A Simple and Robust Method for Sexing Ancient Bovine Bones

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Abstract: We set up a simple method for sexing ancient bovine bones, generally attributed to a specific sex by morphological methods. ZFX/ZFY genes were chosen among the sex-specific sequences available in Genbank. The method, validated on 22 individuals of 11 cattle breeds, was used to analyze 3 roman and 9 medieval cattle bones. In no cases we have found a false attribution of sex. On the 12 ancient remains we always had a consistent outcome. Only in the case of PCR failure we couldn't attribute the sample to the gender, but in no cases we had inconsistent results since in our method the sex is determined by the amplification of both sequences in X and Y (if present) chromosomes.

A short PCR product of 132bp in both chromosomes was amplified and digested with specific enzymes to discriminate X and Y amplicons. Digestion gives fragments of different length according to the sex chromosome.

This method amplifies simultaneously both sex chromosomes, therefore presents the advantage of using less aDNA for the analysis and reduces the possibility of wrong assignments.

This approach combines the advantage of being accurate and successful while it only relies on widely available and cheap equipment. The system is strong enough to be used with aDNA (ancient DNA) and it could be easily extended to other mammals.

Keywords: Ancient DNA, Sexing, ZFX/ZFY, Cattle.

1. INTRODUCTION

The determination of sex of ancient livestock remains is important to reconstruct the demography of ancient populations and possibly their use, giving important insights about post Neolithic civilisations. Only a few of the recovered bones may be attributed to a specific sex by morphological methods [1]. Usually bones are measured and compared to reference collections and patterns but it is possible only for some kind of these, like bovine metacarpals. Generally, bigger animals are males. However, wrong assignments are likely to occur in the case of change in animal size during time, absence of sexual dimorphism, presence of different classes of age, or simultaneous presence of more populations/breeds of different size. Some DNA-based techniques for sexing animals starting from tiny amounts of fresh tissues by polymerase chain reaction (PCR) are described in the literature, but often they do not take into account the level of damage of ancient DNA (aDNA) and the high risk of false positive or negative encountered in the aDNA field. In fact, too long fragments cannot be amplified starting from degraded aDNA [2-4]. Moreover, most of the methods rely on the amplification of a fragment from a single sex chromosome [5-7]. The goal of our work was developing an easy and reliable method suitable for sexing ancient remains, where DNA is typically scarce and fragmented. We developed suitable primers for analysing ancient bovine bones by using *ZFX/ZFY* sequences [8], and here we present the method tested both on fossils of different ages and on various modern cattle breeds. We propose a protocol that is based on *ZFX/ZFY* sequences analysed with a widely available and cheap equipment and produces reliable results incorporating the quality check of an amplification on both sex chromosomes in males.

2. MATERIALS AND METHODS

All fossil bones were excavated in Ferento, located near Viterbo, Central Italy, a site inhabited since Bronze Age that developed mainly from roman age to medieval age.

Stringent standards for the authentication of ancient DNA were applied [9, 10]:

(i) for all samples aDNA was extracted in a dedicated laboratory room and at least two independent DNA extractions were performed; one single sample a day was extracted with a relative extraction control;

(ii) all aDNA amplifications were performed in two separated laboratory; modern DNA was manipulated in a third one. We checked that both extraction and PCR controls produced negative results in each amplification;

(iii) amplification of long DNA fragments, unusual in ancient DNA analyses, was not observed;

(iv) at least two independent amplifications of the same fragment in each sample were performed to validate results.

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1874-4028/12

We set up procedures to avoid samples contamination during excavation and degradation before storage, due to the sudden change in environmental conditions. Particularly, the samples were picked up using latex gloves, put immediately in vacuum-pack and stored at -20°C in order to preserve aDNA [11].

We collected 13 cattle remains, three roman (~2100BP) and ten medieval (~1000BP) bones, excavated in different layers of Ferento site. The dating was made on the basis of pottery found in the same layer. Moreover, two random samples (one roman and one medieval) were carbon-dated using high resolution mass spectrometry. The results confirmed the age estimated by the layers.

Bone powder was collected by drilling, after removing of the outer surface with sand paper. In order to reduce contamination from the external surface and to preserve the bone for future morphological studies, we perforated the bone and dug internally. To prevent cross-contamination, only a sample per day was processed. DNA was extracted according to Yang *et al*., [12] in a dedicated laboratory under flow-hood, using 500mg of powder per bone and including a negative control in each extraction. Samples were extracted at least twice to validate the results.

The extracted DNA was quantified using a DTX Multimode Detector 880 (Beckman) with Picogreen method (Quant-iT, Invitrogen) according to manufacturer instructions. An average of 5 ng per g of powdered bone, was recovered. Also extraction blanks were checked with picogreen, to be sure that no contamination occurred.

Primers were designed to include a portion of each of *ZFX/ZFY* sequences (NM_177490; NM_177491) that is variable across X and Y chromosomes, obtaining a PCR product of 132 bp, a size suitable to be amplified in aDNA. Primer sequences are AGGTTTTCGTCACCCATC (FW) and AGACGCATGTGAAAACTAAGCA (RW) for ZFY, AGGTTTTCGTCATCCATC (FW) and AAACGCATGTA AAAACTAAGCA (RW) for ZFX.

All ancient samples were amplified in a dedicated laboratory. We amplified the portion of the *ZFX/ZFY* gene using 10ng DNA, 0.5 pM of each primer (Sigma), 1x PCR buffer containing 1.5mM $MgCl_2$, *dNTPs* 0.2mM, 1 U of a mix of *Pfu DNA Polymerase* and *GoTaq Polymerase* (Promega) 1:5 in a final volume of 20 µl. A 5 minutes denaturation step was followed by 14 cicles of denaturation at 94°C (30 sec), annealing starting from 62°C and decreasing 0.5°C per cycle (45 sec) and extension at 72°C (40sec), then by 20 cycles of denaturation at 94°C (30sec), annealing at 55°C (45 sec) and extension at 72°C (40 sec); the final extension step was carried out at 72°C for 5 minutes. Each amplification included the extraction blank and a PCR blank, to check that no contamination occurred during extraction and amplification procedures, respectively. 5 µl of amplification product was used as template to perform a second PCR reaction at the same conditions in a final volume of 40 µl. 5µl of both extraction blank and PCR blank of the first amplification were used as template in a second PCR reaction, for a total of three negative controls, to exclude even sporadic DNA contaminations. PCR products were visualized and evaluated on a 2.2% FlashGel (Lonza).

From each extraction at least two amplifications were made, for a total of 4 amplification per sample.

Modern samples were stored and amplified in a separate laboratory. An aliquot of the PCR mix was carried to the modern DNA laboratory and used to amplify one male and one female modern samples, as positive controls, for each aDNA reaction.

Amplification of DNA of 11 different modern breeds was performed using the same protocol described for ancient material.

Amplification products were divided in 3 aliquots. One was kept as undigested control, one was digested with the restriction enzyme PstI (Fermentas), which cuts the Y amplicon, and the other was digested with the restriction enzyme SsiI (Fermentas), which cuts the X amplicon. For each digestion, 15µl of amplification product was added to a mix of enzyme (10u/µl), buffer (1X) and H2O to a final volume of 20 µl, then incubated at 37°C in a thermocycler. Reactions were stopped by heating at 80°C or at 65°C for 20 minutes, according to enzyme manufacturer instructions.

3. RESULTS AND DISCUSSION

Among the sex-specific sequences available in Genebank, we selected the *ZFX/ZFY* genes that are located in the homologous X and Y chromosome region. Sequences were aligned to search for short stretches harbouring chromosome specific variations, suitable for amplification in aDNA. We designed primer pairs that were able to amplify these sequences in both chromosomes. It has been reported in the literature that these genes are highly conserved in mammals [13]. We have experimentally checked that the sequences did not present individual variations within chromosome. We BLASTed the sequences against the *Bos taurus* ESTs database that contains about 1,500,000 entries deriving from several individuals. We found no variation among the three retrieved sequences that match the full sequence length. To further explore the possibility of individual variation, we amplified and digested around 30 modern animals of 11 breeds and we found no variation at all in the amplified fragments.

Of the 13 collected samples, all but one produced quantifiable DNA, with an average of 5 ng per g powdered bone, as from picogreen data.

Ten of the 12 samples were successfully amplified once. Moreover, 8 of the 12 samples, two roman and six medieval, were amplified by two independent PCR.

X and Y chromosome-specific variations are revealed by selective digestion of aliquots of the same PCR product with two restriction enzymes, thus producing sex-specific digestion patterns: one enzyme (PstI) cuts the Y amplicon and another (AciI) the X amplicon (Fig. **1**).

Digestion revealed that, of the 10 amplified samples, 8 were males and only 2 were females (Figs. **2**, **3**).

We have chosen ZFX/ZFY sequences because only if both are present we have the discrimination between males and females. These sequences are known in many mammalian species, so the method can be extended and applied to them.

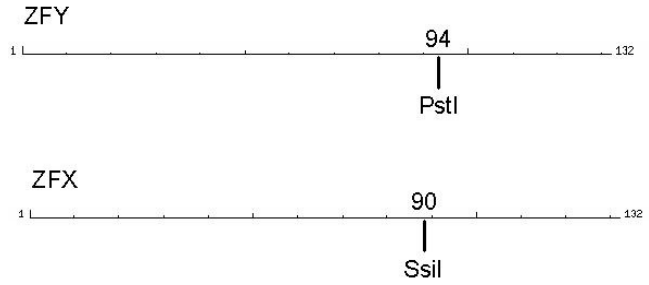

Fig. (1). Examples of digestion with restriction enzymes. Portions of *zfy*/*zfx* genes with male/female specific cutting sites.

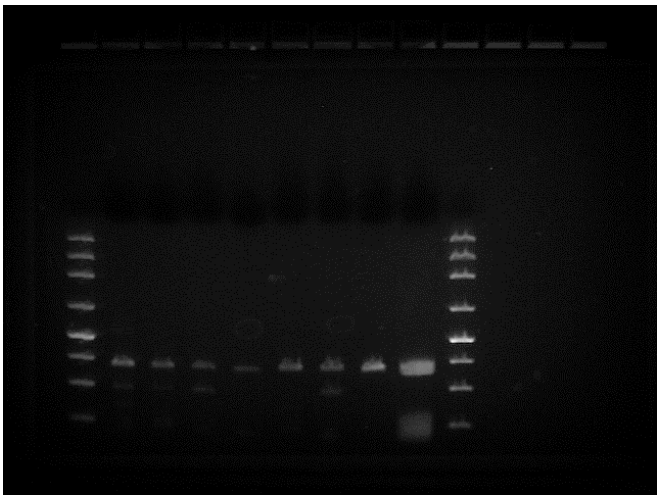

Fig. (2). RFLP analysis of PCR fragments- PstI. Marker 50bp; 1-5:ancient samples; male control; female control; undigested fragment; marker 50bp.

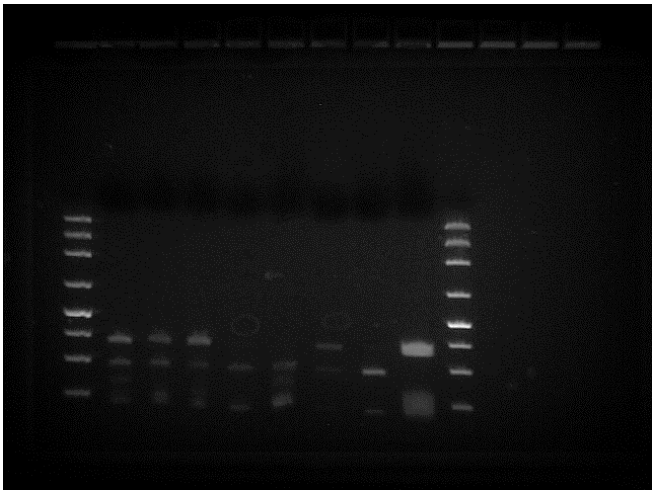

Fig. (3). RFLP analysis of PCR fragments- SsiI. Marker 50bp; 1-5:ancient samples; male control; female control; undigested fragment; marker 50bp.

CONCLUSIONS

Other methods, based on the amplification of specific fragments of Y chromosome [6, 14], rely upon the success of the PCR amplification. This means that in the case of amplification failure the animal is scored as a female. Our method presents the advantage of amplifying simultaneously regions in both sex chromosomes, thus allowing a double check that makes a wrong assignment unlikely. Moreover, amplifying both fragments within the same PCR reaction is an advantage when working with low amounts of aDNA. Methods based on the amplification of both the X and Y chromosomes are described, but the sequences chosen are too long to be successfully amplified in ancient samples [2-, 4, 15].

A method based on pyrosequencing the same *ZFX/ZFY* regions has been recently described by Svensson *et al*., [16]. Our method presents the advantage of being cheaper and feasible even in laboratories not equipped with pyrosequencing equipment.

In conclusion, we described a strong technique for sexing ancient bones and demonstrated that the method is robust enough to be used with scarce and damaged ancient DNA up to 2000 years BP. The technique has been employed for sexing cattle (*Bos taurus*) bones, but it can be easily extended to other mammalian species.

AUTHORS' CONTRIBUTIONS

FG: carried out all the laboratory analysis and drafted the manuscript; EDM: provided fossil samples; AV: participated in developing ideas, in supervision and revision of the manuscript; LP: designed the study and revised the manuscript.

ACKNOWLEDGEMENTS

We wish to thank Paolo Ciorba for technical assistance.

COMPETING INTERESTS

We declare no potential competing interests.

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Received: November 4, 2011 Revised: December 7, 2011 Accepted: December 9, 2011