This study highlights one of the most significant and recurring problems in ancient DNA research: that of authentication of results. While many labs are now careful to use strict experimental controls (as outlined in Cooper and Poinar[4]), the recommendation of phylogenetic verification of results continues to be ignored, despite the relative simplicity and lack of expense of such tests, particularly in comparison to experimental procedures. It should be noted that many of the most embarrassing mistakes in the ancient DNA literature (including the incorrect report of dinosaur DNA in the early 1990s[5]) could have been avoided with a simple phylogenetic test.

Conflict of interest: No conflict of interest to declare.

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Insufficient phylogenetic analysis may not exclude candidacy of typhoid fever as a probable cause of the Plague of Athens (reply to Shapiro et al.)

The cause of the Plague of Athens (430—426 BC) has been debated among scientists, who have relied exclusively on Thucydides' historical narrations[1] to introduce several possible diagnoses.[2,3] The application of DNA analysis on skeletal remains taken from the Kerameikos mass grave[4] has been acknowledged as an ideal material that might provide clues for a definite evidence-based diagnosis of the epidemic.[5] Following a research methodology of proven accuracy and validity ('suicide' PCR),[6] it was shown by analysis of three genes (*osmC*, *clyA*, *narG*) that an ancient strain of *Salmonella enterica* serovar Typhi was present in the investigated dental pulp material of three putative victims of the plague, thus incriminating typhoid fever as a probable cause.[7]

Despite this evidence-documented approach, Shapiro et al.[8] have argued against the validity of these results. Through the application of a simple phylogenetic analysis of the published sequence of one gene (*narG*), Shapiro et al. concluded that, although the sequenced ancient DNA quite possibly corresponds to a *Salmonella* strain, this might not be typhoid. Instead, they argue that this sequence is more closely matched to other *Salmonella* species such as *S. bongori*, *S. arizonae*, and *S. diarizonae* rather than *S. enterica* Typhi. The authors base this assumption on an inferred evolutionary timescale of *Salmonella* and other related bacterial species. Shapiro et al. eventually make the assumption that the identified sequence most probably represents a modern and currently unknown free-living soil bacterium instead of an ancient one.

Against these arguments we must once again state the extreme preventative measures that were taken in our study[7] to exclude any possibility of environmental contamination. These included the absence of the pathogens themselves or a previously attempted extraction or PCR amplification of the target DNA sequences in the implemented laboratories, and also the 'suicide' PCR methodology that was followed, which excluded positive controls from this study. In addition, since possible environmental contamination is a major problem with ancient DNA studies as Shapiro et al. suggest, soil wash was actually used as a negative control in addition to DNA extracts from modern teeth. As is clearly stated in the original publication of our results, no product was yielded following the application of the same primers under the same laboratory conditions on the negative controls as well as on the soil sample washed off the ancient teeth,[7] thus excluding the possibility of any contamination of the investigated ancient material. Besides, *Salmonella* species do not survive for long in soil, which is typically regarded as a transitional environment for this pathogen prior to its infecting a host.[9] Even if the soil of the mass grave was indeed contaminated by a modern *Salmonella* strain, such as a close relative of *S. bongori*, *S. arizonae*, and *S. diarizonae* (which are naturally found in reptiles), as suggested by Shapiro et al.,[8] it would not be possible for any of these pathogens to survive during the long storage of the skeletal material and not be identified in the subsequently conducted investigation of the soil wash.

On the other hand, the application of phylogenetic models, as suggested by Shapiro et al.,[8] undoubtedly constitutes a powerful tool for the introduction of theoretical assump-

tions and hypotheses where clear-cut evidence is not available. Nevertheless, it has been supported that there is no 'golden rule' to fit all cases of phylogenetic analysis, whereas a minimum of 6–10 comparable gene sequences are needed to permit a complete phylogenetic study.[10] In contrast to these guidelines, the use of only one gene sequence for the application of phylogenetic analysis in bacteria, in which lateral gene transfer occurs widely between species,[11] might yield misleading results. Such seems to be the case for Shapiro et al.'s approach[8] that applied their proposed model on only one gene sequence. Besides, while there have been some reports of molecular data, which are useful for construction of accepted phylogenetic trees of *Salmonella* species,[12,13] other reports yield conflicting results. For example, the evolutionary relationships of *Salmonella* species based on synonymous site variation in the *fimA*, *fimI*, and *fimZ* genes, which belong in the same operon, result in diverse phylogenetic trees, possibly because of variable horizontal transmission of all or part of the same gene cluster.[14] Another phylogenetic study of three genes placed most studied *Salmonella* species in monophyletic lineages, but one serotype was placed into two statistically well supported separate lineages which contained either bovine only or avian only isolates.[15]

In brief we doubt Shapiro et al.'s assumptions since:

- Environmental contamination of the investigated ancient material or its surrounding soil was specifically excluded by the precise methodology that was followed in our research study.
- A phylogenetic analysis of only one gene might be insufficient and/or misleading.
- The speculative results of the conducted phylogenetic analysis (''a modern and currently unknown free-living soil *Salmonella* species'') cannot be more valid than the known clear evidence – *Salmonella* species do not survive for long in soil; dental pulp DNA sequences for two other genes have been detected, which not only are closest to *S. enterica* serovar Typhi, but one of these genes is lacking in its closest known relative, *S. typhimurium*.[7]

We agree with Shapiro et al.[8] that there is not yet definite proof that the cause of the Plague of Athens was typhoid fever. The concurrent presence of a plurality of infectious diseases in besieged Athens of 430–426 BC was not excluded in the first place[7] allowing for the variable clinical manifestation of the epidemic as reported by Thucydides.[3]

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