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ORIGINAL ARTICLE

A novel method of male sex identification of human ancient skeletal remains

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Abstract Sex identification of ancient individuals is important to understand aspects of the culture, demographic structure, religious practices, disease association, and the history of the ancient civilizations. Sex identification is performed using anthropometric measurements and molecular genetics techniques, including quantification of the X and Y chromosomes. These approaches are not always reliable in subadult, or fragmented, incomplete skeletons or when the DNA is highly degraded. Most of the methods include the identification of the male and female sexes, but the absence of a specific marker for the males does not mean that the sample obtained was from a female. This study aims (1) to identify new male-specific regions that allow male identification; (2) to contrast the effectiveness of these markers against AMELX/AMELY and anthropometric measurement procedures; and (3) to test the efficacy of these markers in archaeological samples. For the first two aims, we used known sex samples, and for the third aim, we used samples from different archaeological sites. A novel molecular technique to identify male-specific regions by amplification of *TTTY7*, *TSPY3*, *TTTY2*, and *TTTY22* genes of the human Y chromosome was developed. The results showed amplification of the specific DNA regions of Y chromosome in male individuals, with no amplification being observed in any of the female samples, confirming their specificity for male individuals. This approach complements the current procedures, such as the AMELX/AMELY test and anthropometric principle.

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Abbreviations

Chr-X	X chromosome
Chr-Y	Y chromosome

Introduction

The current method used to sex determine ancient human remains is anthropometric measurements of the bones, such as skull, pelvis, vertebrae, teeth, long bones, and the pars petrosa ossis temporalis (Ovchinnikov et al. 1998; Graw et al. 2005; Veroni et al. 2010; Harris and Case 2012; Krishan et al. 2016; Ubelaker and DeGaglia 2017; Hora and Sládek 2018; García-Campos et al. 2018a, b). Sex determination using this method is more accurate in adult whole-body skeletons, than in the subadult skeletons, and very old or fragmented bone remains (Álvarez-Sandoval et al. 2014) because the anatomical differences between men and women are more clearly distinguished after the onset of puberty due to adolescent hormonal changes. These female characteristics begin to develop at different ages and ageing process can be affected by nutritional status and related socioeconomic factors. In many cases, these anthropometric methodologies do not yield conclusive results in subadult individuals. On the other hand, in modern humans, secondary sexual traits usually emerge in the skull or postcranial skeleton during adolescence. Female characteristics begin to develop at different ages with the ageing process also being affected by nutritional status and related socioeconomic factors (Moore 2013; Candelas-González et al. 2017).

Similar procedures are used in the sex identification of newborns, and subadults, although the accuracy of sex estimation is lower in subadult than adult skeletons (Fazekas and Kósa 1978; Schutkowski 1987; Luna et al. 2017). Sexing can be also performed in 66% of the specimens with skulls that have been largely destroyed, using the usually well-preserved pars petrosa ossis temporalis of skulls (Graw et al. 2005). Furthermore, sex was correctly assigned in male maxillary canines of subadults that contained greater amounts of dentine (García-Campos et al. 2018a, b). Still, these methods are useless in very fragmented adult, and subadult bones, or when the adequate bone remains are not available (Álvarez-Sandoval et al. 2014).

There are several molecular methods to determine sex, such as the amelogenin test based on the amplification of the *AMEL* gene in the X and Y chromosomes (*AMELX*/*AMELY*) (Stone et al. 1996; Tschentscher et al. 2008; Gibbon et al. 2009; Álvarez-Sandoval et al. 2014). The AMEL locus has 2 homologous genes: *AMELX*, which is located on the distal short arm of X chromosome (Chr-X) (p22.1-p22.3), and *AMELY*, which is located near the centromere of the Y chromosome (Chr-Y) (p11.2). These genes have 89% homology, with there being a 6-bp deletion in the third intron of *AMELX* which is absent in *AMELY* (Stone et al. 1996; Butler and Li 2014; Álvarez-Sandoval et al. 2014; Dutta et al. 2017). The amplification of other genes, such as *DXYS156*, *SOX3*, *STS*, and *TSPYL2* (Bashamboo et al. 2003; Butler and Li 2014), are also used for the sex determination of ancient remains. However, the limitation of this and other molecular methods is DNA degradation, which depends on the archaeological context and bone sample (Quincey et al. 2013; Álvarez-Sandoval et al. 2014; Gaudio et al. 2019). Sex identification of ancient human remains was recently performed using the quantification of sequences that are aligned with Chr-Y and Chr-X by considering that males have half of the amount of DNA that corresponds to Chr-X compared to females (Green et al. 2010; Skoglund et al. 2012, 2013). Sex determination with this methodology was obtained by massive sequencing and the exclusion of the homologous regions in both chromosomes (Green et al. 2010; Skoglund et al. 2013). However, the read depth must be reasonably high to avoid any confounding effects due to sequencing errors.

Chr-Y typically determines male sex in humans. It is the third smallest chromosome comprising ~2–3% of the haploid genome (Quintana-Murci and Fellous 2001; Halder et al. 2017). It is composed of a pseudoautosomal portion (PAR) that is divided into the two regions: PAR1, located in the terminal region of the short arm (Yp11.32), and PAR2, located in the long arm (Yq12). The pseudoautosomal regions can recombine with the pseudoautosomal region of Chr-X (Xp22.3 and Xq28) during meiosis (Quintana-Murci and Fellous 2001; Bashamboo et al. 2003; Halder et al. 2017). PAR1 and PAR2 represent ~5% of the whole chromosome, with the remaining ~95% corresponding to the non-recombinant region of human Chr-Y (NRY). Genes in the NRY region are classified into two categories. The first region is comprised of genes that are ubiquitously expressed, show homology with Chr-X, and exhibit housekeeping cellular functions. The second region includes genes with specialized functions that are expressed in the testes (Halder et al. 2017).

The development of new and improved methodologies for sex estimation and the revaluation of existing methods is very important for both ancient and forensic researchers to achieve more accurate results. The objective of this study was to develop an alternative

technique. For this, we selected four genes from fifteen Chr-Y-specific genes in contemporary male samples in silico. These male-specific DNA markers were experimentally tested in contemporary and ancient samples from individuals found in Tacotalpa, Tabasco, Mexico. The results confirmed that these regions were specific to the male sex.

Materials and methods

Contemporary samples

The present study contained ten contemporary samples (five males and five females) from peripheral blood obtained from the "Servicio de Medicina Interna" of the Regional Hospital by Dr. Carlos MacGregor Sánchez Navarro from the Instituto Mexicano del Seguro Social (IMSS). The samples were registered to project number 2017-785-071, and the National Ethics Commission (IMSS) ethics committee approved the study with the number and R-2017-785-071. The tests were performed by following the rules established in the Helsinki Declaration. All data were kept strictly confidential, as indicated by national and international regulations. The participants were informed about the procedure prior to providing their written consent.

Archaeological material

The study contained nineteen samples discovered in three different archaeological sites in the municipality of Tacotalpa in Southern Tabasco (Supplementary Table 1). Ten of these samples were found at the Puyil cave located at San Felipe Mountain in Puxcatan town at the coordinates 17° 27′ 38.04″ N and 92° 39′ 28.5″ W; seven samples were located at the Abrigo Rocoso Fidencio López, Ejido Lázaro Cárdenas, at the coordinates 17° 31′ 21.35″ N and 92° 47′ 54.21″ W; and two samples were located at the Sima Cuesta Chica, Ejido la Pila, at the coordinates 17° 26′ 33.59″ N and 92° 44′ 31.53″ W. Figure 1 shows the geographic locations of all ancient samples. The archaeological samples recovered from the Puyil cave were found by Luis Alberto Martos-López in 2007 and Abrigo Rocoso Fidencio López and Sima Cuesta Chica by Eladio Terreros-Espinosa in 2016.

The sex was determined in fifteen of the samples using anthropometric measurements as described previously and according to conservation of the bone (Bass 1987; White and Folkens 2005; Blau and Ubelaker 2009; Ubelaker 2014).

Pretreatment of ancient samples

To eliminate exogenous DNA and surface contaminants, each sample was washed by rinsing in water, and the samples then incubated for 5 min with 10% full-strength Clorox bleach. The supernatant was discarded, the sample was incubated in distilled water for 5 min, and the water was then discarded. After the 3rd rinse with distilled water, the sample was dried by incubation at 37 °C overnight.

All experiments were performed in a clean room, according to the guidelines of working with ancient remains (Adler et al. 2011; Campos et al. 2012). The clean room was routinely cleaned with bleach and equipped with a UV light. All containers were wiped with bleach before and after being placed in the laboratory.

DNA extraction

Contemporary DNA extraction was performed in duplicate using magnetic beads (PerkinElmer, Waltham, MA, USA) with the Prepito DNA Blood250 Kit (PerkinElmer, Waltham, MA, USA) and the Prepito-D instrument (PerkinElmer, Waltham, MA, USA) following the manufacturer's instructions. The extracted DNA was maintained at − 70 °C. One negative control without DNA was used during the entire process.

Ancient DNA (Supplementary Table 1) was extracted from 20 mg of bone powder by incubation in 200 μl of lysis buffer and 6 μl proteinase K under gentle rotation at 56 °C until lysis was completed, following the manufacturer's instructions (PerkinElmer, Waltham, MA, USA). Aliquots of the extracted DNA were maintained at − 70 °C until use. Ancient DNA was also extracted in duplicate from 50 mg of bone powder via incubation in 500 μl ethylenediaminetetraacetic acid (EDTA; pH 8, 0.5 M) and 20 μl proteinase K (0.25 mg/ml) under gentle rotation at 37 °C overnight. The supernatant obtained following centrifugation was used for DNA extraction with silica (silicon dioxide; Sigma) as previously described by Rohland and

Fig. 1 Location of pre-Hispanic samples from Tacotalpa, Tabasco, that belonged to individuals from the Mesoamerican region. The samples were found in the ARLF (Abrigo Rocoso Fidencio López), SCC (Sima Cuesta Chica), and the PC (Puyil cave) at the coordinates 17° 31′ 21.35″ N and 92° 47′ 54.21″ W; 17° 26′ 33.59″ N and 92° 44′ 31.53″ W; and 17° 27′ 38.04″ N and 92° 39′ 28.5″ W, respectively

Hofreiter (2007). Purified DNA was eluted in 50 μl TE buffer, and 20 μl aliquots were stored at − 70 °C until use. This method was also performed in the Insectary at CINVESTAV-IPN, where human DNA is not handled. One negative control without DNA was used during the entire procedure. The ancient DNA extracted, amplified, and then sequenced were used for haplogroup identification and replication in four samples from Puyil cave in the Department of Anthropology, University of Kansas, Lawrence, KS. This laboratory is specific to work with ancient DNA. Results were the same from both laboratories.

Analysis of the Y chromosome in silico

The non-homologous regions of the X and Y chromosomes of the reference sequence CRs_human_hg38/Chr Y (NC_000024.10) were analysed using IGV software (Thorvaldsdóttir et al. 2013; Integrative Genomics Viewer 2018) to identify the regions specific to Chr-Y. Table 1 shows the sequences identified in the following fifteen genes: *TTTY7*, *TSPY3*, *TTTY2*, *TTTY22*, *USP9Y*, *UTY*, *NLGN4Y*, *TTTY14*, *FAM41AY1*, *AC007359.1*, *TTTY4*, *PBY2B*, *DAZ1*, *DAZ3*, and *DAZ4*. The sequences were classified into the following 3 groups:

(1) the sequence was present only in Chr-Y; (2) the sequence showed similarity with Chr-X; and (3) the sequence was also identified in autosomal chromosomes (Fig. 2).

Male identification using specific Chr-Y sequences

To demonstrate that the region identified in this study was specific to male individuals, a pair of primers (F_TTTY7-R_TTTY7) specific for the *TTTY7* gene were designed to amplify a 140-bp fragment from nucleotides 6453443–6453582. A specific pair of primers (F_TSPY3-R_TSPY3) for *TSPY3* amplified a fragment of 121 bp from nucleotides 9400901–9401021. A specific pair of primers (F_TTTY22-R_TTTY22) for *TTTY22* amplified a fragment of 124 bp from nucleotides 9811189–9811312, and a specific pair of primers (F_TTTY2-R_TTTY2) for *TTTY2* amplified a fragment of 133 bp from nucleotides 9738313–9738445 (Table 2). All primer sets were designed using the program PrimerX-Bioinformatics.org. The contemporary DNA samples were tested with the primer set designed to target the Chr-Y-specific regions to confirm the effectiveness of male sex identification. The following PCR conditions using the enzyme Phusion Hot Start II Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) were used: 98 °C for 30 s; 35 cycles at 98 °C for 10 s, 60.5 °C or 61.5 °C (according to the primer pair used; Table 2) for 30 s, and 72 °C for 20 s; and 72 °C for 10 min. After optimization and confirmation of this procedure in the contemporary samples, this assay was tested on the DNA of ancient bone samples at least in triplicate.

Sanger sequencing was performed using the forward and reverse primers (Table 2) following the manufacturer's instructions for the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

Female and male sex identification using the *AMEL* gene (*AMELX/AMELY*)

Sex was determined via amplification of the *AMEL* gene (*AMELX/AMELY*) in contemporary human samples using the specific pairs of primers shown in Table 2. Amplicons of 130 bp and 170 bp indicated female and male sex, respectively. After sex identification of the contemporary samples, the test was performed on the ancient bone samples. PCR amplification was performed using the enzyme Phusion Hot Start II Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with the following conditions: 98 °C for 30 s; 35 cycles at 98 °C for 10 s, 52 °C for 30 s (Table 2), and 72 °C for 20 s; and 72 °C for 10 min (Lin et al. 1995).

Results

Analyses of X and Y chromosomes for male sex identification

The *TTTY7*, *TSPY3*, *TTTY2*, *TTTY22*, *USP9Y*, *UTY*, *NLGN4Y*, *TTTY14*, *FAM41AY1*, *AC007359.1*, *TTTY4*, *PBY2B*, *DAZ1*, *DAZ3*, and *DAZ4* genes specific for Chr-Y (Stelzer et al. 2016; GeneCards-Human Genes n.d.) were analysed using IGV software (Thorvaldsdóttir et al. 2013; Integrative Genomics Viewer 2018) to identify the regions not homologous with Chr-X and autosomal chromosomes (Table 1). The regions specific to Chr-Y, selected to identify the male sex, were in the following genes: *TTTY7* from nucleotide sites 6453472–6453552 and 6457811–6457861; *TSPY3* from nucleotide sites 9712721–9712755, 9340012–9340052, and 9338261–9338286; *TTTY2* from nucleotide sites 9738350–9738414 and 9740557–9740591; and *TTTY22* from nucleotide sites 9811090–9811129 and 9811192–9811267 (Table 1).

Amplification of *TTTY7*, *TSPY3*, *TTTY2*, and *TTTY22* gene-specific regions for Chr-Y in contemporary human samples using PCR

To verify the specificity of the sequences selected for each gene in identifying males, amplicons of 140, 121, 124, and 133 bp were obtained for *TTTY7* (from nucleotides 6453443–6453582), *TSPY3* (from nucleotides 9400901–9401021), *TTTY22* (from nucleotides 9811189–9811312), and *TTTY2* (from nucleotides 9738313–9738445), respectively. Female and male contemporary samples were used to optimize the test. The expected molecular band size for each gene in the male samples and the absence of the band in the female samples were observed, as shown in Fig. 3. This test was also repeated in ten contemporary DNA samples from

Table 1 Description of 15 genes of Chr-Y analysed to select specific sequences that allow the identification of the male sex

[a]Chr-Y Gen	Sequence	Sequence length (bp)	[b]Function of gene	Query cover with Chr-X (bp)	[c]Autosomal Chr's
TTTY7 6449468-6457906 8439 bp	6453472–6453552	81	♦Testis-specific transcript Y-linked 7	0	0
	6457811–6457861	51		0	0
TSPY3 9398421–9401223 2803 bp	9712721–9712755	35	Testis-specific Y-encoded protein 3	0	0
	9340012–9340052	41		0	0
	9338261–9338286	26		0	0
TTTY2 9736286–9758476 22,191 bp	9738350–9738414	65	♦Testis-specific transcript Y-linked 2	0	0
	9740557–9740591	35		0	0
TTTY22 9801153–9813245 12,093 bp	9811090–9811129	40	♦Testis-specific transcript Y-linked 22	0	0
	9811192–9811267	76		0	0
USP9Y 12701231–12860844 159,614 bp	12761218–12761250	33	Ubiquitin-specific peptidase 9 Y-linked	100.0% (33)	0
	12852196–12852244	49		95.91% (47)	0
	12783154–12783189	36		51.28% (20)	0
UTY 13233920–13480673 246,754 bp	13283351–13283407	57	Ubiquitously transcribed tetratricopeptide repeat Y-linked	85.96% (49)	0
	13356296–13356423	128		89.06% (114)	0
	13479887–13479942	56		42.86% (24)	0
	13378902–13378976	75		76.00% (57)	0
NLGN4Y 14522573–14845650 323,043 bp	14564733–14564782	50	Neuroligin 4 Y-linked	90.00% (45)	0
	14641102–14641191	90		92.22% (83)	0
	14530587–14530660	74		85.14% (63)	0
	14602714–14602806	93		43.01% (40)	0
TTTY14 18872501–19077547 205,047 bp	18993204–18993243	40	♦Testis-specific transcript Y-linked 14	82.50% (33)	0
	19040591–19040663	73		89.04% (65)	0
	19051191–19051266	76		71.05% (54)	0
	19076468–19076542	75		28.00% (21)	0
FAM41AY1 17500958–17516742 15,785 bp	18390242–18390268	27	♦Family with sequence similarity 41 member A, Y-linked 1	0	5 and 16
	17514463–17514535	73		0	1, 3, 4, 9, and 18
	18391477–18391549	73		0	1, 3, 4, 9, and 18
AC007359.1 22100814–22147484 46,671 bp	22122477–22122552	76	♦Uncharacterized LOC101929148	0	7, 9, 16, and 17
	22108467–22108519	53		0	7, 11, and 17
	22477887–22477939	53		0	7, 11, and 17
	22102367–22102415	49		0	7, 12, and 17
TTTY4 22936455–22973284 36,830 bp	22960559–22960618	60	♦Testis-specific transcript Y-linked 4	0	2, 7, 8, and 19
	24586517–24586573	57		0	7 and 8
	24587660–24587731	72		0	7, 8, 16, and 19
	25070310–25070384	75		0	7–9, 14, 17, and 20

Table 1 (continued)

[a]Chr-Y Gen	Sequence	Sequence length (bp)	[b]Function of gene	Query cover with Chr-X (bp)	[c]Autosomal Chr's
BPY2B 24607560–24639207 31,648 bp	23005338–23005470	133	Encodes a protein that interacts with ubiquitin protein ligase E3A	0	7 and 8
	24619544–24619592	49		0	21 and 2
	24622023–24622064	42		0	8
	23000633–23000734	102		0	7, 8, and 19
DAZ1 23129355–23199123 69,769 bp	23139613–23139688	76	Encodes an RNA-binding protein that is important for spermatogenesis	0	3 and 4
DAZ3 24763069–24813505 50,437 bp	23267233–23267285	53	Encodes an RNA-binding protein that is important for spermatogenesis	0	3 and 8
	24773336–24773411	76		0	3, 7, and 4
	23257864–23257932	69		0	3
	24771932–24772044	113		0	1,7, and 10
DAZ4 24833800–24907040 73,241 bp	24835630–24835705	76	Encodes an RNA-binding protein that is important for spermatogenesis	0	2 and 3
	24896627–24896702	76		0	2, 7, 8, and 12
	24851394–24851450	57		0	3

[a] The genomic locations of GRCh38.p12

[b] The information of GeneCards (Stelzer et al. 2016)

[c] The sequence was covered in a percentage between 20 and 50% of the autosomal chromosomes

♦This region is transcribed as a non-coding RNA

five males and five females to confirm our results. The minimal amount of DNA needed to obtain a reliable determination of males with contemporary DNA was 3 ng/μl. The PCR products were sequenced by Sanger, which confirmed the specific amplification of the *TTTY7*, *TSPY3*, *TTTY2*, and *TTTY22* gene regions (GenBank accession numbers: MEXCHRYTTTY22, MN385559; MEXCHRYTTTY7, MN385560; MEXCHRYTSPY3, MN385561; MEXCHRYTTTY2, MN385562).

PCR amplification of *TTTY7*, *TSPY3*, *TTTY2*, and *TTTY22* sequences from ancient pre-Hispanic samples

The bone remains of this study were discovered in 2007 and 2016 at the following sites: Puyil cave, Abrigo Rocoso Fidencio López, and Sima Cuesta Chica in Tacotalpa, Tabasco, Mexico. Due to the archaeological context, cave environmental conditions, and body-skeleton integrity, the sex

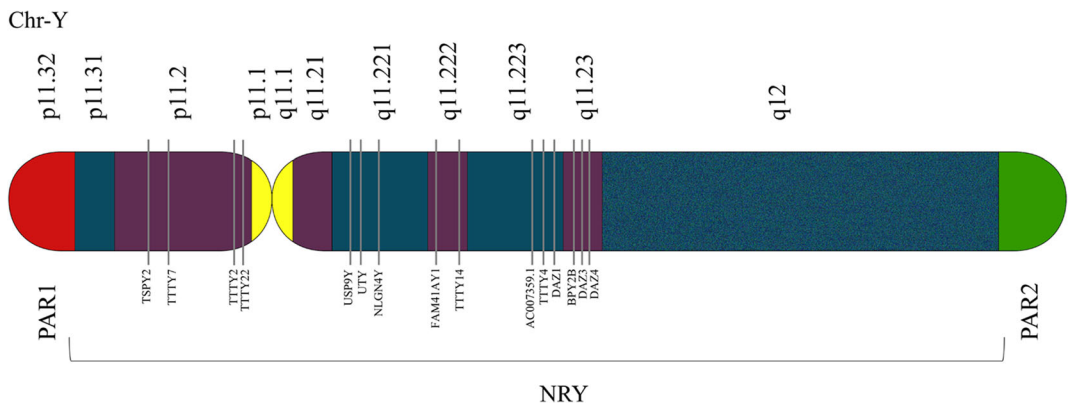

Fig. 2 Schematic representation of Chr-Y shown in the PAR1, PAR2, and NRY regions, and the location of the 15 genes used to find specific sequences to identify the male sex in ancient samples

Table 2 Primers used to identify the male sex in contemporary and ancient human samples

Primer	Sequence 5′ − 3′	Gene	Fragment size (bp)	Tm (°C)	Reference
F_rcaX$_1$ R_rcaX$_2$	AATCATCAAATGGAGATTTG GTTCAGCTCTGTGAGTGAAA	*AMELX*	130	52	Lin et al. 1995
F_rcaY$_1$ R_rcaY$_2$	ATGATAGAAACGGAAATATG AGTAGAATGCAAAGGGCTC	*AMELY*	170	52	Lin et al. 1995
F_TTTY7 R_TTTY7	CTGAGGCTGTGTGTTTGTGC CCTGGGAGTTGTAGGGTTGA	*TTTY7*	140	60.5	Used in this study
F_TSPY3 R_TSPY3	CACAAATGGGGAAGGGATA ACAACTGGGAGTCCCCTAGA	*TSPY3*	121	61.5	Used in this study
F_TTTY22 R_TTTY22	GGGGAGTGATGTAGCTGTGG TTTTTGGTCTTTTCATAAACATCAT	*TTTY22*	124	61.5	Used in this study
F_TTTY2 R_TTTY2	AGAACCCTCAACGACACACC AGGGAGAGGCATTTCCAGAC	*TTTY2*	133	60.5	Used in this study

Primer sequences used for *AMELX* amplification for female sex identification, and *AMELY*, *TTTY7*, *TSPY3*, *TTTY22*, and *TTTY2* amplification for male sex identification

identification was determined in all samples using the new molecular approach developed in this study, the AMEL test, and anthropometric measurements.

The sex of the bone samples from Tabasco was determined via amplification of the *TTTY7*, *TSPY3*, *TTTY2*, and *TTTY22* genes. The minimal amount of DNA needed to amplify the specific region in Chr-Y in human ancient samples was 15 ng/μl. None of the ancient samples showed amplification of the *TTTY22* gene. From a total of nineteen ancient samples, fourteen showed amplification of *TTTY2* (Fig. 4a), eleven showed amplification of *TTTY7* (Fig. 4b), and eight showed amplification of *TSPY3* (Fig. 4c), as shown in Table 3. These results indicated that all samples from Puyil belonged to males (Fig. 4). The samples from Abrigo Rocoso Fidencio López showed amplification of the specific amplicon for Chr-Y in four of the samples, and one sample from Sima Cuesta Chica was a male positive (Fig. 4), which confirms that at least these samples belonged to males. Four of the samples did not show amplification of any of these genes.

Sex identification using the AMELX/AMELY test

To determine the sex of contemporary and ancient samples using molecular techniques, specific regions of the X and Y chromosomes were amplified according to previous publications using the AMELX/AMELY test as described in the "Materials and methods" section (Lin et al. 1995; Ovchinnikov et al. 1998; Tschentscher et al. 2008; Gibbon et al. 2009; Dutta et al. 2017). This procedure produced amplicons of 130 bp for female and 130 and 170 bp for male contemporary DNA samples. The amplicons for Chr-X and Chr-Y in all contemporary samples are shown in Fig. 5a. All ancient samples from the Puyil cave were positive for Chr-X, and four of the samples were positive for Chr-Y (PUXTABMEX001–PUXTABMEX004) (Fig. 5b).

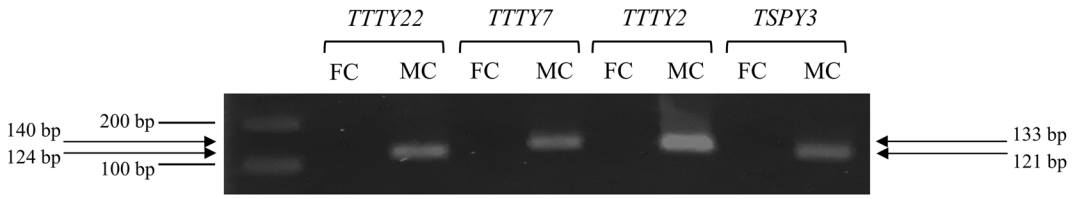

Fig. 3 Amplification of specific regions in the *TTTY22*, *TTTY7*, *TTTY2*, and *TSPY3* genes to identify male sex in contemporary human DNA samples. The DNA was amplified using the specific primers to amplify each region of Chr-Y in contemporary human samples. The specific amplicon size is indicated by the arrows on the right side, and the molecular marker is displayed on the left side. (FC) Female DNA sample (negative control). The specific amplifications for *TTTY22*, *TTTY7*, *TTTY2*, and *TSPY3* genes with sizes of 124, 140, 133, and 121 bp, respectively, are shown as indicated in top of the figure

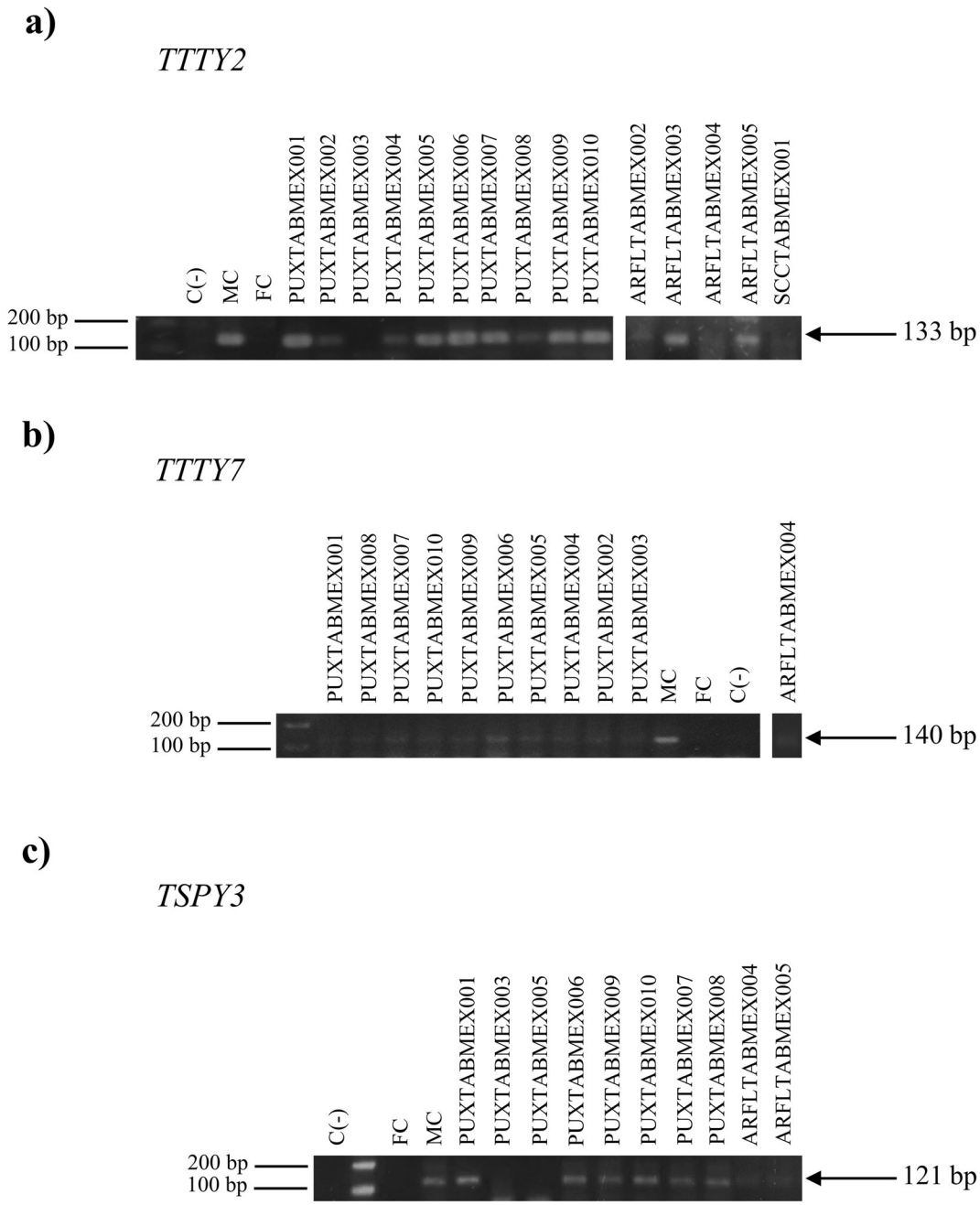

Fig. 4 Amplification of the specific regions in *TTTY2*, *TTTY7*, and *TSPY3* genes to identify male sex in ancient human DNA samples. The pre-Hispanic samples from the geographic areas Puyil cave, Abrigo Rocoso Fidencio López, and Sima Cuesta Chica were tested to determine male sex via amplification of the specific regions in *TTTY2*, *TTTY7*, and *TSPY3* genes. **a** Amplification of the specific region in *TTTY2* gene displaying the specific band of 133 bp. **b** Amplification of the specific region in *TTTY7* gene displaying the specific band of 140 bp. **c** Amplification of the specific region in *TSPY3* gene displaying the specific band of 121 bp. Female ancient DNA was used as a negative control (FC), displayed an absence of any specific band, and male ancient DNA was used as a positive control showing the amplification of the specific band in each gene (MC), and the negative control with no DNA shows the absence of any amplification band (−). The size of the specific amplicon is indicated on the right side of each panel and the molecular weight markers on the left side. The name of each sample is indicated on the top of each line

Table 3 Sex identification using different approaches in ancient samples. Sex identification in pre-Hispanic samples from Tacotalpa, Tabasco, was performed via amplification of the specific regions in the *TTTY2*, *TSPY3*, *TTTY7*, and *AMELX/AMELY* genes and the anthropometric measurement method as indicated in each column

ID	TTTY2	TTTY7	TSPY3	AMELX/AMELY		Sex determine by anthropometric measurement/bone sample	Assigned sex
	Male			X	Y		
PUXTABMEX001	+	+	+	+	+	Male/full skeleton*	Male
PUXTABMEX006[&]	+	+	+	+	–	Male/skull**	Male
PUXTABMEX007	+	+	+	+	–	Male/skull**	Male
PUXTABMEX008	+	+	+	+	–	Male/skull**	Male
PUXTABMEX009[&]	+	+	+	+	–	Male/skull**	Male
PUXTABMEX010	+	+	+	+	–	Female/skull**	Male
ARFLTABMEX004	+	+	+	+	–	Female/fragmented jaw	Male
PUXTABMEX005	+	+	–	+	–	Male/full skeleton*	Male
PUXTABMEX004[&]	+	+	–	+	+	ND/ fragmented skull	Male
PUXTABMEX002	+	+	–	+	+	Male/full skeleton*	Male
SCCTABMEX001	+	–	–	+	+	Male/coxal bone femur	Male
PUXTABMEX003	–	+	–	+	+	Female/Vertebrae	Male
ARFLTABMEX005	+	–	+	–	–	Male /coxal bone femur	Male
ARFLTABMEX002	+	–	–	–	–	Male/coxal bone femur	Male
ARFLTABMEX003	+	–	–	+	–	ND/bone fragment	Male
ARFLTABMEX001	–	–	–	+	+	Male/right iliac	Male
ARFLTABMEX006	–	–	–	+	+	Male/coxal bone femur	Male
ARFLTABMEX007	–	–	–	+	+	ND/bone fragment	Male
SCCTABMEX002	–	–	–	+	+	ND/bone fragment	Male

No label indicates that remains are from the Classic period. Supplementary Table 1 shows the bone remain used to obtain the DNA used in the molecular studies

ND not determined because of bone fragmentation, poor preservation of bone fragment, or the remains were of a subadult very fragmented bone remains

*Skeletons without skulls

**Skulls without skeletons

[&] Samples from the Archaic period

Five ancient samples from Abrigo Rocoso Fidencio López were positive for Chr-X (ARFLTABMEX001, ARFLTABMEX003, ARFLTABMEX004, ARFLTABMEX006, and ARFLTABMEX007), and three of these samples were positive for Chr-Y (ARFLTABMEX001, ARFLTABMEX006, and ARFLTABMEX007) (Fig. 5c). The two ancient samples from Sima Cuesta Chica were positive for Chr-X and Chr-Y (SCCTABMEX001 and SCCTABMEX002) (Fig. 5c). These results suggest that at least nine of the nineteen samples corresponded to the males using the AMELX/AMELY test (Fig. 5). The Chr-X amplicon was observed in eight samples, but this result does not indicate that these samples corresponded to the females because DNA that matches the Chr-Y region may be degraded.

Sex identification by anthropometric measurements

The bone remains used in this study for each sample and the results of the sex identification of fifteen of the samples that were subjected to anthropometric measurements are displayed in Table 3 and Supplementary Table 1. The sex was determined in fifteen of the samples using anthropometric measurements as described in the "Materials and methods" section and according to conservation of the bone remains. Twelve of the fifteen

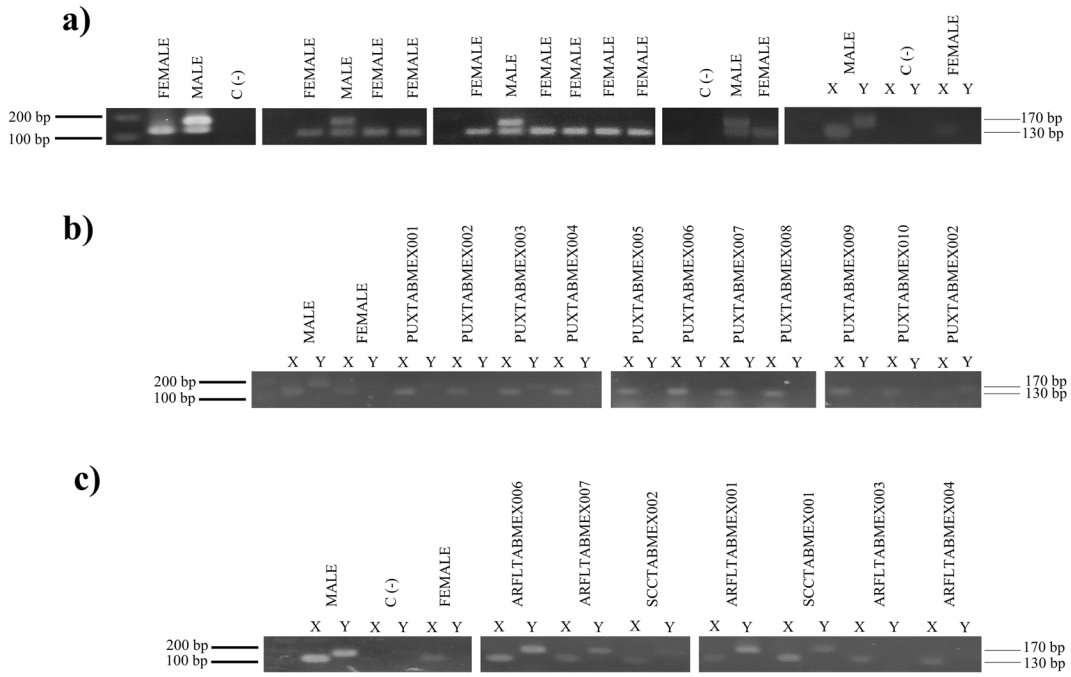

Fig. 5 Sex identification using the AMELX/AMELY test. To determine the sex of contemporary and ancient samples, specific regions of the X and Y chromosomes of female and male genomes were amplified according to previous publications using the AMELX/AMELY test described in the "Materials and methods" section. This procedure showed amplicons of 130 bp for females and 130 and 170 bp for males in contemporary DNA samples. **a** The amplicons for X and Y chromosomes in contemporary samples using the AMELX/AMELY test are shown; the arrows show amplicons of 130 bp for females (Chr-X) or 130 (Chr-X) and 170 bp (Chr-Y) for males. The amplicons in ancient samples from the archaeological sites are shown in **b** the Puyil cave, **c** Abrigo Rocoso Fidencio López, and Sima Cuesta Chica. The amplicon sizes are shown by the arrows on the right side, and the molecular weighs are shown on the left side

bone samples characterized using this approach were found to be from males. The sex identification of five of these ancient samples was confirmed using the amelogenin test, and ten of the samples were confirmed using the anthropometric procedure.

Discussion

To correctly identify males in ancient samples, we developed a new approach to identify males in ancient samples using the amplification of four specific regions in the *TTTY7*, *TSPY3*, *TTTY2*, or *TTTY22* genes of Chr-Y that are absent in the Chr-X and autosomes. The analysis of these four genes using IGV software (Thorvaldsdóttir et al. 2013; Integrative Genomics Viewer 2018) indicated that they are exclusively found in males. The results of this study confirmed the specificity of this region for the male sex in samples of contemporary human DNA (Fig. 3). Confirmation of the specificity for male was demonstrated as well as the absence of the amplification of these genes in contemporary DNA samples from females.

The *TTTY7* gene region encodes a non-coding RNA (Y-linked 7) (Stelzer et al. 2016; GeneCards-Human Genes n.d.) and was amplified from contemporary and ancient human DNA samples from Mexico (Fig. 4b), confirming the male sex in eleven of the ancient samples, nine from the Puyil cave, and two from Abrigo Rocoso Fidencio López.

The *TSPY3* gene, which encodes the male-specific protein Y-linked 3 gene (Stelzer et al. 2016; GeneCards-Human Genes n.d.) showed amplification of the specific region in eight of the ancient samples, six from the Puyil cave, and two from Abrigo Rocoso Fidencio López (Fig. 4c), indicating that they were males.

The *TTTY2* gene, which is transcribed into a non-coding RNA (Y-linked 2) (Stelzer et al. 2016; GeneCards-Human Genes n.d.), was amplified in nine samples from the Puyil cave, four samples from Abrigo Rocoso Fidencio López, and one sample from Sima Cuesta Chica (Fig. 4a).

The *TTTY22* gene, which is transcribed into a testis-specific non-coding RNA (Y-linked 22) (Stelzer et al. 2016; GeneCards-Human Genes n.d.), did not amplify in any of the ancient samples, which may be related to allelic dropout (Takayama et al. 2009; Kim et al. 2013; Stevens et al. 2017) or DNA degradation in ancient DNA samples. Ancient DNA degradation depends on the environmental conditions and the microbial activity in the decaying tissues (Lindahl 1993). Environmental conditions that favour ancient DNA survival from tissues are low temperatures and dryness, which would limit hydrolytic and oxidative processes (Lindahl 1993; Dabney et al. 2013). Overrepresented purines (depurination) at positions adjacent to the breaks in the ancient DNA may also contribute to its degradation (Willerslev and Cooper 2005; Briggs et al. 2007). Consequently, this DNA region is not a good candidate to determine the male sex in ancient samples.

AMELX/AMELY was also tested to specifically identify sex (Stone et al. 1996; Esteve-Codina et al. 2008; Butler and Li 2014; Dutta et al. 2017). The results from using the AMELX/AMELY method indicated that nine of the samples were males, and eight samples may be females.

Anthropometric measurements of bones (Stone et al. 1996; Veroni et al. 2010; Harris and Case 2012; Krishan et al. 2016; Ubelaker and DeGaglia 2017; Hora and Sládek 2018; García-Campos et al. 2018a, b) have been largely used to identify sex. Fifteen of the nineteen samples were tested for sex determination using the anthropometric measurements and results showed twelve males and three females. It is worth emphasizing that the method of sex identification using anthropometric measurements of full adult skeletons, a coxal femur bone, a right iliac bone, and three out of four skulls correctly identified males. These results are in agreement with previous studies that successfully identified the sex of adult full skeletons and coxal femur bones (Ovchinnikov et al. 1998; Veroni et al. 2010; Harris and Case 2012; Krishan et al. 2016; Ubelaker and DeGaglia 2017; Hora and Sládek 2018; García-Campos et al. 2018a, b). Similar approaches have been also used in the sex determination of newborns and subadults. However, they are more laborious than the sex identification of adult skeletons and their accuracy of sex identification estimation is lower in subadult than in adult skeletons (Fazekas and Kósa 1978; Schutkowski 1987; Luna et al. 2017). Therefore, the molecular procedures should be preferred when skeletons or useful bone remains are not available for sex determination.

Amplification of the specific regions in the *TTTY7*, *TSPY3*, or *TTTY2* genes from ancient bone samples was positive in fifteen of the nineteen samples (Table 3). Four of these fifteen samples were also positive using the AMELX/AMELY procedure, and nine samples were positive using the anthropometric measurement method. Four samples that were negative for all the male-specific DNA markers proposed to identify males in the ancient samples were positive for the AMELX/AMELY test, and two of these samples were also positive using the anthropometric measurement method.

The molecular procedures developed in this study used along with the AMELX/AMELY and anthropometric measurements are very useful in confirming the male sex from the samples. From a total of nineteen samples in the present study that were identified as males, seven samples showed the specific amplicons for the three genes of this study; four samples showed two of the specific gene regions; and four samples showed one of the specific gene regions, which gives a total of fifteen samples positive for male identification. Four samples of a total of nineteen which were not amplified any of the three new gene regions proposed in this study tested positive for Chr-Y using the AMELX/AMELY method. Although seventeen samples showed the amplicon for Chr-X, only nine of these samples were identified as males using the AMELX/AMELY method (Table 3). Therefore, the method developed here is complementary and accurate in identifying males from ancient samples and/or useful for forensic analysis. These results also indicate that the AMELX/AMELY method is not always accurate in identifying males. This may be due to deletion of a large fragment and mutations of the amelogenin gene in the Y-homologue, which results in the process known as allelic dropout that leads to gender typing errors (Esteve-Codina et al. 2008; Takayama et al. 2009; Kim et al. 2013; Stevens et al. 2017). This depends on the type of population, possibly specific to the lineage, and inherited deletions (Kashyap et al. 2006). Similarly, failure of the amplification of the *TTTY22* gene may be due to allelic dropout or DNA degradation of ancient DNA samples.

Molecular procedures are very important to identify sex in ancient and forensic samples using the specific *TTTY7*, *TSPY3*, or *TTTY2* genes and the

AMELX/AMELY test (Table 3) in addition to anthropometric measurements when a correct bone sample is available. Therefore, we recommend sex identification by the simultaneous use of molecular procedures for the *TTTY7*, *TSPY3*, or *TTTY2* gene male-specific DNA markers identified in this study and the AMELX/AMELY test.

The molecular identification method has certain advantages over morphometric methods, because it is not affected by individual variation in the size and the morphology of skeletal materials. It can be used to identify sex from foetal and subadult remains and is not restricted by physical fragmentation, requiring only one complete bone or tooth for sex determination. However, molecular methods have several constraints: (a) molecular contamination, which can usually be avoided by using strict laboratory conditions (Llamas et al. 2017); (b) molecular preservation of the specimen, which is the greatest challenge for molecular methods, because of the difficulty in predicting the level of preservation (Lindahl 1993); (c) environmental factors, which can induce molecular degradation that severely impairs the process of obtaining DNA for forensic analysis (Dabney et al. 2013); (d) molecular methods can be costly and thus their use is often restricted to forensic material where other methods are not useful; and (e) molecular methods are destructive in obtaining DNA from an ancient bone remain. However, it is possible to extract DNA from bones with minimal damage to the ancient bone because ancient DNA extraction needs minimal quantities (25–700 mg). Of importance is that bone destruction is justifiable in cases when the results are likely to inform on debates to test hypotheses, and/or when their destruction is not detrimental to other research areas (Kaestle and Horsburgh 2002).

The anthropometric methods are usually based on the assessment of more than one anatomical feature from the skeleton, and their effectiveness depends, to a great extent, on the state of conservation of the anatomical structures, which in many cases are of poor quality, with these methods not yielding conclusive results from subadult skeletons.

Conclusions

The new approach developed to identify male sex in ancient samples via amplification of the male-specific regions of the *TTTY7*, *TSPY3*, or *TTTY2* genes is a useful tool to confirm male sex in ancient human remains and/or forensic studies when sex cannot be determined using anthropometric measurements or the AMELX/AMELY test. This approach is especially important in ancient samples and forensic research when the age of the individual is unknown, the availability of the bone under study is limited, or the DNA is highly damaged. Furthermore, these genes may also be searched in silico to identify sex if massive sequencing is developed. Since male sex identification was not assessed in all used samples, we would like to stress the value of using complementary approaches in sex identification of human remains, especially when methods applied in one study may not be applicable to others with the same level of confidence.

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Code availability Not applicable.

Author contribution MTN-R and M-d-LM conceived and designed the research; MTN_R performed the research; EA- and ET-E contributed with the data of the ancient bones; ED-de-la-Cruz and MAM-G performed amplification experiments; NG-H obtained the contemporary DNA and contributed with data analysis; MTN-R and M-d-LM wrote the paper; all authors read and approved the manuscript.

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Data availability All data generated or analysed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval The samples were registered to project number 2017-785-071, and the National Ethics Commission (IMSS) ethics committee approved the study.

Consent to participate The participants provided their written consent to participate.

Consent for publication The participants provided their written consent for publication.

References

Adler CJ, Haak W, Donlon D, Cooper A (2011) Survival and recovery of DNA from ancient teeth and bones. J Archaeol Sci 38(5):956–964. https://doi.org/10.1016/j.jas.2010.11.010

Álvarez-Sandoval BA, Manzanilla LR, Montiel R (2014) Sex determination in highly fragmented human DNA by high-resolution melting (HRM) analysis. PLoS One 9:e104629. https://doi.org/10.1371/journal.pone.0104629

Bashamboo A, Giran HM, Azfer MA et al (2003) Genomics of the human Y chromosome and molecular diagnosis. Proc Indian Natl Sci Acad B Biol Sci, B69:525–538

Bass WM (1987). Human osteology: a laboratory and field manual. Missouri Archaeological Society

Blau S, Ubelaker DH (2009) Handbook of forensic anthropology and archaeology. Left Coast Press

Briggs AW, Stenzel U, Johnson PLF, Green RE, Kelso J, Prufer K, Meyer M, Krause J, Ronan MT, Lachmann M, Paabo S (2007) Patterns of damage in genomic DNA sequences from a Neandertal. Proc Natl Acad Sci 104:14616–14621. https://doi.org/10.1073/pnas.0704665104

Butler EK, Li R (2014) Genetic markers for sex identification in forensic DNA analysis. J Forensic Investig 2(3)

Campos PF, Craig OE, Turner-Walker G, Peacock E, Willerslev E, Gilbert MTP (2012) DNA in ancient bone—where is it located and how should we extract it? Ann Anat 194(1):7–16. https://doi.org/10.1016/j.aanat.2011.07.003

Candelas-González N, Rascón Pérez J, Chamero B et al (2017) Geometric morphometrics reveals restrictions on the shape of the female os coxae. J Anat 230:66–74. https://doi.org/10.1111/joa.12528

Dabney J, Meyer M, Pääbo S (2013) Ancient DNA Damage. Cold Spring Harb Perspect Biol 5. https://doi.org/10.1101/cshperspect.a012567

Dutta P, Bhosale S, Singh R et al (2017) Amelogenin gene—the pioneer in gender determination from forensic dental samples. J Clin Diagn Res 11(2):ZC56–ZC59. https://doi.org/10.7860/JCDR/2017/22183.9407

Esteve-Codina A, Niederstätter H, Parson W (2008) "GenderPlex" a PCR multiplex for reliable gender determination of degraded human DNA samples and complex gender constellations. Int J Legal Med 123:459–464. https://doi.org/10.1007/s00414-008-0301-z

Fazekas IG, Kósa F (1978) Forensic fetal osteology. Akadémiai Kiadó, Budapest

García-Campos C, Martinón-Torres M, Martín-Francés L, Martínez de Pinillos M, Modesto-Mata M, Perea-Pérez B, Zanolli C, Labajo González E, Sánchez Sánchez JA, Ruiz Mediavilla E, Tuniz C, Bermúdez de Castro JM (2018a) Contribution of dental tissues to sex determination in modern human populations. Am J Phys Anthropol 166:459–472. https://doi.org/10.1002/ajpa.23447

García-Campos C, Martinón-Torres M, de Pinillos MM et al (2018b) Modern humans sex estimation through dental tissue patterns of maxillary canines. Am J Phys Anthropol 167:914–923. https://doi.org/10.1002/ajpa.2371

Gaudio D, Fernandes DM, Schmidt R, Cheronet O, Mazzarelli D, Mattia M, O'Keeffe T, Feeney RNM, Cattaneo C, Pinhasi R (2019) Genome-wide DNA from degraded petrous bones and the assessment of sex and probable geographic origins of forensic cases. Sci Rep 9:8226. https://doi.org/10.1038/s41598-019-44638-w

GeneCards-Human Genes (n.d.) | Gene Database | URL https://www.genecards.org/

Gibbon V, Paximadis M, Strkalj G et al (2009) Novel methods of molecular sex identification from skeletal tissue using the amelogenin gene. Forensic Sci Int Genet 3(2):74–79. https://doi.org/10.1016/j.fsigen.2008.10.007

Graw M, Wahl J, Ahlbrecht M (2005) Course of the meatus acusticus internus as criterion for sex differentiation. Forensic Sci Int 147:113–117. https://doi.org/10.1016/j.forsciint.2004.08.006

Green RE, Krause J, Briggs AW, Maricic T, Stenzel U, Kircher M, Patterson N, Li H, Zhai W, Fritz MHY, Hansen NF, Durand EY, Malaspinas AS, Jensen JD, Marques-Bonet T, Alkan C, Prufer K, Meyer M, Burbano HA, Good JM, Schultz R, Aximu-Petri A, Butthof A, Hober B, Hoffner B, Siegemund M, Weihmann A, Nusbaum C, Lander ES, Russ C, Novod N, Affourtit J, Egholm M, Verna C, Rudan P, Brajkovic D, Kucan Z, Gusic I, Doronichev VB, Golovanova LV, Lalueza-Fox C, de la Rasilla M, Fortea J, Rosas A, Schmitz RW, Johnson PLF, Eichler EE, Falush D, Birney E, Mullikin JC, Slatkin M, Nielsen R, Kelso J, Lachmann M, Reich D, Paabo S (2010) A draft sequence of the Neandertal genome. Science 328(5979):710–722. https://doi.org/10.1126/science.1188021

Halder A, Kumar P, Jain M, Iyer VK (2017) Copy number variations in testicular maturation arrest. Andrology 5(3):460–472. https://doi.org/10.1111/andr.12330

Harris SM, Case DT (2012) Sexual dimorphism in the tarsal bones: implications for sex determination. J Forensic Sci 57:295–305. https://doi.org/10.1111/j.1556-4029.2011.02004.x

Hora M, Sládek V (2018) Population specificity of sex estimation from vertebrae. Forensic Sci Int 291:279.e1–279.e12. https://doi.org/10.1016/j.forsciint.2018.08.015

Integrative Genomics Viewer (2018) | Nature Biotechnology | URL https://www.nature.com/articles/nbt.1754

Kaestle FA, Horsburgh KA (2002) Ancient DNA in anthropology: methods, applications, and ethics. Am J Phys Anthropol Suppl 35:92–130. https://doi.org/10.1002/ajpa.10179

Kashyap V, Sahoo S, Sitalaximi T, Trivedi R (2006) Deletions in the Y-derived amelogenin gene fragment in the Indian population. BMC Med Genet 7:37. https://doi.org/10.1186/1471-2350-7-37

Kim K-Y, Kwon Y, Bazarragchaa M, Park AJ, Bang H, Lee WB, Lee J, Lee KH, Kim BJ, Kim K (2013) A real-time PCR-based amelogenin Y allele dropout assessment model in gender typing of degraded DNA samples. Int J Legal Med 127:55–61. https://doi.org/10.1007/s00414-011-0663-5

Krishan K, Chatterjee PM, Kanchan T et al (2016) A review of sex estimation techniques during examination of skeletal remains in forensic anthropology casework. Forensic Sci Int 261: 165.e1–165.e8. https://doi.org/10.1016/j.forsciint.2016.02.007

Lin Z, Kondo T, Minamino T, Ohtsuji M, Nishigami J, Takayasu T, Sun R, Ohshima T (1995) Sex determination by polymerase chain reaction on mummies discovered at Taklamakan desert in 1912. Forensic Sci Int 75:197–205. https://doi.org/10.1016/0379-0738(95)01789-5

Lindahl T (1993) Instability and decay of the primary structure of DNA. Nature 362:709–715. https://doi.org/10.1038/362709a0

Llamas B, Valverde G, Fehren-Schmitz L, Weyrich LS, Cooper A, Haak W (2017) From the field to the laboratory: controlling DNA contamination in human ancient DNA research in the high-throughput sequencing era. STAR 3:1–14. https://doi.org/10.1080/20548923.2016.1258824

Luna LH, Aranda CM, Santos AL (2017) New method for sex prediction using the human non-adult auricular surface of the ilium in the collection of identified skeletons of the University of Coimbra. Int J Osteoarchaeol 27:898–911. https://doi.org/10.1002/oa.2604

Moore M (2013) Sex estimation and assessment. Research methods in human skeletal biology, In, pp 91–116

Ovchinnikov IV, Ovtchinnikova OI, Druzina EB et al (1998) Molecular genetic sex determination of Medieval human remains from North Russia: comparison with archaeological and anthropological criteria. Anthropol Anz 56(1):7–15

Quincey D, Carle G, Alunni V, Quatrehomme G (2013) Difficulties of sex determination from forensic bone degraded DNA: a comparison of three methods. Sci Justice J Forensic Sci Soc 53:253–260. https://doi.org/10.1016/j.scijus.2013.04.003

Quintana-Murci L, Fellous M (2001). The human Y chromosome: the biological role of a "functional wasteland." J Biomed Biotechnol 1:18–24. https://doi.org/10.1155/S1110724301000080

Rohland N, Hofreiter M (2007) Ancient DNA extraction from bones and teeth. Nat Protoc 2:1756–1762. https://doi.org/10.1038/nprot.2007.247

Schutkowski H (1987) Sex determination of fetal and neonate skeletons by means of discriminant analysis. Int J Anthropol 2:347–352. https://doi.org/10.1007/BF02443994

Skoglund P, Malmström H, Raghavan M et al (2012) Origins and genetic legacy of Neolithic farmers and hunter-gatherers in Europe. Science (New York, N.Y.) 336(6080):466–469. https://doi.org/10.1126/science.1216304

Skoglund P, Storå J, Götherström A, et al. Accurate sex identification of ancient human remains using DNA shotgun sequencing, vol. 40; 2013.

Stelzer G, Rosen N, Plaschkes I, Zimmerman S, Twik M, Fishilevich S, Stein TI, Nudel R, Lieder I, Mazor Y, Kaplan S, Dahary D, Warshawsky D, Guan-Golan Y, Kohn A, Rappaport N, Safran M, Lancet D (2016) The GeneCards suite: from gene data mining to disease genome sequence analyses. Curr Protoc Bioinformatics 54:1.30.1–1.30.33. https://doi.org/10.1002/cpbi.5

Stevens AJ, Taylor MG, Pearce FG, Kennedy MA (2017) Allelic dropout during polymerase chain reaction due to G-quadruplex structures and DNA methylation is widespread at imprinted human loci. G3 (Bethesda) 7:1019–1025. https://doi.org/10.1534/g3.116.03868

Stone AC, Milner GR, Pääbo S, Stoneking M (1996) Sex determination of ancient human skeletons using DNA. Am J Phys Anthropol 99:231–238. https://doi.org/10.1002/(SICI)1096-8644(199602)99:2<231::AID-AJPA1>3.0.CO;2-1

Takayama T, Takada N, Suzuki R, Nagaoka S, Watanabe Y, Kumagai R, Aoki Y, Butler JM (2009) Determination of deleted regions from Yp11.2 of an amelogenin negative male. Legal Med 11:S578–S580. https://doi.org/10.1016/j.legalmed.2009.01.049

Thorvaldsdóttir H, Robinson JT, Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform 14:178–192. https://doi.org/10.1093/bib/bbs017

Tschentscher F, Frey UH, Bajanowski T (2008) Amelogenin sex determination by pyrosequencing of short PCR products. Int J Legal Med 122:333–335. https://doi.org/10.1007/s00414-008-0228-4

Ubelaker DH (2014) Osteology reference collections. In: Smith C (ed) Encyclopedia of global archaeology. Springer New York, New York, pp 5632–5641

Ubelaker DH, DeGaglia CM (2017) Population variation in skeletal sexual dimorphism. Forensic Sci Int 278:407.e1–407.e7. https://doi.org/10.1016/j.forsciint.2017.06.012

Veroni A, Nikitovic D, Schillaci MA (2010) Brief communication: sexual dimorphism of the juvenile basicranium. Am J Phys Anthropol 141:147–151. https://doi.org/10.1002/ajpa.21156

White TD, Folkens PA (2005) The human bone manual. Elsevier Academic Press, San Diego

Willerslev E, Cooper A (2005) Ancient DNA. Proc Biol Sci 272: 3–16. https://doi.org/10.1098/rspb.2004.2813