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We sampled teeth from 53 ancient Sardinian (Nuragic) individuals who lived in the Late Bronze Age and Iron Age, between 3,430 and 2,700 years ago. After eliminating the samples that, in preliminary biochemical tests, did not show a high probability to yield reproducible results, we obtained 23 sequences of the mitochondrial DNA control region, which were associated to haplogroups by comparison with a dataset of modern sequences. The Nuragic samples show a remarkably low genetic diversity, comparable to that observed in ancient Iberians, but much lower than among the Etruscans. Most of these sequences have exact
matches in two modern Sardinian populations, supporting a clear genealogical continuity from the Late Bronze Age up to current times. The Nuragic populations appear to be part of a large and geographically unstructured cluster of modern European populations, thus making it difficult to infer their evolutionary relationships. However, the low levels of genetic diversity, both within and among ancient samples, as opposed to the sharp differences among modern Sardinian samples, support the hypothesis of the expansion of a small group of maternally related individuals, and of comparatively recent differentiation of the Sardinian gene pools.

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Genetic variation in prehistoric Sardinia

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Received: 4 June 2007 / Accepted: 29 June 2007
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Abstract We sampled teeth from 53 ancient Sardinian (Nuragic) individuals who lived in the Late Bronze Age and Iron Age, between 3,430 and 2,700 years ago. After eliminating the samples that, in preliminary biochemical tests, did not show a high probability to yield reproducible results, we obtained 23 sequences of the mitochondrial DNA control region, which were associated to haplogroups by comparison with a dataset of modern sequences. The Nuragic samples show a remarkably low genetic diversity, comparable to that observed in ancient Iberians, but much lower than among the Etruscans. Most of these sequences have exact matches in two modern Sardinian populations, supporting a clear genealogical continuity from the Late Bronze Age up to current times. The Nuragic populations appear to be part of a large and geographically unstructured cluster of modern European populations, thus making it difficult to infer their evolutionary relationships. However, the low levels of genetic diversity, both within and among ancient samples, as opposed to the sharp differences among modern Sardinian samples, support the hypothesis of the expansion of a small group of maternally related individuals, and of comparatively recent differentiation of the Sardinian gene pools.

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Introduction

The population of Sardinia is one of the main European genetic outliers (Cavalli-Sforza and Piazza 1993). When compared with populations from all over the world, Sardinians are clearly part of a European genetic cluster (Rosenberg et al. 2002). However, they differ sharply from their European (Barbujani and Sokal 1990) and Italian (Barbujani and Sokal 1991; Barbujani et al. 1995) neighbours, so much so that they are often excluded from multivariate analyses, lest all other samples appear identical in comparison (Piazza et al. 1988; Semino et al. 2000).

Mitochondrial (Morelli et al. 2000) and Y-chromosome (Semino et al. 2000; Quintana-Murci et al. 2003) haplotypes that are rare elsewhere in Europe occur at higher frequencies in Sardinia, and an extensive linkage disequilibrium has been described for autosomal markers (Tenesa et al. 2004). In addition, unusually strong genetic differences are observed among Sardinian communities, both for allele-frequency (Barbujani and Sokal 1991) and DNA (Fraumene et al. 2003) polymorphisms.

These peculiar features are the likely evolutionary product of the interaction between reproductive isolation and small population sizes, the former historically documented and the latter a consequence of the fragmented habitat. Starting perhaps 10,000 years ago, people of different provenance reached the island (Webster 1996), but strong isolating factors, such as the Mediterranean Sea and mountain ranges, as well as cultural barriers (Barbujani and Sokal 1991), have probably enhanced the evolutionary role of genetic drift, both within the island and between it and the rest of Europe. However the details of these processes are not well understood, and it is well known that ages of particular genealogical lineages present in a geographical region do not contain information on the arrival of the population in that region (Barbujani and Goldstein 2004). Therefore, to gain insight into the biological features of past populations, and into the relationships between modern populations and their ancient counterparts, the direct study of the DNA of ancient individuals is a crucial research priority.

Here we present the first analysis of the hypervariable region I (HVR-I) of mitochondrial DNA (mtDNA) from samples of Bronze and Iron Age inhabitants of Sardinia, who are called Nuragic people from the towers (nuraghi) that they built. Using a strict set of methodological criteria that give a high probability of reproducible results (which forced us to exclude more than half of the initial samples from the analysis) we obtained sequences that were compared with those of modern European populations, and with the sequences of the two pre-classical European populations whose DNA has been described so far, the Etruscans (Vernesi et al. 2004) and the Iberians (Sampietro et al. 2005). In particular, prior to sequencing, the samples were treated with Uracil-N-glicosidase, so as to avoid, as far as possible, artefacts due to post-mortem damage of DNA (Hofreiter et al. 2001; Gilbert et al. 2003).

Materials and methods

DNA molecules are often scarce and damaged in ancient samples. As a consequence, experimental artefacts are possible or even likely, depending on the state of preservation of the material, during the analysis of DNA from fossil remains. The risk to mistake modern contaminating DNA for endogenous genetic material is higher when dealing with relatively recent human samples, whose DNA sequence may be similar to that of the archaeologists, museum personnel and geneticists who manipulated them. For all these reasons, in order to obtain reproducible results, the most stringent available standards were followed in this study, based on criteria listed by Cooper and Poinar (2000) and Hofreiter et al. (2001).

We collected 106 teeth, 2 from each of 53 different individuals, coming from excavations at six archaeological sites of Sardinia (Fig. 1) and dated between 4,300 and 3,000 years before the present (Table 1). The selected teeth had not been washed, which meant they had not been touched by anthropologists for morphological analysis. By analysing dental pulp from inside the tooth we reduced the risk of contamination from the archaeologists’ DNA, and care was taken to choose integer teeth without fractures. As a rule, to avoid the risk of sampling twice the same individual.

![Fig. 1 A map of Sardinia showing the six Nuragic sampling sites. The two-letter codes are used to label the sequences in the text and in Fig. 2](image-url)
whenever the teeth had not been found in place in the skull or mandible, we took only left lower canines and left lower molars. Because of the limited amount of amplifiable DNA present in most specimens, we decided to concentrate our efforts on the typing of the non-coding HVRI region.

Criteria of authenticity: an overview

Tooth specimens were handled (using mask, gloves and laboratory coats) in a space where no modern DNA had ever been extracted or analysed. We used only disposable sterile tubes, filtered tips, sterile reagents and solutions, exclusively dedicated to ancient DNA studies. Different sets of pipettes were used for DNA extraction, PCR amplification and analysis of the PCR products. DNA was extracted and PCR-amplified in separate rooms and under hoods, constantly irradiating with UV rays (254 nm). PCR products from ancient DNA templates were analysed in a third room. In each set of extractions or purifications we included a negative control, represented by all the reagents except the bone powder, and these negative controls, together with blanks (all amplification reagents minus DNA), were regularly analysed in every PCR experiment to control for presence of exogenous DNA.

All phases of the analysis were replicated at least twice. Four samples for which relatively large amounts of tooth powder were available were sent to the Barcelona laboratory, where the whole analysis was independently replicated. To test for preservation of other macromolecules as an indirect evidence for DNA survival (Poinar et al. 1996), we estimated the degree of aminoacid racemisation in each sample, using approximately 5 mg of tooth powder (Poinar et al. 1996). We also quantified the amount of target DNA by competitive PCR. PCR products were cloned, an average 25 clones were sequenced for each individual, and the sequences thus obtained were aligned and compared across clones. The consensus sequences were finally compared with a database of European mitochondrial sequences to test whether the ancient sequences obtained make phylogenetic sense.

DNA was also extracted from cattle (Bos taurus) remains retrieved in one burial (PE25), and we tried to amplify it using both human and cattle primers. The presence of human DNA sequences in extracts from non-human bones would suggest contamination in other bones in the burial, and possibly in other burials as well. All persons who worked in this study were mtDNA genotyped and none of their sequences matched those obtained.

DNA extraction

After brushing and irradiating each tooth surface (1 h under UV light), the root tip of each tooth was removed and the crown was repositioned into its mandibular alveolus. The root was powdered and DNA was extracted by means of a silica-based protocol (modified from Caramelli et al. 2003). For each individual we obtained two independent extracts from different teeth. A negative control was included in each extraction.

Competitive PCR

A competitor was used containing a 94 bp deletion (from position 16,106 to 16,189, nt position according to Caramelli et al. 2003), in the mitochondrial HVR-I. PCR components were the same as described below for the sequencing of mitochondrial HVR-I, and the primers were those used for the second fragment amplification. Thermal cycler conditions consisted of an initial 10-min incubation at 95°C followed by 45 cycles of 50 s at 94°C, 50 s at 48°C, and 50 s at 72°C, with a final extension step at 72°C for 5 min.

Real-time PCR

Real-time PCR amplification was performed to confirm the results of competitive PCR. We used Brilliant SYBR Green QPCR Master Mix (Stratagene) in MX3000P (Stratagene), using 0.5 µM of appropriate primers (forward primer at H 16,107 and reverse primer at L 16,261). Thermal cycling conditions were 95°C for 10 min, 40 cycles at 95°C.
183 95°C for 30 s, 53°C for 1 min and 72°C for 30 s, followed
184 by SYBR® Green dissociation curve steep. Tenfold serial
185 dilutions of the purified and quantified standard were
186 included in the experiment to create the standard curve,
187 thus estimating the number of initial DNA molecules in the
188 samples.
189
189 UNG treatment
190 Uracil-N-Glycosylase (UNG) reduces sequence artefacts
191 caused by post-mortem damage of DNA, resulting in appar-
192 ent C to T and G to A mutations and subsequent errors in
193 the sequence (Hofreiter et al. 2001). For each specimen
194 considered, 10 µl of DNA extract were treated with 1 U of
195 UNG for 30 min at 37°C to excise uracil bases caused by
196 the hydrolytic deamination of cytosines.
197
197 Amplification
198 Two microliters of DNA extracted from the bone and
199 treated with UNG was amplified with this profile: 94°C for
200 10 min (Taq polymerase activation), followed by 50 cycles
201 of PCR (denaturation , 94°C for 45 s, annealing, 53°C for
202 1 min and extension, 72°C for 1 min) and final step at 72°C
203 for 10 min. The 50-µl reaction mix contained 2 U of Amp-
204 liTaq Gold (Applied Biosystems), 200 µM of each dNTP
205 and 1 µM of each primer. The 360-bp long HVR-I was sub-
206 divided in three overlapping fragments using the following
207 primer pairs: L15,995/H16,132; L16,107/H16,261;
208 L16,247/H16,402 (Vernesi et al. 2004). Each extract was
209 amplified at least twice. Since overlapping primers were
210 used throughout the PCR amplifications, it is highly
211 unlikely that we amplified a nuclear insertion rather than
212 the organellar mtDNA.
213
213 Cloning and sequencing
214 PCR products were cloned using TOPO TA Cloning Kit
215 (Invitrogen) according to the manufacturer’s instructions.
216 White recombinant colonies were screened by PCR, trans-
217 ferring the colonies into a 30-µl reaction mix [67 mM Tris–
218 HCl (pH 8.8), 2 mM MgCl2, 1 µM of each primer, 219 0.125 mM of each dNTP, 0.75 U of Taq Polymerase] con-
220 taining M13 forward and reverse universal primers. After
221 5 min at 92°C, 30 cycles of PCR (30 s at 90°C, 1 min at
222 50°C, 1 min at 72°C) were carried out and clones with
223 insert of the expected size were identified by agarose gel
224 electrophoresis. After purification of these PCR products
225 with Microcon PCR devices (Amicon), a volume of 1.5 µl
226 was cycle-sequenced following the BigDye Terminator kit
227 (Applied Biosystems) supplier’s instructions. The sequence
228 was determined using an Applied Biosystems 3100 DNA
229 sequencer. Finally, to test for possible contamination within
230 the laboratory, four teeth were subjected to DNA extrac-
231 tion, amplification, cloning and sequencing in Barcelona. In
232 this lab the following primer pairs were used: L16,022/233
234 H16,095; L16,055/H16,218; L16,209/H16,401.

234 Amplification of associated animal remains
235
235 We tried to amplify the DNA extracted from cattle bones
236 using both human-specific and bovine-specific primers,
237 namely primers for a 152-bp fragment of the Bos taurus
238 mtDNA D-loop, and for a fragment of the human D-Loop
240 The PCRs were performed with 2 µl of DNA, 1 µM of each
241 primer, 200 µM of each dNTP, 1× reaction buffer (Applied
242 Biosystem), 1.5 mM MgCl2, and 2 U of AmpliTaq Gold
243 (Applied Biosystem) in a total volume of 50 µl. The cattle
244 D-Loop was amplified using the following thermal cycle:
245 initial denaturation at 94°C for 10 min, followed by 40
246 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min,
247 and final extension at 72°C for 5 min. The conditions
248 described above for human mitochondrial DNA amplifica-
249 tion were used in the amplification of bovine DNA with
250 human-specific primer. After a run on a 1.5% agarose gel,
251 bands of the appropriate size were excised from the gel
252 and purified with Ultra Free DNA (Amicon, Beverly, Ma).
253 Cloning and sequencing of the PCR products were as
254 described above.

255 Detection of long amplificates
256 Appropriate molecular behaviour was also tested by ampli-
257 fication of longer mtDNA fragments (443 and 724 bp),
258 which have been reported to be very unusual for ancient
259 DNA. PCR conditions were those described for mtDNA
260 analysis above, the primers used were L15,995 and
261 H16,401 (for the 443 bp fragment), and L16,247 and
262 H00,360 (for the 724 bp fragment).

263 Data analysis
264 We estimated three measures of intrapopulation diversity in
265 the Nuragic sample. Nucleotide diversity (π) is the average
266 number of nucleotide differences per site (Nei and Kumar
267 2000), over the 360 sites screened [from position 16,024 to
268 16,383 of the Cambridge reference sequence (CRS)]. The
269 haplotype diversity (h, often referred to as heterozygosity)
270 is defined as $h = 1 - \sum q_i^2$, where $q_i$ is the frequency
271 of each haplotype and summation is over all haplotypes
272 observed (Nei and Kumar 2000). The average pair wise
273 sequence difference or average mismatch (k) is the average
274 difference between sequences in the sample.

275 Phylogenetic trees, i.e. reduced median networks
276 (Bandelt et al. 1995), summarising mtDNA variation were
estimated by means of Network 4.1, using a reduction
threshold = 2 and the same weights for all loci. To put
Nuragic DNA diversity in the proper evolutionary context
we used a database of HVR-I sequences from 60 popula-
tions, namely 57 modern populations from Europe, Central
Asia and the Southern Mediterranean shores, plus the two
pre-classic European populations typed so far at the DNA
level, the Etruscans (Vernesi et al. 2004) and Iberians
(Sampietro et al. 2005), and a medieval sample from Spain
(Casas et al. 2006). The modern database includes a sample
generically labelled as Sardinia (Di Rienzo and Wilson
1991), and two samples from two regions of Sardinia,
namely Ogliastro in the Southeast (Fraumene et al. 2003)
and Gallura in the Northeast (Morelli et al. 2000). Com-
plete references are available at this URL: http://web.
unife.it/progetti/genetica/pdata.htm.

Genetic distances between populations, $F_{ST}$ distances,
were calculated by the Arlequin ver 3.01 software
(Excoffier et al. 2005), based on Kimura’s two-parameter
model and using $\theta = 0.26$. A two-dimensional repre-
sentation of population relationships was obtained by multi-
dimensional scaling (MDS), using the software Statistica
5.5 (Statsoft Inc.).

### Results

For 10 of the 53 samples the D enantiomer of Asp repre-
sented more than 10% of the total amino acid (Table 2),
implying that there was little hope to retrieve well-pres-
served DNA molecules from those samples (Poinar et al.
1996; Serre et al. 2004). Therefore, we extracted the DNA
from the remaining 43 samples. Sporadic contamination is
considered unlikely when the number of molecules that
PCR will use as template (target DNA) is greater than 1,000
(Poinar et al. 1996); in 7 samples quantitative PCR showed
that the DNA available was less than that, whereas 36 sam-
ples yielded sufficient DNA for amplification, cloning

<table>
<thead>
<tr>
<th>Initial sample size (individuals)</th>
<th>Test</th>
<th>Eliminated</th>
<th>Kept</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>AA racemisation</td>
<td>10</td>
<td>43</td>
</tr>
<tr>
<td>43</td>
<td>Quantitative PCR</td>
<td>7</td>
<td>36</td>
</tr>
<tr>
<td>36</td>
<td>Cloning, sequencing</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>30</td>
<td>Sequence comparisons</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>23</td>
<td>Replication in a second lab</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>23</td>
<td>Amplification of non-human</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>

Table 2 Elimination of the specimens that did not comply with quality standards

* This test was carried out for four specimens only

### Table 3 DNA sequences of 23 Nuragic specimens

<table>
<thead>
<tr>
<th>CRS</th>
<th>ACTGGTCCCTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>(AL07)</td>
<td>. . . . . . . . (Alghero)</td>
</tr>
<tr>
<td>(CA02)</td>
<td>. . . . . . . . (Carbonia)</td>
</tr>
<tr>
<td>(CR14)</td>
<td>. . . . . . . . (Carbonia)</td>
</tr>
<tr>
<td>(FL04)</td>
<td>. . . . . . . . (Fluminimaggiore)</td>
</tr>
<tr>
<td>(PE11)</td>
<td>. . . . . . . . (Perdasdefogu)</td>
</tr>
<tr>
<td>(PE15)</td>
<td>. . . . . . . . (Perdasdefogu)</td>
</tr>
<tr>
<td>(PE20)</td>
<td>. . . . . . . . (Perdasdefogu)</td>
</tr>
<tr>
<td>(PE23)</td>
<td>. . . . . . . . (Perdasdefogu)</td>
</tr>
<tr>
<td>(PE25)</td>
<td>. . . . . . . . (Perdasdefogu)</td>
</tr>
<tr>
<td>(SE01)</td>
<td>. . . . . . . . (Seulo)</td>
</tr>
<tr>
<td>(SE02)</td>
<td>. . . . . . . . (Seulo)</td>
</tr>
<tr>
<td>(SE13)</td>
<td>. . . . . . . . (Seulo)</td>
</tr>
<tr>
<td>(SE60)</td>
<td>. . . . . . . . (Seulo)</td>
</tr>
<tr>
<td>(SE81)</td>
<td>. . . . . . . . (Seulo)</td>
</tr>
<tr>
<td>(SE84)</td>
<td>. . . . . . . . (Seulo)</td>
</tr>
<tr>
<td>(ST08)</td>
<td>. . . . . . . . (S. Teresa di Gallura)</td>
</tr>
<tr>
<td>(ST10)</td>
<td>. . . . . . . . (S. Teresa di Gallura)</td>
</tr>
<tr>
<td>(ST15)</td>
<td>. . . . . . . . (S. Teresa di Gallura)</td>
</tr>
<tr>
<td>(ST16)</td>
<td>. . . . . . . . (S. Teresa di Gallura)</td>
</tr>
<tr>
<td>(ST30)</td>
<td>. . . . . . . . (S. Teresa di Gallura)</td>
</tr>
<tr>
<td>(ST38)</td>
<td>. . . . . . . . (S. Teresa di Gallura)</td>
</tr>
<tr>
<td>(ST47)</td>
<td>. . . . . . . . (S. Teresa di Gallura)</td>
</tr>
<tr>
<td>(ST54)</td>
<td>. . . . . . . . (S. Teresa di Gallura)</td>
</tr>
</tbody>
</table>

CRS Cambridge reference sequence
was successful only using primers specific for *Bos taurus*, and not human primers.

Ten sites appeared polymorphic in the Nuragic samples, with nine transitions and one transversion, leading to identification of ten distinct haplotypes. Nucleotide diversity was 0.0041, and haplotype diversity a low 0.83, close to the value observed in Ogliastra, 0.78. By contrast, haplotype diversity values of 0.96 (Di Rienzo and Wilson 1991) and 0.97 (Morelli et al. 2000) were observed in the other modern Sardinian samples and ≥0.93 in all other European samples with the exception of Saami. The low haplotype diversity among the Nuragic people is due to the occurrence at relatively high frequencies of five haplotypes, including the CRS (9 individuals, 39% of the total). The other five haplotypes, AL07, PE15, SE02, SE60 and ST16, are observed once in the Nuragic sample, and so, despite the small sample sizes, different Nuragic populations share one or more haplotypes (Table 4). The average difference between pairs of sequences, or average mismatch, was a low 1.43 ± 0.90 compared to the Etruscans (3.90; Vernesi et al. 2003) and the modern samples (average 4.68), but close to the Iberians’ 2.12 (Sampietro et al. 2005) and to the modern Ogliastra 2.63 (Fraumene et al. 2003).

The phylogenetic relationships of the Nuragic sequences were summarised in a network comparing them with the modern Sardinian samples and other European, African, Caucasian, Near Eastern and North African populations, including Tuscany and the Ladin linguistic isolate of Northern Italy. Two modern samples from Sardinia (Di Rienzo and Wilson 1991) and Ogliastra (Fraumene et al. 2003) share four haplotypes each with the Nuragic sample, and, in particular, the Ogliastra sample shares three haplotypes with the ancient samples from the same area (Seulo and Perdasdefogu). The CRS, which represents 39.1% of the ancient Sardinians, represents 61% of the ancient individuals. Four and two haplotypes, representing respectively 52 and 48% of the total Nuragic sequences, is the level of sharing between the Nuragic people and both Etruscans and ancient Iberians. The minimum allele sharing was one for the Sardinian sample from Gallura (and for a few European, Caucasian, Near Eastern and North African populations, including Tuscany and the Ladin linguistic isolate of Northern Italy). Two modern samples from Sardinia (Di Rienzo and Wilson 1991) and Ogliastra (Fraumene et al. 2003) share four haplotypes each with the Nuragic sample, and, in particular, the Ogliastra sample shares three haplotypes with the ancient samples from the same area (Seulo and Perdasdefogu). The CRS, which represents 39.1% of the

Table 4 Number of haplotypes shared between the population of Nuragic Sardinia and other regions of Eurasia (relative frequencies between parentheses)

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>North Africa</th>
<th>Near East</th>
<th>Caucasus</th>
<th>Central Asia</th>
<th>Europe</th>
<th>Ancient Iberians</th>
<th>Etruscans</th>
<th>Sardinia</th>
<th>Ogliastra</th>
<th>Gallura</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA02, PE11, PE20, PE23, SE81, SE84, ST10, ST38, ST47</td>
<td>39.1</td>
<td>44 (9.6)</td>
<td>18 (6.9)</td>
<td>56 (14.1)</td>
<td>11 (5.4)</td>
<td>451 (13.3)</td>
<td>3 (17.6)</td>
<td>2 (7.4)</td>
<td>15 (20.5)</td>
<td>78 (44.5)</td>
<td>5 (18.5)</td>
</tr>
<tr>
<td>ST16</td>
<td>4.4</td>
<td>11 (2.4)</td>
<td>4 (1.0)</td>
<td>2 (1.0)</td>
<td>89 (2.6)</td>
<td>1 (3.7)</td>
<td>3 (4.1)</td>
<td>12 (7.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL07</td>
<td>4.4</td>
<td>2 (0.4)</td>
<td>3 (0.8)</td>
<td>2 (1.0)</td>
<td>46 (1.4)</td>
<td>1 (1.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE02</td>
<td>4.4</td>
<td>1 (0.4)</td>
<td>1 (0.3)</td>
<td>5 (0.1)</td>
<td>2 (1.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE60</td>
<td>4.4</td>
<td>1 (0.4)</td>
<td>11 (0.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE25, ST08</td>
<td>8.7</td>
<td>1 (0.4)</td>
<td>1 (0.3)</td>
<td>1 (0.0)</td>
<td>1 (3.7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE01, ST30</td>
<td>8.7</td>
<td>1 (0.4)</td>
<td>1 (0.3)</td>
<td>22 (0.6)</td>
<td>2 (11.8)</td>
<td>2 (7.4)</td>
<td>3 (1.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA14, SE13</td>
<td>8.7</td>
<td>7 (1.5)</td>
<td>1 (0.3)</td>
<td>1 (0.0)</td>
<td>1 (1.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE15</td>
<td>4.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL04, ST15, ST54</td>
<td>13.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The three modern Sardinian samples are from Di Rienzo and Wilson (1991) (Sardinia, SR in the legend to Fig. 3), Fraumene et al. (2003) (Ogliastra, SO), and Morelli et al. (2000) (Gallura, GR).
Nuragic sequences and only 13.3% all over Europe, reaches similarly high frequencies only in the modern Sardinians (globally 35.5%), and especially in Ogliastra. An analysis of molecular variance (Excoffier et al. 2005) showed no difference among the Perdasdefogu (n = 5), Seulo (n = 6) and Santa Teresa di Gallura (n = 8) samples, with a negative estimate of the among-population variance component (−0.60%). Among the Etruscans, the differences among populations, albeit insignificant, accounted for 1.11% of the overall variance.

The multidimensional scaling graph (Fig. 3) summarises the genetic relationships inferred from $F_{st}$ distances between populations. Saami were eliminated from this graph, because their high level of differentiation drastically reduces the apparent differences among all other populations. A mild stress value was observed in the analysis, 0.19, meaning that the projection of the data on a plane distorted population relationships only to a limited extent. In this global representation, Nuragic Sardinia falls in a large European cluster, where little or no geographical structure is apparent. All outliers are either populations separated by large geographic distances from the other Europeans (mainly North Africans and Central Asians), or well-known genetic outliers, such as the modern population of Ogliastra (Fraumene et al. 2003) and the ancient, non-Indo-European speaking Etruscans.

**Discussion**

The first archaeological evidence for the Nuragic civilisation dates back to the Middle Bronze Age, 3,800–3,300 years ago. Populations previously scattered in small units integrated in larger communities, still having only sporadic contacts with non-Sardinian people (Webster 1996). In the Late Bronze Age pre-existing nuraghi were enlarged and metallurgy intensified, leading to increased trade across the Mediterranean Sea. Population growth and warfare can be inferred from the archaeological record of that period, the former being a possible cause of the latter.

The Late Bronze Age Sardinians whose mtDNA we could type show a remarkably low level of genetic differentiation. Despite sampling covering an area of approximately 24,000 km², and a 700-year time interval (up to the early Iron Age, 2,700 years BP), the same sequences were repeatedly found at different locations, and all belong to...
only 3 or 4 of the 20 haplogroups documented in Western Mediterranean populations (Sampietro et al. 2005). Only another ancient population, the sixth to second century BC Iberians (Sampietro et al. 2005), showed a comparable mitochondrial diversity, much lower than in all modern samples and in the Etruscans.

Genetic variation in Sardinia appears limited across time as well. Six haplotypes observed in the Nuragic sample are still present in the modern Ogliastro and Sardinia samples, and they include not only the widespread CRS (whose frequency is higher in moderns as well as in ancient Sardinians compared to other samples), but also rarer sequences such as those that we labelled AL07, SE02, ST16, SE01-ST30, and CA14-SE13. Three such sequences, AL07, ST16, and CA14-SE13 also occur in North Africa; their presence in both modern and Nuragic Sardinia suggest the effects of common ancestry or ancient gene flow, rather than those of gene flow in historical times. In particular, modern people from Ogliastro share three haplotypes with Nuragic people from the nearby localities of Perdasdefogu and Seulo, i.e. half of both the modern (83/175) and the ancient (12/23) sequences (Table 4). On the contrary, the modern population of Gallura appears genetically distant from both ancient and modern Sardinian samples, in agreement with the effects of relatively recent immigration from continental Italy, known to have affected Gallura more than Central and Southern Sardinia (Morelli et al. 2000).

The other pre-Roman Italic population described so far at the genetic level, the Etruscans (Vernesi et al. 2004), showed very different patterns of mtDNA diversity. The Etruscans appear more closely related to modern Tuscans than to any other European or Near Eastern population, but only 2 of 23 different Etruscan sequences were found to have an exact match in the modern Tuscan populations, including some that were selected for their supposed Etruscan origins (Achilli et al. 2007). The difference with Nuragic Sardinia (where shared sequences are seven out of ten) is highly significant ($\chi^2 = 13.21$, 1 d.o.f., $P < 0.001$). Plausible explanations for the low resemblance between Etruscans and modern Tuscans include loss of haplotypes by genetic drift and/or extensive immigration, possibly but not necessarily related to the social changes that occurred after the Roman assimilation (Belle et al. 2006), or in modern times. By contrast, the results of the present study show a much clearer genealogical continuity in Sardinia, presumably reflecting the island’s higher degree of isolation.

Despite this clear genealogical continuity, there are differences between ancient and modern Sardinians, notably in their levels of internal differentiation, much higher in the latter, and very high at the allele-frequency level (Cappello et al. 2005).
et al. 1996). A previous genetic analysis excluded pre-
Bronze-Age expansions similar to those that occurred in
continental Italy and other European regions (Barbujani
et al. 1995). Although mitochondrial and autosomal varia-
tion are not necessarily correlated, this finding suggests that
Sardinian populations differentiated recently, probably
because of local immigration and population subdivision.
Consistent with a recent differentiation is the fact that zones
of increased genetic change in Sardinia often correspond to
linguistic boundaries, which cannot be more than
2,000 years old (Barbujani and Sokal 1991). The simplest
way to reconcile these findings with the archaeological evi-
dence of demographic growth in the Late Bronze Age is to
envisage the expansion of a small group of geographically
dispersed but maternally related individuals. The succes-
sive subdivision in geographically and linguistically differ-
entiated isolates enhanced the evolutionary effects of
geographic drift.

In the multidimensional scaling of Fig. 3, Nuragic
Sardinians cluster with the majority of the European popu-
lations. Given the small sample size, inevitable in ancient
DNA studies, it is at present impossible to infer their evolu-
tionary relationships from mtDNA affinities. Neverthe-
less, in relation with ancient samples, Nuragic Sardinians
appear more related to the Iberians than to the Etruscans,
whose position in the graph is eccentric. Three data points
are not enough for a robust generalisation. However, one
can at least conclude that Sardinians and Iberians show a
greater genealogical continuity with the Bronze-Age
inhabitants of the same regions than the Tuscans. To better
understand the processes leading to these differences it
will be necessary to genetically characterise people who
lived in those areas between 2,000 years ago and the pres-
tent time. At present, a study of paternal genealogies in
Holland shows that gene flow in the last 3 centuries
resulted in a massive population displacement (Manni
et al. 2005). We do not know to what extent this finding
can be generalised to other European populations and to
maternal genealogies, although female migration is known
to be higher than male migration in most human societies
(Sieielstad et al. 1998;Dupanloup et al. 2003). However,
the Dutch data raise the possibility that mildly isolated
populations, such as those of Holland and Tuscany, have
recently experienced drastic demographic changes, a pro-
cess that has affected only marginally more isolated
regions, such as Sardinia.

GENBANK accession numbers for the ancient Sardinian
sequences are: This paper was supported by funds from the
Italian Ministry of the Universities (PRIN 2006) and by
funds from the Universities of Ferrara and Florence. We
thank Antonio Torroni for a thorough preliminary discus-
sion of the results of this study, and three anonymous refer-
rees for several useful suggestions.

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