idation of ancient DNA sequences have been followed" (Vernesi et al. 2004 [p. 703]) is not quite correct, since one of the most important criteria of Cooper and Poinar (2000)—that is, that of independent replication in another lab—has not been followed for 25 of 28 of the reported HVS-I sequences or for any of the RFLP tests. Moreover, the 20 excluded sequences were not displayed. The claim that the "Etruscan" sequences "all belong to lineages that are still present in Europe" (Vernesi et al. 2004 [p. 702]) is not justified, in view of the unusual mutational pattern, especially as the basal haplogroup status (U, JT, pre-HV, N1, W, X, or other) was not determined in half of the data set. Under these circumstances, it is unclear to what extent the "Etruscan" data represent severely damaged or partly contaminated mtDNA sequences; therefore, any comparison with modern population data must be considered quite hazardous.

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## On the Etruscan Mitochondrial DNA Contribution to Modern Humans

*To the Editor*:

The growing number of ancient human mtDNA samples sequenced in recent years has given rise to the problem of correspondence between distributions of mutations in ancient and modern mtDNA sequences. It has been suggested that mtDNA nucleotide sequences obtained from human remains may include some artifacts, for multiple reasons, such as contamination with modern DNA; artifacts induced by cytosine deamination during multiple amplification of ancient DNA via PCR; and postmortem damage in DNA, occurring as hydrolytic deamination and depurination, double-strand breaks, and oxidative nucleotide modification (Hofreiter et al. 2001*a*). Therefore, to determine the nature of the DNA sequences amplified, each amplified product should be cloned, and the obtained clones should be sequenced (Pääbo 1989; Handt et al. 1996). The consensus sequence from each sample is determined from the sequences shared between all clones, and intraclone nucleotide differences represent the postmortem data set (Gilbert et al. 2003). Therefore, cloned sequences of ancient DNA samples may show a pattern of a shared consensus (haplotype), with many singleton substitutions corresponding to postmortem DNA changes. It has been suggested that the consensus sequence should be part of the original sequence (Hofreiter et al. 2001*b*).

In this study, we reanalyzed nucleotide sequences of the mtDNA HVS-I region in 575 clones derived from bone samples of 28 Etruscans (7th–3rd centuries B.C.),

recently published by Vernesi et al. (2004). To determine whether the Etruscan samples indeed represent mtDNA sequences similar to modern human ones, we compared mutational spectra derived from ancient mtDNAs and from mtDNA sequences characteristic of present-day Europeans, Asians, and Africans. Figure 1 shows the distribution of mutations found in both mtDNA data sets. For modern human populations, we analyzed the distribution of mutations found in ~8,000 HVS-I sequences belonging to 90 mtDNA haplogroups (Malyarchuk and Rogozin 2004). The number of parallel mutations that have independently arisen at certain nucleotide positions in different mtDNA haplogroups (monophyletic clusters) was used as a measure of DNA variability in modern populations (for details, see Malyarchuk et al. 2002). In the case of ancient mtDNA data, we assumed that mutations found in cloned sequences of each sample may be considered as having independently arisen in different DNA templates—that is, we do not suggest that cloned sequences are the products of a single template. Comparative analysis of data sets shows that, among 261 variable nucleotide positions seen in both spectra, 222 nucleotide positions are variable in the mutational spectrum of modern humans, 147 positions are variable in the Etruscan spectrum, and 108 positions appear to be shared between the two spectra. The frequency of variable positions in the Etruscan spectrum is 147/342 = 0.42 (the sequence length is 342 bases). The frequency of variable positions in the mutational spectrum of modern humans is 222/342 = 0.64. If we assume a random distribution of variable positions along the HVS-I sequence, the expected frequency of shared variable positions is 0.64 × 0.42 = 0.28. The observed frequency of shared variable positions is 0.31; this value is not significantly different from the randomly expected 0.28 ($\chi^2 = 2.3$, $P(\chi^2) = .13$).

This result suggested that the mutational spectrum of the Etruscan mtDNA is characterized by a large fraction of the Etruscan-specific mutations; moreover, the fraction of shared variable positions is not different from the random expectation. According to the database of HVS-I sequences combined with mtDNA coding region markers that was used for comparison (fig. 1), 26.5% (39 of 147) of variable positions observed in the cloned ancient mtDNA sequences contain unique mutations—that is, mutations that have not been found in modern humans. A similar value (27.7%) was found by comparison of the HVS-I region variation data in modern humans and the Cro-Magnon–type individuals, with a sample date of 23,000–25,000 years ago (Caramelli et al. 2003). In 165 cloned sequences of these two ancient individuals, 13 of 47 variable positions were found to be unique. Among them, only five variable positions (16057, 16059, 16112, 16139, and 16158) were shared by the Etruscan and Cro-Magnon HVS-I data sets. Meanwhile, other ancient and modern data sets on HVS-I variability show no or small deviation from the mutational spectrum of modern humans used in this study. For example, no differences were found between this spectrum and the HVS-I sequences of mtDNAs extracted from the skeletal remains of 44 specimens of the Xiongnu tribe (from the Egyin Gol necropolis, northern Mongolia, 3rd century B.C. to 2nd century A.D.) (Keyser-Tracqui et al. 2003), and small differences (<3%) were found in comparison with HVS-I sequence variation in modern populations of the Roma (1 of 64 variable positions) (Gresham et al. 2001), the Egyptians (2 of 71 variable positions) (Stevanovitch et al. 2004), and the Italians from Bologna (1 of 58 variable positions) (Bini et al. 2003).

Although the screening of the Mitomap database for polymorphic nucleotide positions in the HVS-I region has shown that 11 of 39 Etruscan-specific positions were previously found as variable in different individuals, the frequency of the variable positions specific to the mutational spectrum of the cloned sequences of the Etruscans (positions 16044, 16045, 16056, 16057, 16060, 16072, 16073, 16083, 16091, 16098, 16100, 16101, 16112, 16118, 16123, 16130, 16139, 16151, 16158, 16159, 16237, 16282, 16306, 16315, 16334, 16339, 16345, and 16348) remains very high (19%). It is likely that these nucleotide positions represent a mutational spectrum of the mtDNA molecules altered by postmortem damage. Comparison of these positions with the list of nucleotide positions suggested as sites with postmortem damage in the study of ancient DNA from northwestern European samples (Gilbert et al. 2003) shows that only position 16072 is shared between the two data sets. In addition, positions 16131, 16144, and 16325, which have an increased mutation rate of postmortem damage, according to Gilbert et al. (2003), were found as singleton mutations in the Etruscan cloned sequences.

It is important that some of the Etruscan mutations, which are rare or absent in modern humans, were found in multiple clones of ancient individuals and therefore were assigned by Vernesi et al. (2004) to the consensus haplotypes, suggesting that these mutations should represent the original mtDNA sequences of the Etruscans. The most noticeable position is 16334, which was found in 15 cloned Etruscan sequences belonging to two different haplotypes (3V and 22T). However, this position is invariable in almost 8,000 of the HVS-I sequences of modern humans. Nucleotide positions 16228 and 16229 are also among the most conservative positions in modern human data sets, but mutations at these positions were found frequently in the Etruscan nucleotide sequences—mutation C16228T was present as a consensus variant in haplotype 21T and as a singleton mutation in two cloned sequences belonging to another specimens, and mutation T16229C was observed as a consensus

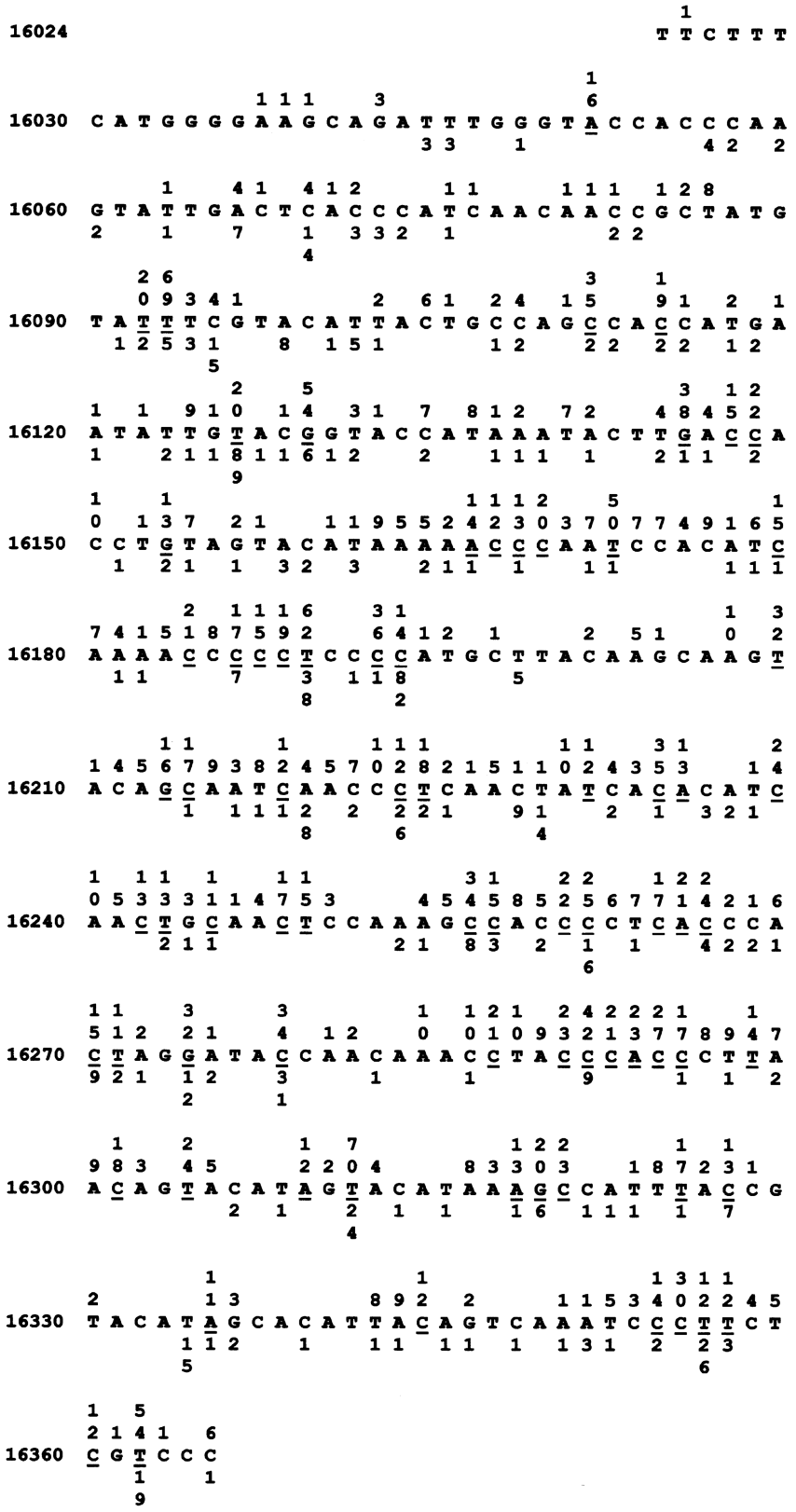

**Figure 1** Comparison of HVS-I mutational spectra (between nucleotide positions 16024 and 16365) in ancient (Etruscan) and modern humans. Mutations are shown relative to the Cambridge reference sequence (Anderson et al. 1981). Numbers above the sequence are numbers of parallel mutations (both transitions and transversions) observed in mtDNA haplogroups from modern human populations; predicted mutational hotspots are underlined (for details, see Malyarchuk et al. [2002] and Malyarchuk and Rogozin [2004]). Numbers under the sequence are numbers of mutations found in cloned Etruscan HVS-I sequences.

variant in haplotypes 20T and 21T and as a singleton mutation in another sample. Haplotype 21T is represented by a combination of variants 16228T and 16229C, which has not been found in modern human HVS-I sequences.

Therefore, the mutational spectrum derived from the Etruscan mtDNA sequences shows some degree of similarity to modern human mtDNA sequences. However, many of the singleton mutations, as well as some consensus mutations found in the cloned sequences, represent substitutions that are very rare in living individuals or do not even exist. The possibility that these haplotypes underwent extinction (Vernesi et al. 2004) cannot be excluded. However, many of these mutations might be due to postmortem damage of mtDNA. The assignment of postmortem mutations in consensus variants of the haplotypes can lead to misidentification of mtDNA sequences. In addition, some phylogenetically informative nucleotide positions are highly susceptible to postmortem damage (Gilbert et al. 2003). These problems may lead to misassignment of mtDNA sequences to haplogroups and, consequently, to biased opinions about genetic history of human populations.

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## Electronic-Database Information

The URL for data presented herein is as follows:

Mitomap: A Human Mitochondrial Genome Database, http://www.mitomap.org/

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## Etruscan Artifacts: Much Ado about Nothing

*To the Editor:*

Malyarchuk and Rogozin (2004 [in this issue]) and Bandelt (2004 [in this issue]) question the authenticity of