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Use of next-generation molecular tools in archaeological neotropical deer sample analysis

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ABSTRACT

Taxonomic identity and genetic variation of three bone samples from a late Holocene earth mound in the eastern Uruguayan lowlands were analyzed. Samples were initially identified by morphology as being from pampas deer (*Ozotoceros bezoarticus*), a middle-sized cervid that occupies open environments forming herds of up to 50 animals. Prehistoric human communities may have developed herd management systems for this species. The aim of this study was to evaluate usefulness of a Next Generation Sequencing (NGS) strategy using pooled samples to explore genetic relationships between specimens. Two mitochondrial *D-loop* haplotypes were found which surprisingly identifies another deer species (*Mazama gouazoubira*). Our findings confirm that NGS is a powerful tool for taxonomic identification of ancient Uruguayan samples, also indicating that *M. gouazoubira* may be underrepresented in the zooarchaeological record of eastern Uruguay. Finally, since both species have different niches and behaviors it is necessary to formulate new questions in order to understand prehistoric management of Neotropical deer involving larger sample sizes and analysis of other genetic markers.

1. Introduction

The Uruguayan lowlands located in southeastern South America, were occupied by mound building groups dating back from the mid-Holocene until the European conquest. The mounds are known locally as "*Cerritos de Indios*" (López Mazz, 2001; Bracco, 2006). Within this region archaeological studies indicate that diverse socio-economic features were developed by these groups, including domestication of plants and horticulture, a trend towards sedentarization and social complexity, villages and public architecture, territorial control and formal cemeteries (López Mazz, 2001; Bracco, 2006; Iriarte, 2006). Paleoenvironmental studies suggest the beginning for establishing these villages and activities initiated ca. 2500 years BP (Bracco et al., 2005a; Bracco et al., 2005b; Del Puerto et al., 2011).

Zooarchaeological studies of *cerritos* suggest the exploitation of cervids including pampas deer (*Ozotoceros bezoarticus*), marsh deer (*Blastocerus dichotomus*) and in a lesser degree brown brocket deer (*Mazama gouazoubira*), small and medium-sized rodents (*Myocastor coypus*, *Cavia* sp. and *Hydrochoeris hydrochaeris*), ñandú (*Rhea americana*), pinnipeds (*Otaria flavescens* and *Arctocephalus australis*), and several fish species (siluriformes, *Pogonias cromis* and *Micropogonias furnieri*). Fragmentation rates of the assemblages are around 90%. As such, 70% of taxonomic determinations of remains cannot be made beyond the class level in zooarchaeological collections (Pintos, 2000; Iriarte, 2006; Moreno, 2014).

In spite of the variety of species found in assemblages, Pintos (2001) proposed that the base of the animal economy were cervids and pinnipeds. Within the ungulates, the pampas deer (*O. bezoarticus*), a middle-sized cervid that occupies open environments forming herds of up to 50 individuals (Cosse and González, 2013), was found most frequently (Moreno, 2014). The social complexity proposed for the archaeological groups who built these *cerritos* allows for the formulation of hypotheses on how they controlled and exploited wild animals such as the pampas deer (*O. bezoarticus*), as a stage in the intensification process going from hunting to domestication (Vigne, 2011). One form of control could imply the exclusive use of the resources within a given territory, possibly including herd management.

The control of wild animals does not necessarily imply or result in morphological changes visible at an osteological level (Vigne, 2011).

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Testing hypotheses on animal control methods by ancient people relies on development of alternative tools such as those based on genetics. Ancient DNA research in archaeological faunal remains is focused on answering questions related to the domestication and management of various species (Leonard et al., 2002; Cai et al., 2007; Deguilloux et al., 2009; Røed et al., 2011; Sykes et al., 2011; Gravlund et al., 2012; Thalmann et al., 2013). In addition, the recent development of Next Generation Sequencing (NGS) and its application to the taxonomic identification of bulk bone (pooled samples) offers a cost-effective methodology to deal with this challenge (Murray et al., 2013). This approach could be a powerful tool to understand the relationship between human and animal communities in Uruguayan prehistory.

The goal of this study was to evaluate the NGS methodology with pooled samples, to obtain highly informative genetic data from cervid archaeological remains recovered from excavations in eastern Uruguay.

2. Materials and methods

2.1. The site

The analyzed remains were recovered from the Ch2D01 archaeological site in the *bañado* (wetlands) of San Miguel, Rocha, Uruguay (Fig. 1). The site has two earth mounds (named A and B), with an occupation ranging from 2090 ± 90 years BP (KR139) to 220 ± 50 years BP (URU0014) (Bracco, 2006). The site has an extensive zooarchaeological record, and it is very well preserved in comparison to other assemblages excavated in the region (Moreno, 2014).

Bone fragments of three specimens recovered from the central excavation in the mound A (named IA) were processed. These samples were morphologically identified as pampas deer (*O. bezoarticus*) (Fig. 2) and dated at ca. 1300 years BP through stratigraphic association. Molecular studies of human remains excavated at the site yielded good results

Fig. 1. Location of the Ch2D01 archaeological site in southeastern Uruguay.

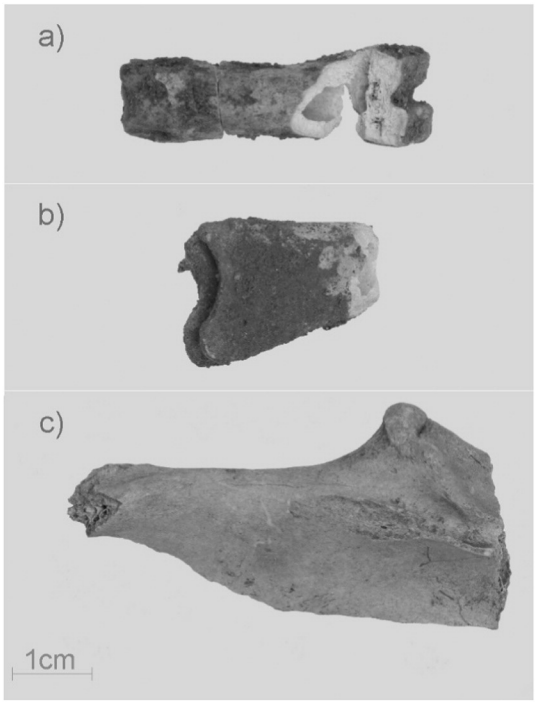

Fig. 2. Three specimens recovered at the site, with a 1 cm scale: a) first phalanx, sample GO19; b) second phalanx, sample GO16; c) innominate bone, sample GO15. Samples were labeled according to the human burial found in the proximity.

(Bertoni et al., 2004; Sans et al., 2012, 2015) allowing us to have confidence that faunal ancient DNA can be recovered as well.

2.2. Ancient DNA extraction

The DNA extraction was carried out in the *Laboratorio de ADN Antiguo* of the *Facultad de Humanidades y Ciencias de la Educación* (*Universidad de la República*, Uruguay). The laboratory has several safeguards against contamination with modern DNA including positive air pressure, laminar flow chambers, UV lamps and UV crosslinkers, and dedicated equipment and reagents which are used neither for modern DNA nor post-PCR applications. In addition, the fact that the laboratory had never previously been used for the extraction and analysis of non-human DNA provided an additional guarantee against the possibility of having similar contaminating DNA in extracted DNA from within bone.

Before extraction, bone samples were mechanically fragmented and submerged in 5% bleach in order to remove all exogenous DNA from the surface of the bone (Kemp and Smith, 2005). Afterwards, approximately 0.5 g of bone fragments from each ancient sample were rinsed with PCR-grade water and put in the UV crosslinker until dry. The first step of the extraction was an overnight EDTA-proteinase K digestion of bone samples in order to liberate the DNA, after which a combined thiocyanate guanidine-silica column protocol was used to purify DNA from the supernatant of the digestion (Höss and Pääbo, 1993; Yang et al., 1998). In this case, a commercial kit was used (GeneClean® for Ancient DNA, MP Biomedicals).

2.3. PCR

The real-time PCR amplifications were done in the *Laboratorio de Biodiversidad y Genética* of the *Instituto de Investigaciones Biológicas* "*Clemente Estable*" (IIBCE-MEC). A 158 bp fragment of the Hypervariable Region I of the mitochondrial *D-loop* (Saccone et al., 1987) was amplified using a novel primer pair spanning nucleotide positions 15,643–15,801 of the *M. gouazoubira* mitochondrial genome (GenBank ID KJ772514.1): DLRHB 5′-GCATGGGGCATATAATGTAATG-3′ and DLFHB 5′-GAGCTTAATTACCATGCCGC-3′. This *D-loop* fragment was selected due to its utility for establishing the taxonomic identity and the phylogeographic association in pampas deer (*O. bezoarticus*) (González et al., 1998; González and Duarte, 2007). In addition, the size of the fragment is suitable for amplification of low quality and low quantity of DNA usually recovered from archaeological specimens. Adapter and barcode sequences were added to the primers, the former in order to sequence the amplicons using the Ion PGM™ system (Thermo Scientific), the latter to distinguish this set of samples from other samples included in the same run.

The PCR reactions were set up in a type IIA laminar flow chamber with UV light in a room separate from that with the real time PCR thermal cycler (Knapp et al., 2012). The positive control, a DNA sample from a contemporary brown brocket deer (*M. gouazoubira*) labeled MSG145 was added in a separate room. PCRs were performed in 10 μL reactions with 1× Sensimix SYBRgreen PCR Buffer containing a hot-start DNA polymerase (Bioline) for high specificity and sensitivity PCR, 0.3 pM of each primer, and 0.7–1.5 μL of DNA extract. Two replicates were prepared for each sample and PCR negative controls were included for each set of reactions. PCR was done in a Corbett 6000 (Rotor Gene) thermal cycler using the following conditions: an initial denaturation at 95 °C for 10 min, followed by 45 cycles of 15 s at 95 °C, 15 s at 55 °C and 15 s at 72 °C. The final extension was 10 min at 72 °C. A melting step at 70–90C° was added at the end of the PCR. The samples for NGS were selected based on the amplification pattern and melting analysis. We used the threshold cycle (Ct) values obtained during the real-time PCR for positive controls.

The selected samples were purified with DNA Clean & Concentrator™-5 (Zymo Research) and diluted to an equal final concentration using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientfic). Subsequently, equal volumes of each PCR product were mixed in a single tube containing an equivalent amount of DNA molecules from each PCR product. This pooled sample carries a unique Ion PGM™ barcode for downstream NGS protocols according to Murray et al. (2013). The sample was quantified using the High Sensitivity double stranded DNA kit in a Qubit® 2.0 fluorometer. The quantified libraries were diluted to an concentration of 26 pM (following the kit protocol) for clonal amplification by emulsion PCR using the Ion OneTouch™ 2 thermal cycling system with the Ion PGM™ Template OT2 400 kit (Thermo Scientific). The amplified libraries were sequenced using the Ion PGM™ system with the Ion PGM™ Sequencing 400 kit in an Ion 316™ Chip v2 (Thermo Scientific).

2.4. NGS output analysis

The data generated by the Ion PGM™ system was transferred to the Torrent Server in order to obtain read sequences in FASTQ format. Primers were removed from the reads using a home-made bash scripts. Sequences were further trimmed based on quality with SICKLE (Joshi and Fass, 2011), and using phred scores 27, 28, 29, and 30 as quality thresholds. The SICKLE tool was used to trim low quality regions in each read using a sliding windows approach. We obtained four groups of reads with phred scores greater than or equal to 30, 29, 28, and 27. Sequences within each group were aligned using MUSCLE (Edgar, 2004). Finally, redundant sequences were identified using CD- HIT- EST (http://weizhongli-lab.org/cd-hit/; Fu et al., 2012). With the reads obtained for each quality cluster, a minimum spanning haplotype network was constructed using PopART (http://popart.otago.ac.nz). This strategy allowed us to identify the most frequent sequence motifs occupying a central position (the ancient sequence) with respect to other motifs (Bandelt et al., 1999; Forster et al., 2013). Sequences identified using this criterion for each score grouping were selected for downstream analyses.

2.5. Sequence analysis

Sequences identified as ancient samples were aligned with three sequence sets of cervids present in Uruguay (*M. gouazoubira*, *O. bezoarticus* and *Blastocerus dichotomus*) including *Cervus elaphus* as outgroup. The alignment was carried out using ClustalW in MEGA6 (Tamura et al., 2013). The evolutionary history of the sequences was inferred through the UPGMA method in MEGA6, using a Tamura 3-parameter substitution model with uniform distribution and 1000 bootstrap replicates. Taxonomic identity determinations of ancient sequences were based on visual inspection and the identification of diagnostic sites from the amplified region (Sinding et al., 2015).

Once assigned to a taxonomical group, archaeological haplotypes were compared with a set of sequences obtained from living individuals of the same species (n = 36) coming from various localities within the species' range of distribution (i.e. southern Brazil, Bolivia, Paraguay, northern Argentina and Uruguay). We estimated genetic distances between haplotypes using the Kimura 2-parameter model assuming a gamma distribution of nucleotide substitution (Kimura, 1980). We used DnaSP v. 5 (Librado and Rozas, 2009) to calculate nucleotide diversity (π: average number of differences per site between any 2 sequences chosen randomly from the sample population) and haplotype diversity (H: a measure of the uniqueness of a particular haplotype in a given population) (González et al., 2015). We also

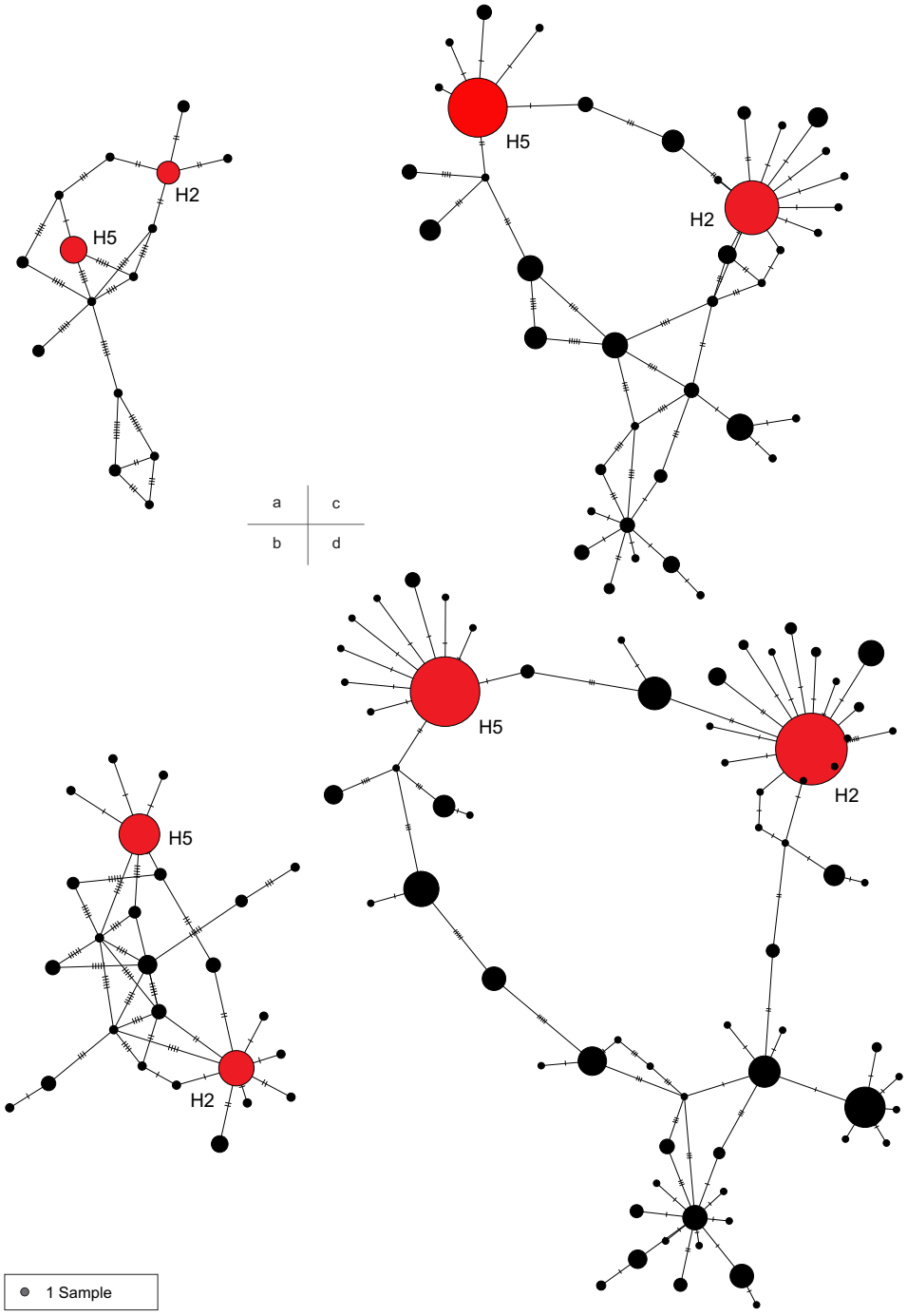

Fig. 3. Median-joining networks (Bandelt et al., 1999) constructed with the reads for each phred score: a) phred score ≥ 30, b) phred score ≥ 29, c) phred score ≥ 28, d) phred score ≥ 27. The size of the circles indicates the frequency of each haplotype in the pooled sample. The crosshatch bars between haplotypes represent nucleotide sequence differences. The haplotypes chosen as the most likely based on their frequency and central position in the network are marked in red. The haplotypes selected for further analysis (H2 and H5) are shown.

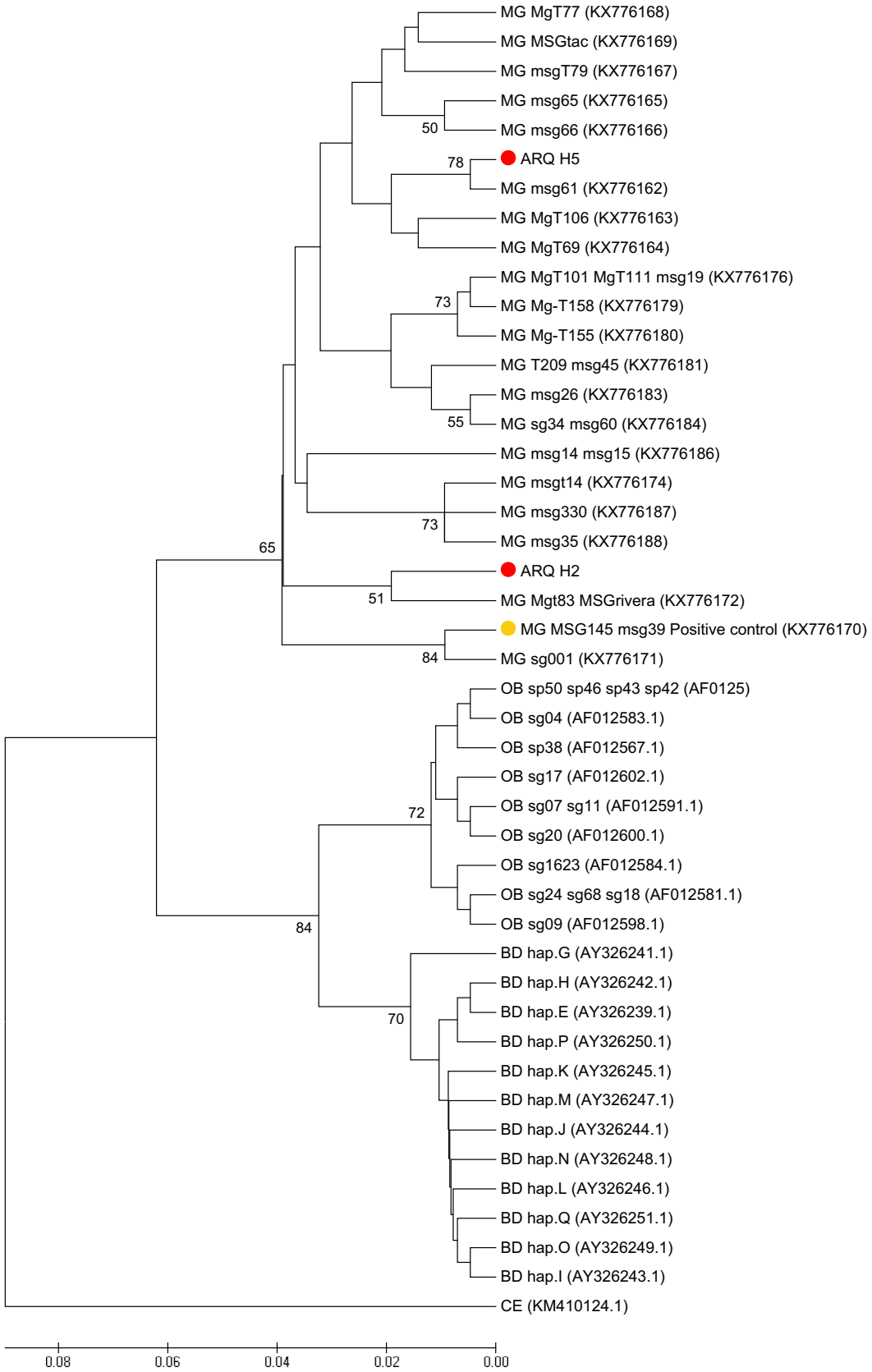

Fig. 4. Phylogenetic tree inferred using the UPGMA method of the archaeological sequences (●) compared to sequences from the three species of cervids found in Uruguay: twenty-two *M. gouazoubira* haplotypes (MG; n = 28, including the positive amplification control MSG145 (●); nine *O. bezoarticus* haplotypes (OB; n = 15); twelve *B. dichotomus* haplotypes (BD); a *C. elaphus* (CE) was included as an outgroup. Bootstrap values ≥50% are shown next to the branches and GeneBank access numbers are in parentheses.

constructed a median-joining network (Bandelt et al., 1999) using the PopART software to analyze the haplotype connections and to infer intraspecific phylogenies.

3. Results

Three PCR products from the real time PCR assay were selected based on Ct values. Once purified, the initial DNA concentrations were 715 ng/μL, 704 ng/μL, and 687 ng/μL, respectively. The three samples had an A260/A280 ratio of between 1.82 and 1.85. Samples were diluted to a final concentration of 30 ng/μL. The final pooled sample contained an equal mix of each PCR product with 100 ng of DNA in a total volume of 35 μL.

The 158 bp amplicon of the *D-loop* region was successfully amplified in pooled archaeological sample. The following results were obtained from the NGS output: 547 reads with a ≥27 phred score, 256 sequences with a ≥28 phred score, 84 sequences with a ≥29 phred score and 36 sequences with a phred score of ≥30 were recovered. For each phred score grouping (≥30, ≥29, ≥28, and ≥27, respectively) 15, 25, 40 and 99 non-redundant sequences (representing different haplotypes) were found by sequence similarity. The final fragment size after trimming adaptors, primers, and barcodes was 109 bp excluding length mismatches. Haplotype networks including all reads in each quality group are shown in Fig. 3. Two haplotypes (named H2 and H5) are central and most frequent in the four quality groups. At 30 phred score H2 was represented with 8 reads (26.7%) and H5 represented with 11 reads (36.7%), the 13 other haplotypes were represented by only one or two sequences.

Phylogenetic analysis of haplotypes H2 and H5 together with the different haplotypes from neotropical cervids species included in our dataset determined that the archaeological haplotypes belong to brown brocket deer (*M. gouazoubira*) (Fig. 4).

We detected three diagnostic sites in the 109 bp sequence which were distinct between modern samples from GenBank of pampas deer (*O. bezoarticus*) (based on 9 haplotypes in 15 sequences) and brown brocket deer (*M. gouazoubira*) (21 haplotypes in 28 sequences). All 90 haplotypes with phred score ≥ 27 from archaeological samples are consistent with *M. gouazoubira* diagnostic sites (Fig. 5).

Analysis of the 109 bp *D-loop* region of archaeological samples together with that of present-day brown brocket deer (*M. gouazoubira*) samples resulted in 29 haplotypes with 31 polymorphic sites, 22 of them being parsimony-informative. Haplotype diversity was 0.984. The average number of differences (k) was 6.61 with a nucleotide diversity $\pi = 0.06121$. The median-joining network (Fig. 6) does not show phylogeographic correspondence.

4. Discussion

Several post-mortem processes result in DNA damage, and these processes are more significant for ancient DNA. This ultimately results in the reduction of overall DNA amounts, fragmentation of the DNA molecule and different types of nucleotide modification (Pääbo et al., 2004). As a result, ancient DNA extracts contain molecules with chemical modifications that may be responsible for sequencing artefacts. The pool of molecules consists of the same fragment but each with differences from the original sequence. Besides the miscoding lesions in ancient DNA, *Taq* DNA polymerase errors may occur in any PCR reaction (Pääbo et al., 2004). NGS allows us to obtain from hundreds to millions of parallel reads of the different sequence variants found in a sample; it is finally more accurate, cost-effective and less time-consuming than the traditional Sanger sequencing-based approach (Taberlet et al., 2012).

The NGS output consists both of the original archaeological sequences plus various similar sequences with nucleotide misincorporations. Analysis of haplotype networks constructed with the reads grouped by phred score allowed us to assume that the branching and frequency pattern of the different nodes in the network could be used in order to track the root sequence of the tree (Bandelt et al., 1999). We furthermore assumed that these root sequences were the valid sequences of the archaeological samples, while the other variants were due to DNA degradation and methodological artefacts (Pääbo et al., 2004). Additionally, more distant nodes can be seen in the network; although one of these nodes could represent a highly degraded sample and its respective artefact-derived sequences. As such these sequences were not considered in subsequent analyses due to the difficulty in reconstructing original sequences. It is possible either that one of the three archaeological samples yielded very little or no amplification product (which may even correspond with pampas deer), that two of the individuals shared one and the same haplotype or that two of the bone specimens belonged to the same individual. In order to avoid the latter bias, we could perform DNA extractions from non-repeated anatomical elements (e.g. right humerus), and use primers with a unique barcode for each sample. In any event, the maximum potential number of haplotypes to be found is three.

The NGS analysis of a 109 bp mitochondrial *D-loop* fragment of the zooarchaeological remains turned out to be efficient for their taxonomical determination. The samples which had been analyzed morphologically were thought to belong to pampas deer (*O. bezoarticus*), however, they are now reassigned to brown brocket deer (*M. gouazoubira*). Given the high fragmentation rate and the low taxonomic level to which most of the specimens could be assigned, it becomes clear that NGS is a very powerful tool for addressing questions related to the taxonomical identity of remains (Murray et al., 2013; Grealy et al., 2015).

The work presented here is the first reported using NGS for analysis of zooarchaeological remains of neotropical cervids. In perspective, increasing the number of genetic markers used and the application of a bulk-bone metabarcoding methodology (Murray et al., 2013) should enhance the utility of this strategy. This would allow us to assess the presence of hitherto undetected species of cervids as well as to improve the frequency estimates of different species for each archaeological site.

Our results may suggest that the low frequency of brown brocket deer (*M. gouazoubira*) reported for the zooarchaeological record of eastern Uruguay could be an underestimate. There are difficulties in distinguishing the brown brocket deer (*M. gouazoubira*) from pampas deer (*O. bezoarticus*) based only on morphological data. In this regard, NGS will allow us to estimate the relative abundance of each species. To better establish the relative presence of one or the other is of great importance to more fully understand the prehistoric animal economy in this region. Since the habits and habitats of the two species are different (Table 1), differential exploitation of the two deer also indicates the use of different hunting strategies,

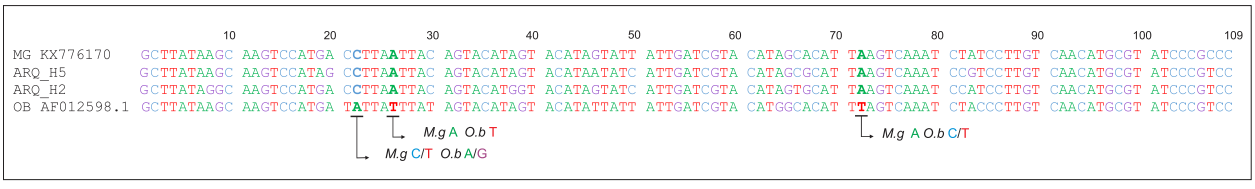

Fig. 5. Alignment of the *D-loop* amplicon of ancient samples (ARQ_H5 and ARQ_H2) and GenBank (GB) sequences of *M. gouazoubira* (MG; GB ID KX776170) and *O. bezoraticus* (OB; GB ID AF012598.1) showing three diagnostics sites. Each diagnostic sites is noticeable with an arrow pointing the substitutions on *M. gouazoubira* (*M.g*) and *O. bezoraticus* (*O.b*).

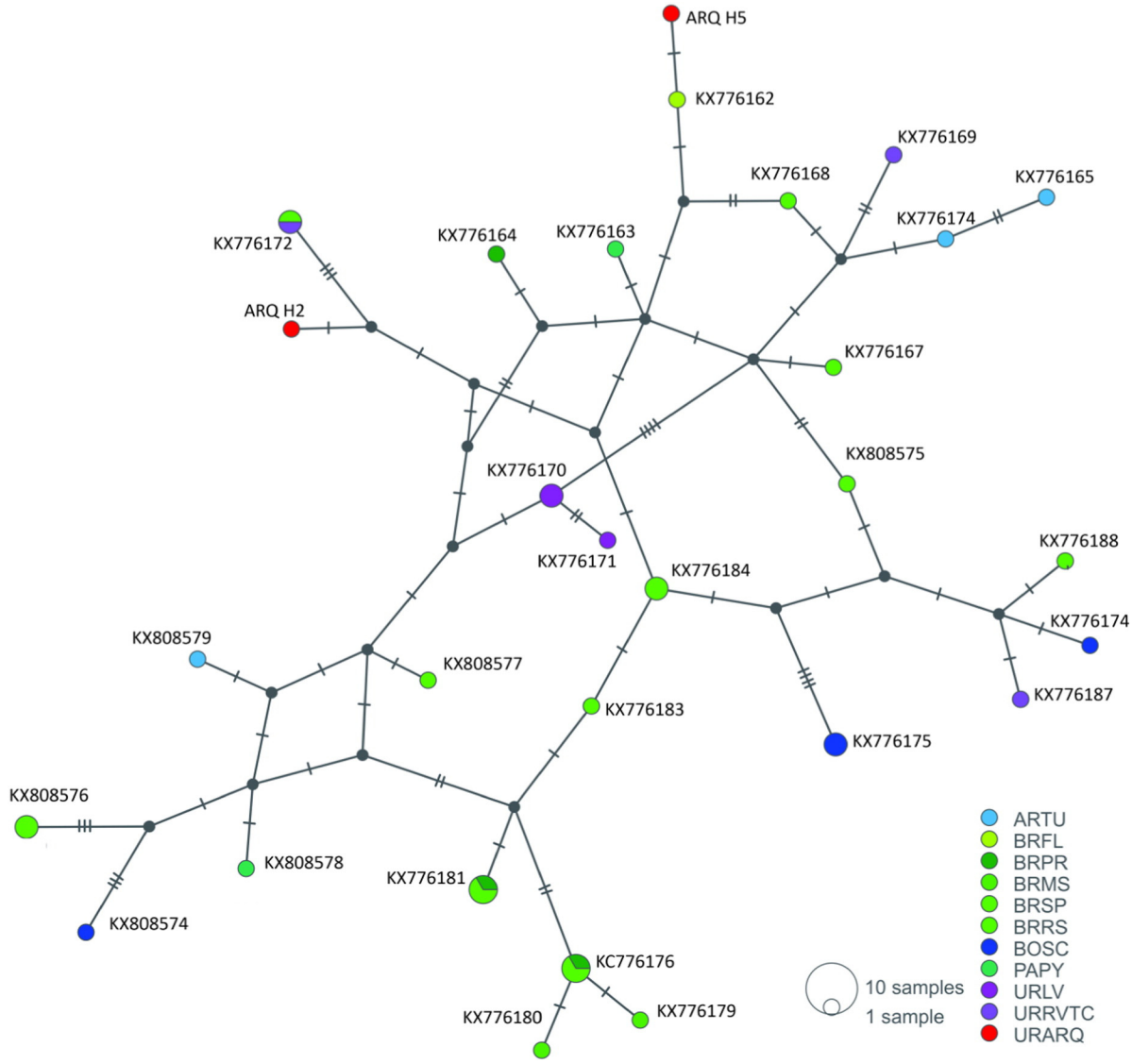

Fig. 6. Median-joining network (Bandelt et al., 1999) based on the number of substitutions in *D-loop* haplotypes using archaeological samples (ARQ 2 - ARQ 5) and modern *M. gouazoubira* samples. The modern samples are identified with their GeneBank access numbers. The size of the circles shows the frequency of each haplotype in the sample. Different origins of samples are indicated with different colours (ARTU: Tucumán, Argentina; BRFL: Florianopolis, Brazil; BRPR: Paraná, Brazil; BRMS: Mato Grosso do Sul, Brazil; BRSP: Sao Paulo, Brazil; BRRS: Río Grande do Sul, Brazil; BOSC: Santa Cruz, Bolivia; PAPY: Asunción, Paraguay; URLV: Lavalleja, Uruguay; URRVTC: Rivera-Tacuarembó, Uruguay; URARQ: archaeological specimens, Uruguay). The crosshatch bars between haplotypes represent nucleotide sequence differences.

different technologies and different yields, which in turn carries social implications in terms of territory and mobility, among other things.

The *D-loop* fragment used in this study is very polymorphic and informative for Neotropical deer studies (González et al., 1998). However, this region shows a much higher variation in brown brocket deer (*M. gouazoubira*) (Márquez et al., 2006; Duarte et al., 2008). As such, it was not possible to distinguish phylogeographic groups for the zooarchaeological haplotype network of brown brocket deer (*M. gouazoubira*) in this study (Fig. 5).

The selection of a mitochondrial region with an adequate and comparable level of variation for each species is of primary importance for characterization of prehistoric management of cervids. This would allow for the identification of groups related by descent potentially

Table 1
Ecological and ethological characteristics of the pampas deer (*O. bezoarticus*) and brown brocket deer (*M. gouazoubira*). Data extracted from Duarte and González (2010).

	Niche	Home range (Km^2, mean value)	Density (Km^2, mean value)	Antler	Weight (kg)	Shoulder height (mm)	Group size (individuals)	Diet
Pampas deer	Open grassland	7.3	6	Male, minimum three tines	20–40	600–700	Gregarious 5–17	Mixed grass feeder
Brown brocket deer	Low secondary woodlands and riparian forest	0.3	1.55	Male, single tine	11–25	500–650	Basically solitary	Browser and mostly frugivorous in the wet season

associated with distinct archaeological occupations. Caparroz et al. (2015) compared genetic diversity of different mitochondrial regions in *M. gouazoubira*, establishing regions with less variation than the *D-loop*. Some of these regions could be more appropriate for distinguishing genetic lineages in this species. This in turn could help to answer questions related to human management of brown brocket deer (*M. gouazoubira*).

The present work is exploratory and the next step is increase the number of samples as well as to include different archaeological sites and remains with different chronologies. This would allow us to determine the correspondence (if any) between the degree of genetic variation and the origin of each sample. This should be expected considering that animals in the same herd would have some degree of kinship. This could also shed some light on possible herd management by different domestic groups and potential management of other cervids such as the pampas deer (*O. bezoarticus*).

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