

Sex assessment in a hominin upper second molar (RV-350) from the "Ruidera-Los Villares" Middle Pleistocene paleoanthropological site: a proteome-based analysis of the amelogenin

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Estimating sex in fossil human remains is crucial for understanding ancient populations' evolutionary anatomy, demographic structure, social behaviors, and evolutionary dynamics. Traditionally, this estimation combined morphological and metric analyses, leveraging features like pelvic and skull characteristics for sexual dimorphism assessment [1]. Modern advancements in technology, such as CT scanning and 3D imaging, have greatly improved accuracy by enabling precise virtual reconstructions and measurements of skeletal features. These methods enhance our ability to reconstruct evolutionary narratives and gain insights into biological diversity in human fossils, contributing significantly to our understanding of human evolution. Molecular analyses, particularly proteomic analysis focusing on the amelogenin protein in dental enamel, have become essential in sex estimation for fossil specimens [2]. Amelogenin, a sexually dimorphic protein encoded by genes on the X and Y chromosomes, can be analyzed using mass spectrometry and proteomic techniques from preserved enamel peptides. This method offers advantages in cases where traditional methods are inconclusive due to fragmentary remains, providing direct molecular evidence of biological sex without damaging samples. Proteomic analysis of amelogenin has been successfully used in paleoanthropological studies, enhancing our understanding of ancient populations' sex composition alongside traditional methods [3].

In this work, we aimed to use proteomics methods to estimate the sex of a second upper molar RV23-350 found in the 2023 campaign from the Middle Pleistocene paleoanthropological site of Ruidera-Los Villares (Castilla-La Mancha, Spain, Iberian Peninsula) [4]. Before studying the RV tooth, we tested the amelogenin extraction protocol using a double-blinded approach: a sex-balanced sample of two contemporary teeth from documented sex, and two archaeological teeth from a Nubian Early Meroitic site in Northern Sudan (3rd-1st cent. BCE) of estimated sex through DSP were subjected to amelogenin extraction with a 100% of success. Once this protocol was accepted, we proceeded with the RV teeth. The specimen sex was determined by mass spectrometry using MALDI as described elsewhere. Tooth enamel was etched with 5% HCl acid for two minutes after washing the tooth sequentially with 3% H₂O₂, two times ddH₂O, and 5% HCl. Cleaning peptides in reverse chromatography were applied to a MALDI plate, and spectra were acquired in positive and reflector modes. The detection of peak 1079.55 Da was characteristic of the peptide from chromosome X amelogenin copy, and the detection of peak 879.44 Da from chromosome Y amelogenin copy. Our sample showed both peaks, so it was assigned as a male individual.

To the best of our knowledge, this study represents the first estimation of sex in Middle Pleistocene teeth using amelogenin analysis, which holds significant implications for understanding anatomical variability within hominins of this period, potentially named *Homo heidelbergensis* (sensu [5]). The utilization of amelogenin as a molecular marker provides a novel and direct approach to sex determination in fossil remains, overcoming potential challenges associated with morphological assessments alone. The data derived from the Ruidera-Los Villares site contribute significantly to this field, shedding light on the demographic structure and population dynamics of hominins inhabiting the Iberian Peninsula during the Middle Pleistocene. By integrating proteomic analysis into our toolkit for paleoanthropological investigations, we enhance our ability to reconstruct the evolutionary history of ancient populations and gain deeper insights into the biological and behavioral aspects of human evolution during this critical period.

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