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Anatomical and genetic study of an ancient animal tooth showing brachyodont and hypsodont mixed taxonomical characteristics

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A non-human dental piece was found in a Roman Empire tomb dated the 3rd century A.C. in Zaragoza (Spain). The morphology of this piece showed mixed brachyodont (carnivores) and hypsodont (herbivores) characteristics. As a result, the taxonomical assignation of the piece was impossible. Therefore, a protocol based on the DNA sequence of the cytochrome c oxidase subunit 1 mitochondrial region (COI) was applied. For this purpose, a pair of primers able to amplify this region in a large variety of animals was designed. The results point to a species of the Genus Bos *(Family Bovidae). This assignation was later confirmed by the sequencing of a short fragment of the mitochondrial D-loop region. A complete morphological description of the tooth is presented together with the DNA sequence study and comparison protocol.* (Folia Morphol 2013; 72, 2: 167–170)

Key words: ancient tooth, ambiguous taxonomical identification, DNA study, mitochondrial DNA

INTRODUCTION

In 1988, a Roman necropolis dated the 3rd century A.C. was discovered in the city of Zaragoza (Spain) [5]. A non-human dental piece was found in one of the tombs (Fig. 1). As described below, it presented only minimal deteriorations.

The specimen showed mixed brachyodont (short crown, long roots and closed apexes), and hypsodont (no cuspids and smalt crests in the occlusal surface) characteristics. As a consequence, the assignment of the piece to a particular species was unclear, even for the experts in animal anatomy.

Therefore, molecular genetics identification was applied, based on DNA from the internal dentine and on the "DNA Bar Coding" concept (Consortium for the Bar Coding of Life, Smithsonian Institution, 2010 available at http://www.barcoding.si.edu/DNABarCoding.htm) [4]. DNA Bar coding tries to identify animal specimens mainly by analysing the sequence of the cytochrome c oxidase subunit 1 mitochondrial region (COI). Multiple copies of the mitochondrial genome exist and are protected by the mitochondrial membranes in each cell. Dental pieces provide a resistant support, so that mitochondrial DNA can be recovered from dentine in sufficient amounts for the laboratory analysis. The usual approach is based on consensus polymerase chain reaction (PCR) primers suitable to allow COI amplification and sequencing in a set of species.

We designed PCR primers suitable to amplify and sequence COI in a wide range of camelids, ruminants and horses. Mitochondrial D-loop analysis was later studied in order to confirm the first results.

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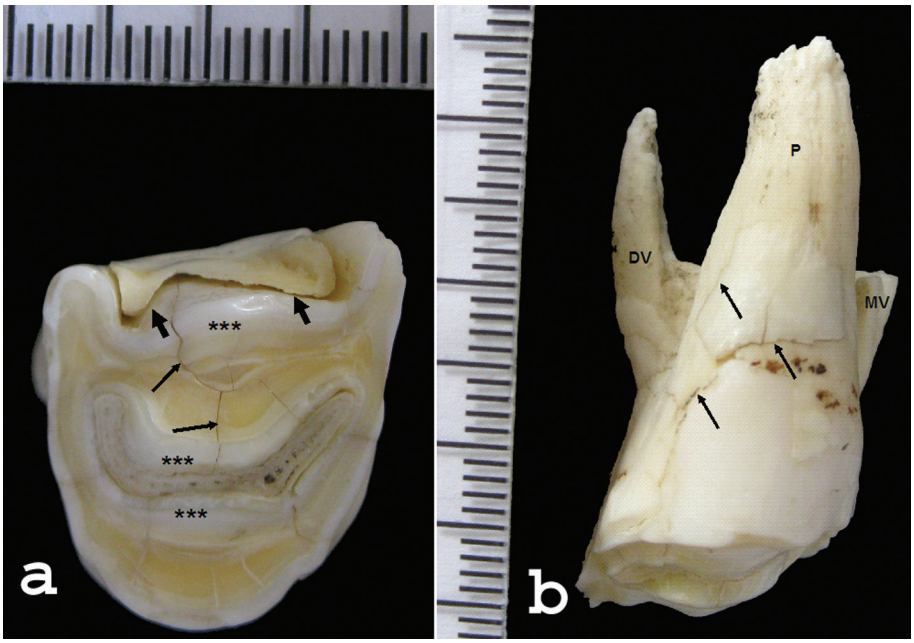

Figure 1. A. Occlusal surface presenting smalt crests (***) and two lines of fracture: vestibular-lingual (thin arrows) and mesial-distal (thick arrows); **B.** The mesial-vestibular, the distal-vestibular and the palatal root (MV, DV and P, respectively). All of them are conical shape and present closed apexes. Noticeably, the mesial-vestibular root is fractured. Several fracture lines are observed in the palatal surface (arrows).

CASE REPORT

The tooth presents a short crown. Half-moon shaped smalt crests are visible in its occlusal surface. Dentine is exposed among the crests. The crown is also crossed by fracture lines running in vestibular to lingual direction. A deeper fracture line follows a mesial to distal direction in the vicinity of the vestibular surface.

The vestibular surface is plain and presents a smalt defect as a result of a fracture. The mesial and distal surfaces are plain, while the palatal surface is half--moon shaped.

The smalt shows some deterioration in all surfaces (except the mesial one). This results in longitudinal, transversal and oblique fracture lines, especially in the distal surface.

The neck of the tooth is clearly marked. A horizontal fracture line runs its distal and palatal surfaces.

Three independent divergent roots converge into a common radicular trunk close to the neck line.

The mesial-vestibular root is fractured. The distal--vestibular root is conical, has a smooth surface and closed apex. Finally, the palatal root is the largest one. Its vestibular surface is tuberculated in the area surrounding the neck. Its apex is closed.

According to the roots morphology, the tooth is a permanent piece from the left superior dental arch.

The assignment of the piece to a particular species of origin by means of morphological analysis was unclear, even after consulting with experts in domestic and wild animal anatomy. On the one hand, the anatomy of the crown and roots could be compatible with a carnivore or an omnivore (humans, canids, felids, ursids, suids, simians), since it presents a brachyodont aspect due to its short crown, long roots and closed apexes.

However, the occlusal surface should points to the opposite assignment. As it presents no cuspids and shows smalt crests, a hypsodont ruminant species (bovids, camelids, horses) should be a logical origin for the piece.

MATERIALS AND METHODS

DNA extraction

DNA extraction was performed in the facilities of the Parasite Diseases Section at the Faculty of Veterinary Sciences, in a building different from the one used for PCR and agarose electrophoresis. Mitochondrial DNA from domestic animals had never been studied at this place. The working area was previously irradiated by the UV light.

The dentine extraction followed a previously reported protocol [6]. To avoid using cooling systems,

the Hedström files were hand operated. For DNA extraction, the dentine was transferred to a 1.5 mL microtube and submitted to two decalcification washes by 0.5 mol/L EDTA (0.5 M pH 8.0), for 5′ each. It was then lysed in a buffer containing 10 mM Tris-HCl, 50 mM NaCl, 2% (w/v) sodium dodecylsulfate (SDS) (pH 8.0), and 15 μL of proteinase K (10 mg/mL) at 56°C for 16 h.

After spinning, the same volume of Chelex (10% in TE) was added to the supernatant. The mix was incubated at 100ºC for 5′, vortexed and submitted to 100ºC for 20 min. After centrifugation at 13.000 g, the supernatant was used as mould DNA (4 μL/10 μL of final PCR volume).

PCR and sequencing

Consensus PCR primers were designed by means of Primer 3 to amplify a 362 bp segment of COI in a wide range of camelids (Arabian camel and Bactrian camel), ruminants (cattle, sheep and goat) and horses. The following Genbank entries were used for such purpose: EU159113, EF212038, EF212037, EF507801, EF507800, EF507798, EF507799, AP003423, NC_001941, NC_001640.1, and AF533441. Primers sequences were: Dien-F: 5′-tcgctgattattctcaaccaa-3′ and Dien-R: 5′-cctgtacctgctcctgcttc-3′.

Amplification protocol for COI

Different annealing temperatures and Mg^{++} concentrations were checked by using a Multigene Gradient Thermal Cycler (Labnet International Inc.). For purpose of sequencing, we chose the PCR product obtained by using a 3 mM final Mg^{++} concentration and the following PCR cycles: 94ºC × 5′ + 35 × (94ºC 30′′ + 54.4ºC 30′′+ 72ºC 45′′) + 72ºC 10′. Studies on COI had never been performed in the laboratory where DNA extraction and PCR amplification were made. After the verification in agarose gel, the PCR products were sequenced at the Central Sequencing Service (University of Zaragoza).

Verification by means of mitochondrial D-loop sequencing

Mitochondrial D-loop was partially PCR-amplified by using the primers Ach-F:5′-cctaagactcaaggaagaaactgc-3′ and Ach-R: 5′-aacctagagggcattctcactg-3′ according to the previously published protocol [1]. The PCR products were sequenced too.

The software Blastn [2] was used to align sequences with those available in the Genbank database.

RESULTS AND DISCUSSION

The odontometric study of the specimen produced the following measurements:

— in the crown, the mesiodistal width is 17 mm, while the vestibule-lingual width is 20.05 mm and the distance between the occlusive surface and the cervix is 13 mm;
— in the mesiovestibular root, the mesiodistal width is 7 mm, while the vestibule-lingual width is 8.5 mm. Since this root is fractured, its height could not be determined;
— in the distovestibular root, the mesiodistal width is 5 mm, the vestibule-lingual width is 7 mm and the cervix to apex distance is 20 mm;
— in the palatal root the mesiodistal width is 10 mm, the vestibule-lingual width is 12 mm and the cervix to apex distance is 24.5 mm.

In all the cases, the diameters were measured at the root bifurcations.

The total height of the specimen from the occlusive surface to the apex is 37.5 mm.

Visual examination of the piece allowed us to disregard a possible human origin, even if the morphological details did not allow us to identify the origin of the specimen.

A reliable 312 bp COI sequence was obtained and deposited at GenBank (Accession number HQ739078). According to Blastn alignment, it is 100% concordant with the sequence of COI in 2 species: *Bos taurus* (for instance with GenBank entry HM045018.1 and others), and *Bos javanicus* (GenBank entry FJ997262.1). Small differences exist with other *Bos* genus species, such as *Bos primigenius* (309 coincidences, entry GU985279.1), *Bos indicus* (entry AY126697.1, 307 coincidences) and *Bos grunniens* (entry GQ464268.1, 299 coincidences).

Since the alignment with other herbivores' COI sequence (horse, camel, goat, sheep, red deer and roe deer) provides coincidences around 80%, they are disregarded as possible sources of the tooth. The coincidence is similarly low for the Ursidae, Felidae, Suidae and Canidae families.

Only a short reliable 123 bp partial fragment from the D-loop region was obtained (GenBank HQ739079). The species and families tested for COI alignment were also studied for this D-loop segment. Only the genus *Bos* provided the high alignment with it. In fact, the Blastn alignment confirmed the bovine origin of the specimen, since the coincidence is 100% with *Bos taurus* (entry AY700556.1 among others), over 99% with *Bos grun-*

niens (entry AY428641.1 among others), and with *Bos indicus* (FJ800951.1 among others), and 98% with *Bos primigenius* (entry GU434123.1 among others).

Taxa different from *Bos* provided only very little alignment coincidences, usually in short fragments.

In all, the analysis of the DNA sequences obtained in this work points to the genus *Bos* as the most suitable origin for this dental piece.

Among the possible extinct *Bos* species, complete mitochondrial DNA sequence information is only available for the auroch (*Bos primigenius*). The comparison with all the bovine species (present and extinct) is thus impossible. Moreover, the phylogeny of the *Bos* taxon is still under revision [3].

Different factors affect the morphology and size of the teeth. Despite the extinction of many cattle breeds, a wide range of body sizes is still observed. The adult body weight of cows from many local breeds conserved all over the old world is below 300 kg. In the past, such limited weight could had been a very common characteristic [7].

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