deletion (InDels) polymorphisms in two couples of individuals buried together in this necropolis.

Although preliminary, our results highlight a very low genetic diversity of the Piceni population compared to modern Italian populations and reveal a possible matrilineal genetic continuity between the inhabitants of Novilara and the current Italian population living in the same geographic area.

Materials and methods

Sample information

The human skeletal remains of 27 individuals from the Iron Age necropolis of Novilara (Supplementary Table 1) were collected based on the archaeological information and on the state of preservation of the samples. All the graves have been dated to the 8th–7th century BC by the stratigraphic sequence coupled with the recovered grave goods and general historical reconstruction of the site. The sampling of teeth and petrous bones was carried out during archaeological excavations in the 2012–2013 field season with all the necessary precautions described in Fortea et al. (2008) to minimise the occurrence of contamination from modern human DNA (Supplementary Appendix S2.1). More details about the archaeological site and sampled burials are reported in Supplementary Appendix S1.

Ancient DNA procedures

DNA extractions and PCR set-up were performed in physically isolated work areas dedicated to ancient DNA analysis at the Laboratories of Physical Anthropology and Ancient DNA, Department of Cultural Heritage (DBC), University of Bologna, according to rigorous aDNA standards to avoid contaminations (Cooper & Poinar, 2000; Fulton, 2012; Knapp et al., 2012, 2015). Suitable disposable clothing (coverall suit, double pair of gloves, over-shoes, face mask and plastic face shield) were worn during the handling and extraction of materials. The worktop and instruments were regularly cleaned with 5% commercial NaClO, 96% ethanol, DNA-ExitusPlus™ solution (AppliChem GmbH, Darmstadt, Germany) (Esser et al., 2006) after each experiment and exposed to ultraviolet radiation ($\lambda = 254$ nm) until the next activity. Sterile materials and dedicated pipettes with aerosol resistant tips were used at each step of work. In addition, all the reagents were screened for modern DNA and stored in small volume aliquots before use. Multiple blank extractions were processed in parallel and negative controls were included in all reactions. The HVS-I sequences of the personnel involved in this study (archaeologists, anthropologists and laboratory researchers) were compared with the genetic profiles obtained from the ancient specimens to make sure of the absence of modern contamination (Supplementary Table 2). PCR and post-PCR laboratory procedures (libraries preparation of amplicons and next-generation sequencing) were carried out in a separate building at the Laboratory of Molecular Anthropology and at the Centre for Genome Biology, Department of Biological, Geological and Environmental Sciences (BiGeA), University of Bologna.

DNA extraction and mtDNA amplification

Before DNA extraction, the samples were superficially cleaned with a sterile drill bit and exposed to ultraviolet radiation ($\lambda = 254$ nm) for 15–30 min on each side, in order to remove the outer surface contamination. Successively, 150–300 mg of bone powder was digested in 1 mL proteinase K and EDTA lysis buffer and DNA was extracted through a silica-based method (Dabney et al., 2013a) with a few modifications described in Supplementary Appendix S2.2.

The first hypervariable segment (HVS-I) of the mtDNA control region was amplified by PCR using three (L15995-H16132, L16107-H16261, L16247-H16402) overlapping fragments (Caramelli et al., 2003) in order to obtain 360 bp, spanning from nucleotide position (np) 16024 to np 16383. The amplification of each fragment was carried out in independent PCR reactions. To verify the repeatability of the mitochondrial data and to confirm the authenticity of the results, for a set of randomly selected individuals, the whole experiment from DNA extraction to DNA amplification was performed twice (Hervella et al., 2015; Lorkiewicz et al., 2015), starting from different anatomical elements and by different researchers. Furthermore, all DNA extracts were screened to test their appropriate molecular behaviour (Cooper & Poinar, 2000) with L15996-H16401 primers pairs (Vigilant et al., 1989), which amplify a larger fragment (~400 bp), in order to detect possible contaminations, given that ancient DNA molecules are often fragmented to very short pieces encompassing between 60 and 150 bp (Prüfer et al., 2010).

A total of 22 SNPs in the mtDNA coding region (4216 L, 4529 L, 4580 L, 7028 L, 10398 L, 10400 L, 10873 L, 12308 L, 12705 L, 14766 L, 3010 L, 3915 H, 3936 H, 3992 L, 4310 L, 4745 L, 4336 L, 4769 H, 4793 H, 6776 H, 13708 L, 13759 L) (Herrnstadt et al., 2002; Richards et al., 2000) were selected to confirm the haplogroup assignment preliminarily inferred with the HVS-I haplotype motifs. The genotyping was performed by means of two different multiplex-PCR reactions

Table 1. mtDNA sequences of samples from Novilara site. mtDNA haplotypes were numbered according to the rCRS (Andrews et al., 1999).

Sample name	Investigated sample	Burial number	Manipulators[c]	rCRS position	HVS-I haplotype (NGS)	HVS-I haplotype (Sanger)	SNP in coding region	Haplogroup
NOR3a[a]		171	M1, M2, M3	16024–16383	rCRS	rCRS	7028C	H*
NOR3b[a]		171	M1, M2, M3	16024–16383	16291T	16291T	7028C	H*
NOR8[a]		67	M2, M4	16024–16383	16069T, 16126C, 16362C	16069T, 16126C, 16362C	3010A, 4216C	J1
NOR10a		155	M2, M4	16024–16261	16222T	–	7028C	H*
NOR10b[a]		155	M1, M2, M4	16024–16383	16192T, 16298C	16192T, 16298C	7028C	H*
NO12		85	M2, M4	16024–16383	16069T, 16126C	–	3010A, 4216C	J1
NO13		89	M2, M3	16024–16383	rCRS	–	3010A	H1*
NO19[b]		125	M2, M4	16024–16383	16224C, 16311C	–	–	K
NO20[b]		83	M2, M3	16024–16383	16356C	–	–	HV1
NO21[b]		128	M2, M4	16024–16383	16145A, 16234T, 16270T	–	–	H1

[a]Samples analysed two times and sequenced with both Sanger and NGS methods.
[b]Haplogroup predicted with Haplogrep2 software.
[c]For HVS I motifs of the researchers see Supplementary Table 2. For autosomal analysis (Globalfiler™ kit), performed in the last part of the project, one more extraction was carried out for NOR3a, NOR3b, NOR10a and NOR10b samples.

followed by a single-base extension assay carried out with the SnaPshot® Multiplex Kit (Applied BioSystems, Foster City, CA) (Bertoncini et al., 2012). Capillary electrophoresis reaction was performed at the Department of Diagnostic and Laboratory Services and Legal Medicine (University of Modena and Reggio Emilia) on an ABI PRISM™ 3130 DNA Genetic Analyzer (Applied BioSystems). Details about the amplification and multiplex PCR reactions are reported in Supplementary Appendices S2.3 and S2.4.

Autosomal analysis

A potential kinship between two couples of individuals buried in the same grave (grave 155: NOR10a and NOR10b; grave 171: NOR3a and NOR3b) was investigated using two commercial forensic PCR kits: the Globalfiler™ assay kit (Applied BioSystems) comprising 23 STRs and the DIPplex® kit (Qiagen GmbH, Hilden, Germany) including 30 InDels biallelic markers. The autosomal markers were amplified at the Institute of Forensic Sciences Luis Concheiro, University of Santiago de Compostela, according to the manufacturer's protocol for the treatment of degraded and problematic DNA samples (see Supplementary Appendices S2.5 and S2.6). The analysis of STRs in ancient or degraded specimens raises major problems of validation of the data. To support the results, for each sample, the Globalfiler™ analyses were conducted on two different extracts, for each of which an independent amplification was performed.

DNA library preparation and sequencing

All the PCR products were converted into blunt-end sequencing libraries using the Ion Plus Fragment Library Kit (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions.

Sequencing of the libraries was performed using the Ion PGM Hi-Q™ Sequencing Kit (Life Technologies) and by setting 500 flows (i.e. 125 cycles) on the Ion PGM™ System (Life Technologies, Grand Island, NY). Details on the library preparation and sequencing reaction are indicated in Supplementary Appendix S2.7. For the sub-set of samples tested twice (see DNA extraction and mtDNA amplification), we performed Sanger sequencing experiment on a 3730 DNA Analyzer (Applied BioSystems).

Data analysis

Ion Torrent data for each barcoded library was processed using tools implemented in the Galaxy 16.01 platform (https://usegalaxy.org/) (Blankenberg et al., 2010; Giardine et al., 2005; Goecks et al., 2010) (see Supplementary Appendix S2.8). All merged and quality filtered sequences of Novilara samples were edited and aligned to the revised Cambridge Reference Sequence (rCRS) (Andrews et al., 1999) with DNA Alignment (fluxus-engineering.com), BioEdit v7.2.5 (Hall, 1999) and MEGA7 (Kumar et al., 2016) software. Afterwards, we checked the types and frequency of nucleotide variations among the collapsed reads for every single individual, such as C→T transitions, which represent the prevalent signal of *post-mortem* miscoding lesions in authentic aDNA (Bollongino et al., 2013; Dabney et al., 2013b; Stiller et al., 2009). Mitochondrial haplogroups were determined based on the PhyloTree mtDNA phylogeny, built 17 (www.phylotree.org) (van Oven and Kayser, 2009) and Haplogrep2 software (Kloss-Brandstätter et al., 2011).

In order to investigate the possible genetic relationships between Piceni and other modern and ancient populations, we used mtDNA sequences belonging to 12 European prehistoric populations available from the literature (populations ranging from the Bronze Age to the Iron Age period), as well

as those belonging to 1833 present-day unrelated individuals from continental Italy, Sicily and Sardinia, clustered into eight macro-areas following the strategy approach described in Boattini et al. (2013). References used to collect these databases are available in Supplementary Tables 3 and 4. Diversity parameters and pairwise Fst distances were computed on HVS-I data (nps 16024–16383) using Kimura 2 P distance option (Kimura, 1980), as implemented in Arlequin software ver. 3.5 (Berne, Switzerland) (Excoffier & Lischer, 2010). Slatkin Fst-values (Slatkin, 1995) were used to reconstruct non-metric Multidimensional scaling (MDS) plot visualised in bi-dimensional space using R "*MASS*" package (R-DevelopmentCoreTeam, 2008).

Results and discussion

Authenticity of results

The procedures performed in this study and the strict criteria used to estimate the reliability of aDNA results allowed us to exclude any modern contamination and certify the authenticity of our ancient data with a high degree of confidence. First, we followed rigorous laboratory precautions for ancient DNA study to avoid contamination with modern DNA (see Materials and methods). Second, we utilised recently excavated skeletal remains, a circumstance that has been suggested to facilitate the discrimination between endogenous and contaminant DNA (Pruvost et al., 2007; Sampietro et al., 2006). Third, no contamination was observed in any of the blank extractions or negative controls included in each reaction. Fourth, none of the ancient samples screened with the L15996-H16401 primer pair yielded any amplification products, indicating the absence of intact modern exogenous DNA. Fifth, all HVS-I sequences obtained from Novilara samples showed different haplotypes from those of operators involved in this study (Supplementary Table 2). Sixth, we considered our aDNA data as genuine whenever a clear sequence was reproduced in all the overlapping portions of each adjacent fragment. Seventh, no recurrent mutations were highlighted in the sequences obtained from ancient individuals, excluding a systematic exogenous contamination. Eighth, we performed DNA extraction and amplification in a sub-set of Novilara samples ($n = 4$) twice, starting from different bones from the same individuals, and the HVS-I mtDNA sequences obtained by Sanger sequencing experiment confirmed the same haplotype derived from NGS sequencing reaction (Table 1). Ninth, the assay (PCR coupled with amplicon sequencing in NGS) has been shown to be highly sensitive for sequencing limited DNA amounts and to analyse biological mixtures of samples, allowing to detect low level variants (Berglund et al., 2011). Moreover, this approach was useful to study damage patterns and point out contaminations from exogenous sources, by means of deep coverage data (Palencia-Madrid & de Pancorbo, 2015). In this study, 13 207 764 bases were obtained on average for samples, producing a mean of 92 007 reads, 99.44% of which were on target. Regarding base coverage data, each base was read 29 044.8 times and uniformity of base coverage was 90.17%.

Genetic diversity

HVS-I mitochondrial consensus sequences were successfully obtained in 10 out of the 27 ancient specimens (37.03% of the total) (Supplementary Figure 1 and Supplementary Table 1). The remaining 17 samples were excluded from subsequent analyses because they yielded no amplification products ($n = 11$) or produced ambiguous sequence results ($n = 6$).

A partial HVS-I consensus sequence was obtained for NOR10a (np 16024–16261), making this sample useless for the population genetic analysis.

Haplogroups preliminarily inferred by HVS-I mutation motif were confirmed by the genotyping of 22 mtDNA SNPs (see Table 1). In three samples the multiplex amplification failed (NO19, NO20 and NO21). By combining sequence and genotyping analyses, the analysed samples were classified as belonging to six different mtDNA lineages: H* (NOR3a, NOR3b, NOR10a and NOR10b), J1 (NOR8 and NO12), K (NO19), H1* (NO13), H1 (NO21) and HV1 (NO20) (see Table 1). All these lineages have been reported to be typical of the West Eurasian area (Richards et al., 2000). It is worth noting that the four samples belonging to the same paragroup H* were found in shared graves (grave 155 for NOR10a and NOR10b and grave 171 for NOR3a and NOR3b), but carried different mutation motifs.

Interestingly, the nucleotide diversity of ancient Piceni (0.0087 ± 0.0056) was lower (albeit not significantly) than the diversity reported for modern Italian populations (Supplementary Table 3). Given the small sample size of the Piceni, which could mislead the interpretation of the intra- and inter-population statistics, a resampling procedure was performed. We randomly extracted, without replacement, 1000 sub-samples of nine individuals each, from all the 34 Italian extant populations of our dataset and then we re-calculated the nucleotide diversity for each sub-set. The resulting distributions were then compared with the value observed for the ancient Novilara sample. Although with no statistical robustness, by the resampling procedure we confirmed the results. In fact, the nucleotide diversity values of the ancient Piceni always fall within the 1st quartile or even outside (Lecce) the distributions obtained through the resampling of the extant Italian populations (see Supplementary Figure 2).

Kinship assessment

The autosomal analysis returned partial profiles for a number of the attempted samples: NOR10a and NOR10b (grave 155) being the most successful ones, followed by NOR3a and NOR3b (grave 171).

The samples of the grave 155 allowed the amplification of an almost full profile with InDels kit (Supplementary Table 5a) and a fairly complete Globalfiler kit STR profile (Supplementary Table 5b), resulting in a 93.3/60% of completeness (DIPplex/Globalfiler) for NOR10A and 83.3/44% of completeness for NOR10B. Both profiles showed a different allele combination in comparison with the other successfully amplified samples and with respect to the laboratory staff's profiles. The comparison of the Globalfiler results from the two independent amplification experiments confirmed the obtained

profiles and pointed out three drop-outs in two loci for NOR10a (TH01 and FGA) and one locus for NOR10b (D22S1045) (Supplementary Table 5b). For the subjects of grave 171, no results were obtained with the Globalfiler analysis, while we achieved a partial profile through DIPplex kit (Supplementary Table 5a). The different completeness percentage observed among InDel and STR results was to be expected due to the shorter amplicon length sported by InDels when compared to what STR typing may offer, including the miniSTR mode amplification. It has to be noted that, in parallel to the severe degradation of the samples, the issue of low copy number DNA may also contribute to the observed results. This leads to a certain degree of randomness in the observed results compared to the expected, serving as an explanation for the partial amplification obtained in one assay but not in the other, and for the absence of successful replication for these profiles.

The amelogenin locus indicated that NOR10a, NOR10ba and NOR3a subjects were females. Due to the failure of the Globalfiler reaction, the only DIPplex analysis for the NOR3b sample does not allow us to confirm with a high degree of confidence the sex of this individual. In fact, the presence of a peak (sz 79.85; ht 150) at the Y position, completely unbalanced in comparison with the X one (sz 76.96; ht 1612), should be confirmed by additional analyses. Accordingly, we do not feel confident enough to assign with certainty the sex of the individual NOR3b, which the archaeological and anthropological data suggested as male. Anyway, in the other three samples analysed with autosomal markers, the sex determination through archaeological, osteological and genetic analysis showed complete concordance.

In order to give clear statistical support to the Novilara individuals' kinship investigations, the genealogical relationship between the two pairs of individuals buried in the same grave (i.e. NOR3a-NOR3b for grave 171 and NOR10a-NOR10b for grave 155) were estimated using the software Familias, Version 3.2.1 (Egeland et al., 2000; Kling et al., 2014). Considering the mtDNA data, according to which the four samples belong to the same haplogroup, but with different haplotypes, we inferred the lack of a maternal relationship between the two pairs of subjects. Blind Search module, a new tool in this version of Familias, was run to perform an unspecific relationship search for a set of persons with some DNA data. The Direct-Match, Parent-Child, Siblings, Half Siblings, Cousins, 2nd Cousins relationships were tested. For NOR10a and NOR10b, the LRs were calculated using both available autosomal STRs (eight loci) and InDels (21) markers, while for NOR3a and NOR3b only the 14 obtained InDels were considered (for the STRs allele frequencies: Presciuttini et al., 2006; Previderè et al., 2013; InDels frequencies: QIAGEN © 2010 Population Data for analysis of results from the Investigator DIPplex Kit). For both pairs of individuals, LR values were below zero (Supplementary Tables 6a and 6b), so these results are not supporting the different tested kinship hypotheses. Moreover, the values are gradually increasing inversely to the degree of kinship, as expected.

The genetic data presented here provide a first attempt to support archaeological hypotheses based on material culture data (such as graves distribution and manufacture styles) in the interpretation of funerary practices and kinship relationships. These data derive from a limited number of genetically analysed samples, with regard to those originally present in the sampling design of the project (see Supplementary Figure 1), selected on the basis of archaeological data in particular areas of the necropolis, as potentially linked to familiar or clan-related groups (see Delpino et al., 2016). Unfortunately, the 10 individuals for which the mitochondrial data passed the stringent criteria adopted in this study were located in burials physically scattered across the necropolis (see Supplementary Figure 1). This random distribution of the genetic results did not allow speculation about specific funerary practices that occurred in contiguous groups of graves. However, we can only state that the mitochondrial data seem to exclude a matrilineal relationship between them (see Table 1).

Moreover, autosomal analyses were provided for only four individuals buried in the two bisome graves (Supplementary Figure 1). Regarding burial 171, the two adult individuals, identified as a woman and probably a man, were buried in the same burial place in two different times: first was buried "individual b" (sample NOR3b—the alleged man) and then, right above the first one, was buried "individual a" (sample NOR3a—a women). Although buried together, autosomal data did not support a family relationship between them, a situation that could lead us to hypothesise that the same burial place was reused for two subjects unrelated or related-in law. The particular funerary set discovered in this grave pushes archaeologists to believe that individuals NOR3a and NOR3b were somehow connected together. The grave goods of this burial belong almost completely to "individual a", a woman, and they include, among the vases, typical female brooches and other female adornments. However, upon the woman's body, there were also some fragments of typical male brooches, probably wrapped in a kind of a textile such as a shroud. It seems that when the first grave was re-opened to place inside the additional female corpse, the first funerary set was collected and put up on the second individual, along with her proper personal grave goods.

The autosomal data obtained from the individuals of the bisome grave 155, containing two females buried simultaneously, completely invalidate the idea of the existence of a kinship between them and leave several scenarios open to interpretation of the burial of two adult females that, apparently, did not show signs of violent death or infectious diseases (at least for those diseases that leave signs on the bones) and for which the data in our possession cannot supply an interpretation.

Population genetics

A multidimensional scaling (MDS) analysis comparing the HVS-I mitochondrial variability (np 16024–16383) of the Novilara individuals, 12 European pre-historic populations and 1833 present-day Italians, was carried out to provide a two-dimensional plot of the Fst genetic distances matrix (Supplementary Tables 7–9). A first exploratory MDS was performed between Novilara samples and current Italian

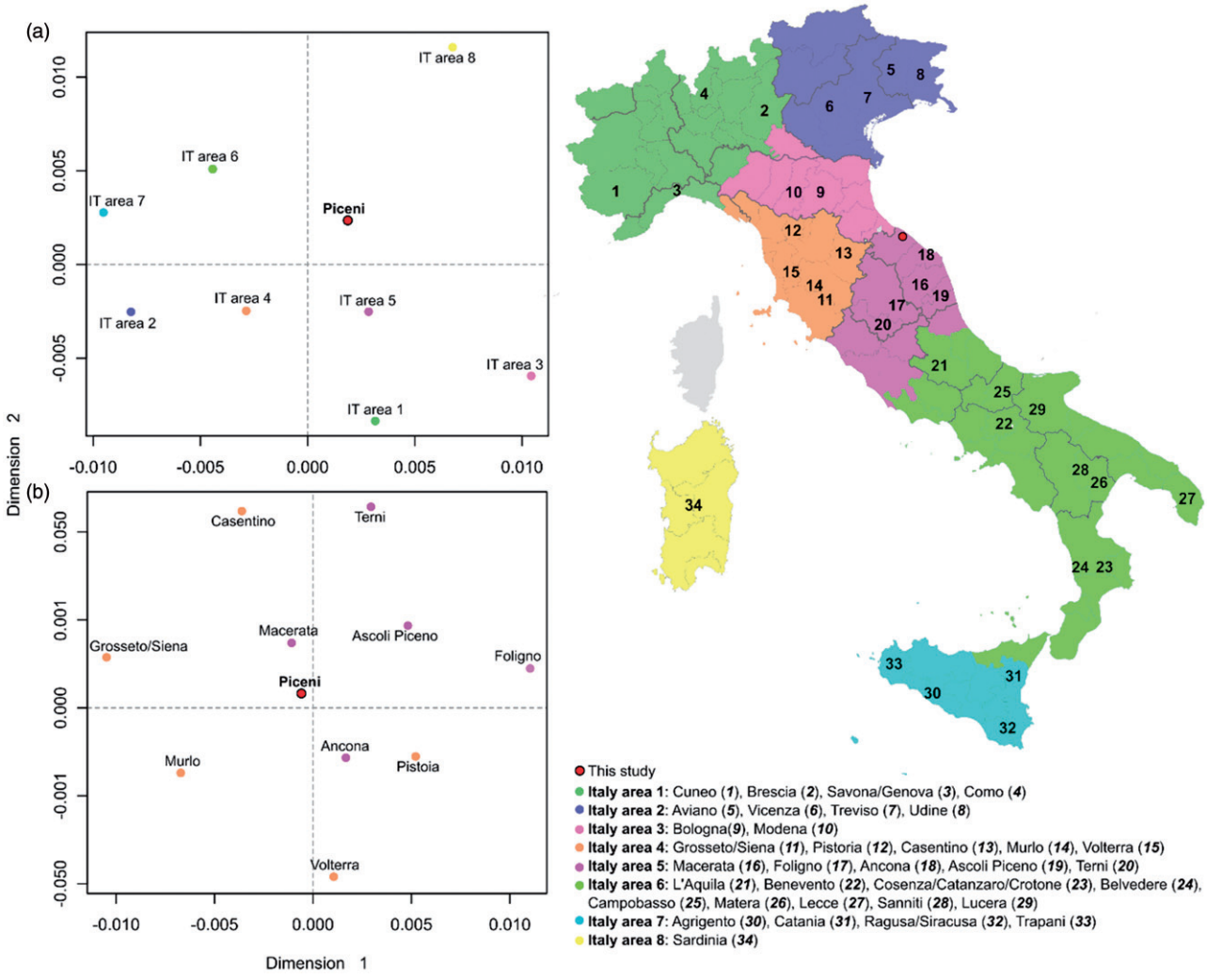

Figure 2. Two-dimensional MDS plots of pairwise Fst values from HVS-I showing relationships among the 34 populations from continental Italy, Sicily and Sardinia: (a) MDS performed between individuals from Novilara and current Italian populations grouped in eight macro-areas; (b) MDS performed between ancient Piceni and 10 populations from central Italy areas (areas 4 and 5). Red circle represents the population from Novilara.

populations grouped in eight macro-areas (see Supplementary Table 3 and Materials and methods section). The obtained plot (Figure 2(a)) generally showed separation of Sardinia (area 8) from all the other populations of continental Italy and Sicily (areas 1, 2, 3, 4, 5, 6 and 7). In this context, Piceni from Novilara appear at the centre of the plot, occupying a position very close to the nearby Italian populations from area 5 (Macerata, Foligno, Ancona, Ascoli Piceno and Terni). To provide further insight, a second MDS analysis was carried out with only the 10 populations belonging to the macro areas that in the previous plot were positioned near to Piceni (areas 4 and 5). The results of the new MDS plot (Figure 2(b)) highlight a genetic affinity, thus suggesting a certain degree of genetic continuity, between Piceni from Novilara and the present-day inhabitants of the Marche region: the nearest ones being Ancona and Macerata populations, followed by the Ascoli Piceno population, encompassing specimens collected in small towns of the Ascoli Piceno province (the "Piceni" of Montefortino, Castorano and Offida available from Brisighelli et al. (2012)). A second resampling procedure was performed to formally test for the higher genetic affinity of the ancient Novilara group with the present-day inhabitants of Central Italy. To do so, we extracted 1000 sub-samples of $n = 9$, without replacement, from three Central Italian populations (Ascoli Piceno, Foligno and Macerata), three Northern Italian populations (Brescia, Udine and Savona/Genova) and three Southern Italian populations (Lecce, Matera, Ragusa/Siracusa). We then calculated the Fst value between each of these sub-populations and the Ancona sample, the geographically closest population to Novilara included in our dataset. The resulting distributions were then compared to the Fst value obtained between Novilara and Ancona. This approach allowed us to test for a model of genetic affinity represented by the comparison between Ancona and Central Italian populations, and of population differentiation represented by Ancona vs Northern and Southern Italian populations. The resampling procedure was performed using the script by Anagnostou et al. (2017).

The resampling procedure highlighted that the genetic distance between Novilara and Ancona falls adequately within the Fst distributions obtained with the genetic affinity rather than with the population differentiation model (see Supplementary Figure 3).

Overall, our data indicate that the mtDNA diversity of Piceni of Novilara falls in the geographical cline of the mtDNA Italian genetic variability, which in general highlights a weaker genetic structure in the modern Italian population compared to Y-chromosome genetic diversity (Boattini et al., 2013; Brisighelli et al., 2012). Although these results are preliminary and being aware that the mtDNA perspective may disclose only a part of the population history, our results seem to suggest that probably there was not such a strong reshuffling in the maternal genetic pool of the investigated area during historical periods, when, for instance, migrations due to Celts, Romans and Goths are attested. Indeed, as suggested by previous studies on the present-day Italian population, the actual sex-biased genetic structure in Italy is possibly the result of different demographic histories for males and females, with the more homogenous pattern of mtDNA variability probably tracing back to more ancient times, and the Y-chromosome structure being instead shaped by more recent migration events (Boattini et al., 2013). Whether this genetic continuity involved other genomic loci remains to be elucidated.

In order to include the Piceni of Novilara within the genetic makeup of the European continent in the same temporal frame, we performed a third MDS plot (see Supplementary Figure 4) based on the Fst genetic distances between Piceni and several ancient populations of the Bronze and Iron Age. No particular geographic cluster has been observed from this comparison, but, interestingly, the Piceni resulted to be in the centre of the MDS plot indicating a genetic affinity with different ancient populations of continental Europe. This result could probably be due to the scarce molecular data availability of ancient populations from this time frame, in particular from the Italian Peninsula. Moreover, given that some data originate from pioneering studies obtained with classical methods (cloning and Sanger sequencing), it will be desirable to acquire more genetic information through high resolution methodologies, such as deep sequencing with NGS, in order to detect with more confidence exogenous contamination by modern DNA (Rizzi et al., 2012).

Conclusions

This study contributes to enlarging the knowledge of the Italian populations of the Iron Age period, for which few genetic data are currently available, focussing on the necropolis of Novilara (8th–7th centuries BC). Despite being aware that more informative and reliable technologies are becoming progressively available and suitable for aDNA analysis (e.g. the mitochondrial DNA capture), we retain that the methodologies and the strict criteria adopted in this study could constitute a good compromise between deep sequencing of entire mitochondrial or nuclear genomes, and a classical low resolution approach constituted by cloning and Sanger sequencing (Beau et al., 2017; Csősz et al., 2016; Kefi et al., 2018; Krzewińska et al., 2015; Le Roy et al., 2016; Nikitin et al., 2017; Palencia-Madrid et al., 2017; Rivollat et al., 2015; Simòn et al., 2017; Vai et al., 2015).

The apparent matrilineal genetic continuity between the ancient and modern populations in the region analysed here suggests that the different migratory events involved in this area probably did not influence the maternal gene pool of their inhabitants. It remains to be seen whether this continuity is also maintained for other loci.

This study also provides the first genetic data about the correlations between kinship and specific funerary traits in Novilara necropolis, as already analysed in previous studies (Le Roy et al., 2016). The few burials examined in this study, represented by two bisome burials, do not seem to reveal a direct genetic relationship between the inhumates. However, a greater richness of genetic data, generated from NGS methods and/or from independent analyses, coupled with stringent validation procedures, would be desirable in order to completely confirm our data.

In conclusion, this study provides a preliminary genetic analysis of the Piceni from the Iron Age necropolis of Novilara, but, in the future, it will be advisable to analyse more ancient samples from various necropolises of Piceni, belonging to different chronological period and localities, in order to better understand their funerary practices and to what extent they can be considered as single populations or smaller communities, which only partly recognised themselves in a wider organisation (Carfagna, 2016).

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