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SEX IDENTIFICATION OF SLAVE SACRIFICE VICTIMS FROM QIN STATE TOMBS IN THE SPRING AND AUTUMN PERIOD OF CHINA USING ANCIENT DNA*

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Ancient DNA (aDNA) analysis was employed to determine the sex of slave sacrifice victims from Qin State tombs during the Spring and Autumn Period of China. It is difficult to obtain sex information from fragmentary skeleton samples with the aid of skeletal morphology methods. aDNA was extracted from the dentine in selected tooth samples of sacrificial slaves using a modified traditional method, which combines together phenol/chloroform extraction, silicon dioxide adsorption and ultrafiltration concentration. Based on the sequence differences between the amelogenin homologous gene in the X and that in the Y chromosome, a pair of specific primers was designed to identify the sex of the selected samples. In the selected eight typical samples, the aDNA analytical results revealed that three were males and two were females. These findings indicate that molecular sex identification might provide more valuable information for archaeological research on the institution of slave sacrifice in the Spring and Autumn Period of China.

KEYWORDS: AMELOGENIN GENE, ANCIENT DNA, SEX IDENTIFICATION, SLAVE SACRIFICE VICTIMS, MOLECULAR ARCHAEOLOGY

INTRODUCTION

Slave sacrifice victims, either buried alive with their masters or killed using a broad array of intentionally brutal methods, have been found in many graves in Chinese history (Yang 2000; Yuan and Flad 2005). Slave sacrifice is sometimes regarded as a cruel practice carried out by a few slave societies (Green 1998; Sutter and Verano 2007; Wilson *et al*. 2007). It has been demonstrated that the number of slave sacrifice victims reached a peak in the Spring and Autumn Period, which represents an era in Chinese history from 770 BC to 476 BC. For example, 164 slave sacrifice victims were excavated from a large grave of the Qin State (Gan 2008). There were many battles for annexation of about 170 smaller states during this period, and most of the prisoners of war and criminals of the state became nobles' slaves. Slaves were usually selected as sacrificial victims at the funerals of their masters. Slave sacrifice victims were a special category of slave in the Spring and Autumn Period. The study of this category is helpful for investigations into law systems, social relationships and sacrificial burial institutions in Chinese slave society.

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In the process of archaeological excavation, archaeologists have always focused much of their attention on studying the masters of the graves, and hardly paid any attention to the slave sacrifice victims in the same grave. Slave sacrifice victims should be considered by archaeologists, anthropologists and historians in order to provide more valuable information on grave investigation. In archaeological practice, the sex of slave sacrifice victims could possibly play a very important role in understanding grave composition, grave grade, social structure and social relationships. In our study, it is necessary and interesting to investigate the sex information of slave sacrifice victims in graves during the Spring and Autumn Period.

Traditional sex identification usually depends on the morphology of the skeleton. The morphometric sex identifying method, a traditional method for archaeological research, has to depend on whole skeletons. Unfortunately, morphometric sexing methods are not applicable to infant skeletons, sub-adult skeletons, fragmentary skeletons disturbed by grave robbers, a few disarticulated bones of a secondary burial and incomplete skeletons. There were no infant skeletons or sub-adult skeletons, but there were incomplete adult skeletons and fragmentary adult bones in Spring and Autumn Period sacrificial slave victim burials. Most of the slave sacrifice victims in the graves of Qin State during the Spring and Autumn Period died with their suffering reflected in the shape and contortions of their bodies, which has made it very difficult for archaeologists to obtain sex information directly, using the morphometric sex identification method.

Molecular sex identification methods serve as emerging approaches that do not require a complete skeleton, in contrast to traditional osteological sex identification. After extracting DNA, the genotype surviving in the bones is analysed using molecular sex identification methods that mainly rely upon the sequence differences of special genes located in the human X and Y chromosomes. Therefore, this promises to become an important method in archaeological research.

We mainly applied the PCR test for the amelogenin gene to obtain sex information in tooth samples of slave sacrifice victims, whose skeletons were excavated at an archaeological site called the Qin State's cemetery of the Spring and Autumn Period, in Fengqiang County, Shaanxi Province, China, in 2002.

MATERIALS AND METHODS

Archaeological samples

All tooth samples of slave victims came from an archaeological site called the Qin State's cemetery of the Spring and Autumn Period in Fengqiang County, Shaanxi Province, China. The archaeological site (107°14′E, 34°28′N) was located on the first terrace of the Qian River, which is one of tributary rivers in the Wei River system (Fig. 1).

According to Chinese archaeological experience, slave sacrifice victims could be identified by the grave structure. The master, with funerary objects, was laid in a coffin in the main room of the tomb, while slave sacrifice victims were deposited haphazardly in small rooms in the side walls of the tombs. Eight typical graves, identified as M05, M10, M42, M43, M45, M76, M90 and M96, were included in our study. For example, the body in grave M42 was flexed within a small room, with no funerary objects (Fig. 2). In some graves, the skeletons of slave sacrifice victims were incomplete (no legs or no arms), as shown in Table 1.

In the process of archaeological excavation, all skeletons of slave victims were collected by archaeologists using sterile gloves. According to the preservation conditions of the skeleton, eight

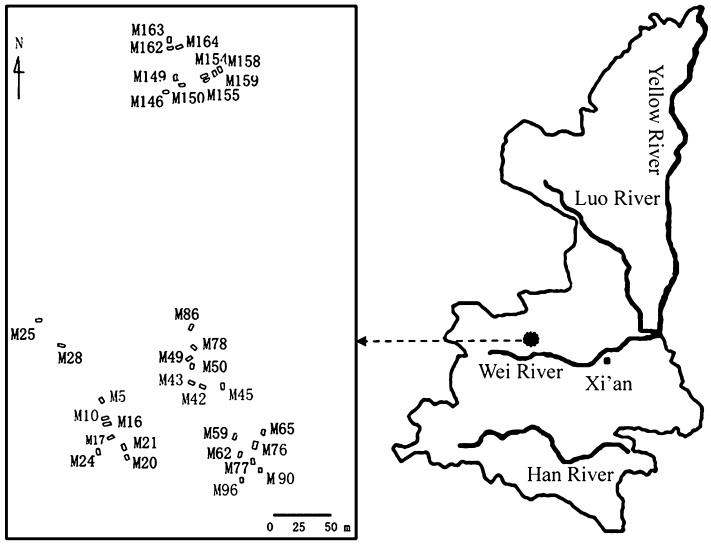

Figure 1 *A sketch map of the cemetery location of the Spring and Autumn Period in Fengqiang County, Shaanxi Province, China.*

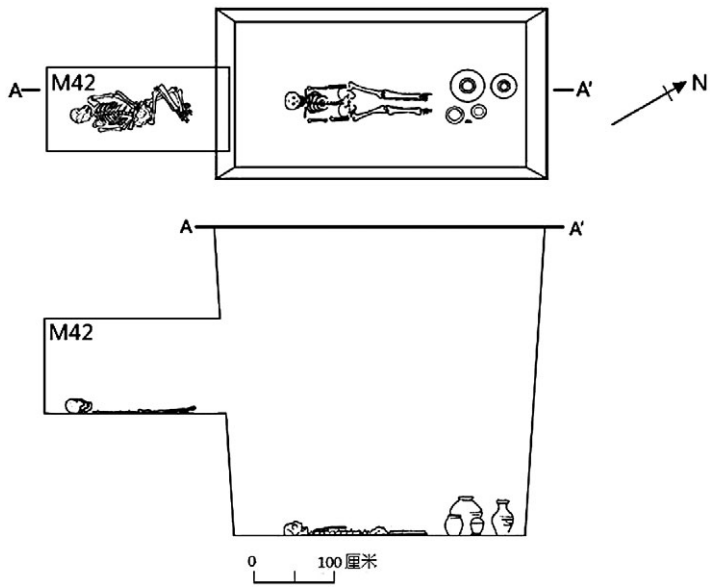

Figure 2 *A schematic illustration of the grave structure in the tomb with slave sacrifice victim M42.*

tooth samples were chosen from the eight typical graves of M05, M10, M42, M43, M45, M76, M90 and M96 *in situ*. Dental identification of slave sacrifice victims' tooth samples was determined according to dental anatomy. The total tooth length (TTL), mesiodistal (MD) diameter and buccolingual (BL) crown diameters were measured, respectively. Data of tooth samples from M05, M10, M42, M43, M45, M76, M90 and M96 are shown in Table 2. Tooth samples without

Table 1 *The taphonomy characters of slave sacrifice victims*

	Master	*Slave sacrifice victims*
Number	Only one	One or several
Location	The tomb's main room	The tomb's small room on the side wall
Burial type	Extended burial	Contracted burial
Funerary type	In coffin	No
Funerary objects	Jade, bronze, pottery, etc.	No
Skeleton shape	Complete (general)	Incomplete or complete

pores or cavities were selected. The eight tooth samples were labelled as M05t, M10t, M42t, M43t, M45t, M76t and M90t, respectively.

Ancient DNA extraction

The silicon dioxide was purchased from Sigma and the proteinase K from Merck KGaA Corporation (Germany). The ultrafiltration tubes (30 kDa) were purchased from the Zhonghao Shidai Biotechnology Company (Beijing, China). The DNA Extraction Kit was purchased from Qiagen (Beijing, China). After the removal of surface clay or dirt with a bistoury or brush and polishing with sandpaper, the exterior 2–3 mm of the tooth samples were scraped with a bistoury. In order to eliminate contaminant DNA as much as possible, the tooth surface had to be irradiated by UV (254 nm) for 30 min. Then, the tooth samples were treated using the following procedures in turn, after being split with a fretsaw: 5% bleach purgation, cleaning with 80% ethanol twice, air drying for 24 h, UV irradiation (254 nm) for 30 min, trituration with the aid of liquid nitrogen, marking, and then preserving in a 4°C environment. The experiments were carried out in a specially controlled laboratory to prevent contamination with modern DNA. The ancient DNA fragments were extracted using a modified phenol/chloroform – silicon dioxide – ultrafiltration method. The detailed procedures were as follows: 0.5 g of tooth powder was added into 5 ml of extraction buffer (50 mM Tris pH 8.0, 0.2 M EDTA pH 8.0, 1 mM NaCl, 1.2 mg ml^{-1} DTT (DL-dithiothreitol), 0.5% SDS, 500 µg ml^{-1} proteinase K), vibration at 37°C overnight, incubation at 55°C for 6 h, and then the mixture was extracted with phenol/chloroform twice. The supernatant liquid was collected and concentrated into 300 µl in the vacuum rotatory concentrator. The iced 80% ethanol with equal volume was added into the concentrated supernatant liquid and the mixture was absorbed using a silicon dioxide column. After the columns were centrifuged at 12 000 rpm for 30 s, the filtered liquid was discarded. Having been washed twice with iced 80% ethanol, the silicon dioxide column was centrifuged at 12 000 rpm for 2 min and placed at room temperature for 10 min to dry. After that, the silicon dioxide columns were shifted into a disinfected centrifuge tube and 200 µl of heated TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0, 60–70°C) was added at room temperature for 5 min. aDNA in the silicon dioxide columns was eluted by centrifuging at 12 000 rpm for 30 s. The eluent was added into an ultrafiltration tube (30 kDa), which was centrifuged at 5000 rpm for 30 s, and the filtered liquid was discarded. Then 100 µl of TE buffer was added into the ultrafiltration tube (30 kDa) for 2 min at room temperature and the eluent was gathered. The blood mtDNA of the technicians (T1, T2 and T3) and the archaeologists (A1 and A2) was extracted, this being conducted in a separate laboratory.

Table 2 *Data of tooth samples from slave sacrifice victims in Qin State tombs in the Spring and Autumn Period of China*

Feature	*M05*		*M10*		*M42*		*M43*		*M45*		*M76*		*M90*		*M96*	
Location	$P1^U$	$P2^U$	$I1^U$	$M2^L$	C^L	$M1^U$	$I2^U$	$P1^L$	$M1^U$	C^U	$M1^L$	$I1^U$	$M2^U$	$P1^U$	$M3^U$	$P2^L$
TTL (mm)	20.49	18.54	20.64	16.09	22.77	18.31	21.01	19.11	19.01	25.21	15.41	21.98	19.19	20.85	17.52	18.64
MD diameter (mm)	6.64	6.69	8.45	10.86	6.72	9.86	6.46	6.66	9.98	7.68	9.33	8.97	9.65	6.73	9.74	6.49
BL diameter (mm)	9.06	8.82	7.13	10.16	6.98	10.48	6.76	7.68	10.64	8.05	9.62	7.56	10.86	9.87	10.34	7.41

Note: TTL stands for total tooth length; MD diameter stands for mesiodistal diameter; and BL diameter stands for buccolingual crown diameter. I, incisor; C, canine; P, premolar; M, molar; U, upper; L, lower.

Table 3 *The primers of amplification for fragments of HVR1*

Fragments of HVR1	*Primers*	*PCR conditions (30 cycles)*
L16055-H16139 (84 bp)	5′-GAAGCAGATTTGGGTACCAC-3′ 5′-TACTACAGGTGGTCAACTAT-3′	94°C 1 min, 55°C 1 min, 72°C 1 min
L16131-H16218 (87 bp)	5′-CACCATGAATATTGTACGGT-3′ 5′-TGTGTGATAGTTGAGGGTTG-3′	94°C 1 min, 53°C 1 min, 72°C 1 min
L16209-H16356 (147 bp)	5′-CCCCATGCTACAAGCAAGT-3′ 5′-GTCATCCATGGGGACGAGAA-3′	94°C 1 min, 55°C 1 min, 72°C 1 min
L16313-H16410 (97 bp)	5′-CCCTTAACAGTACATAGTAC-3′ 5′-GCGGGATATTGTTTTCACGG-3′	94°C 1 min, 53°C 1 min, 72°C 1 min

Amplification of the HVR1 fragment

The HVR1 fragment (16055-16569) of ancient mtDNA was amplified with four pairs of overlapping primers (Handt *et al*. 1996) and under the polymerase chain reaction (PCR) conditions shown in Table 3 (numbering according to GenBank accession number J01415). The thermal cycler conditions consisted of an initial 4 min incubation at 94°C, followed by 30 cycles, with a final extension step at 72°C for 10 min. The 50 μl reaction mix contained 2.5 U of *Taq* polymerase, 200 mM of each of the dNTPs, 1.5 mM of $MgCl_2$, 20 mM of KCl, 20 μM of each primer and 2 mg ml^{-1} of bovine serum albumin. The PCR products were directly sequenced by the Beijing Genomics Institute, using the same primers as for PCR. The amplicons were detected by comparison with a 50 bp (base pair) DNA ladder, and amplification products of the correct size were directly sequenced by the Beijing Genomics Institute, using the primers shown in Table 3. The HVR1 fragments of mtDNA were extracted and sequenced from M05t, M42t, M45t and M90t.

PCR amplification for the amelogenin gene

The PCR reaction was carried out using a total volume of 50 μl on a Thermo Hybaid PCR Amplifier. The *Taq* PCR Kit and primers were from Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd. The primer sequences were as follows (Sullivan *et al*. 1993): primers 1 and 2 were 5′-CCCTGGGCTCTGTAAAGAATAGTG-3′ (upstream) and 5′-ATCAGA GCTTAAACTGGGAAGCTG-3′ (downstream), respectively. The PCR was performed as follows: initial denaturing was at 95°C for 5 min, followed by another denaturing at 94°C for 1 min, annealing at 60°C for 45 s and extending at 72°C for 30 s with 35 cycles, and finally prolonging at 72°C for 5 min. The concentration of Mg^{2+} was 2.0 mmol l^{-1} in our study. Substitution of aDNA template with sterile water served as a blank control.

Polyacrylamide gel electrophoresis (PAGE)

The electrophoresis was performed in 8% polyacrylamide gel with a Hoefer miniVE system and the PCR amplification products were inspected on the white/ultraviolet transilluminator. The 50 bp DNA ladder was purchased from Qiagen (Beijing, China), with the following DNA molecular weight standard fragments, including 50 bp, 100 bp, 150 bp, 200 bp, 250 bp, 300 bp, 350 bp, 400 bp and 500 bp. The initial voltage was established at 70 V and readjusted to 65 V

after 10 min. After dyeing in 0.5 μg ml^{-1} ethidium bromide solution for 20 min, the gel was cleaned with distilled water and then inspected on the Olympia imaging system.

Authentication

In order to eliminate subjective factors, aDNA sex identification was performed independent of grave structure information, including skeleton number and burial contexts. All experimental phases such as aDNA extraction and PCR were performed in a different work area to ensure the credibility of the results. The extraction of aDNA and the amplification of PCR were carried out in two different work areas; and the PCR procedures, including the preparation of reagents, and PCR for target gene and amplicon detection, were also carried out in different areas to avoid contamination of the exogenous DNA. In our study, during the PCR procedure to detect the amelogenin gene, the blank control was involved in ensuring the specificity of PCR in order to screen for contaminants entering the process at any stage. Owing to DNA degradation in ancient samples, we treated the ancient DNA with caution during extraction to ensure the successful amplification of the DNA fragments. In order to ensure consistent results, the extraction of aDNA and amplification of the target gene fragments was performed three times. In addition, the replicated experiments were performed by other independent technicians in the molecular biology laboratory of the Fourth Military Medicine University (Xi'an, China), using the same extraction method, PCR parameters and electrophoresis conditions in order to confirm the accuracy and reproducibility of all of the experiments (Pääbo *et al*. 2004; Gilbert *et al*. 2005a).

In our study, standard precautions and measures for ancient DNA analysis were followed strictly in order to demonstrate the authenticity of the obtained data (Cooper and Poinar 2000; Pääbo *et al*. 2004; Gilbert *et al*. 2005a,b; Willerslev and Cooper 2005). We conducted DNA extraction, PCR mixture preparation, PCR amplification and post-PCR analysis in separated areas and carefully tried to avoid the imperceptible carrying of DNA aerosols on clothes or skin into the ancient laboratory during the experiments (MacHugh *et al*. 2000). Cross-contamination was avoided by using—and frequently changing—disposable tools, masks, gloves, laboratory coats and filter-plugged tips. To detect possible contamination, negative controls were implemented for each sample for extraction and PCR. To trace possible contamination, mtDNA sequences were obtained from the authors and from other laboratory members who had manipulated the teeth. Only independent extractions and amplifications yielding identical sequences with all controls being negative were included in the subsequent analyses. Primers covering four overlapping fragments were used to reduce the likelihood that a nuclear insertion rather than the organelle mtDNA was amplified (Wallace *et al*. 1997; Willerslev and Cooper 2005; Caramelli *et al*. 2007). Nevertheless, for the confirmation that DNA was present in the sample, we relied on replicated extraction and amplification both within and between laboratories, as done in various other studies (Lalueza-Fox *et al*. 2003; Ricaut *et al*. 2005; Topf *et al*. 2006).

RESULTS

Microstructure of samples

The exterior surface and interior microstructure of the teeth were observed with an optical microscope. The exterior surface of all of the selected teeth was clear, smooth and slightly yellow. There was a compact porous network structure consisting of hydroxyapatite (Hu *et al*. 2001) in the inside of the tooth samples, with some slight changes in colour (Fig. 3).

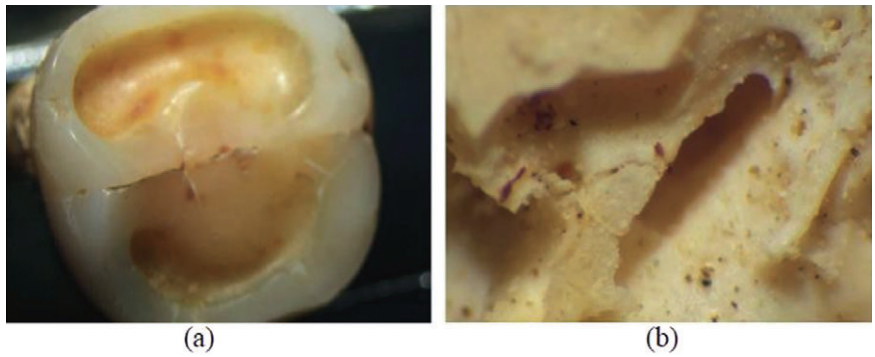

Figure 3 *Microscopic photographs of a tooth from sample M42: (a) exterior shape of tooth (×13); (b) interior structure of tooth (×80).*

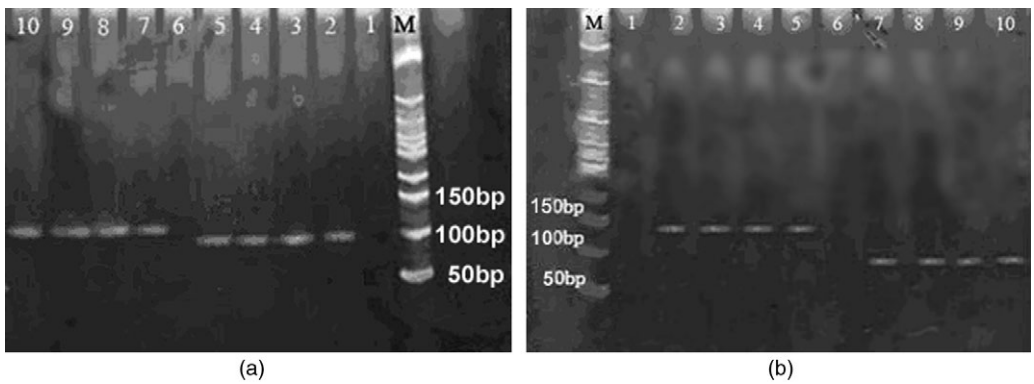

Figure 4 *The results of PCR for fragments in HVR1. (a) Lane 1, negative control for L16055-H16139; lanes 2–5, positive PCR results for L16055-H16139 in M05t, M42t, M45t and M90t, respectively; lane 6, negative control for L16131-H16218; lanes 7–10, positive PCR results for L16131-H16218 in M05t, M42t, M45t and M90t, respectively; M, DNA molecular weight standard. (b) Lane 1, negative control for L16209-H16356; lanes 2–5, positive PCR results for L16209-H16356 in M05t, M42t, M45t and M90t, respectively; lane 6, negative control for L16313-H16410; lanes 7–10, positive PCR results for L16313-H16410 in M05t, M42t, M45t and M90t, respectively; M, DNA molecular weight standard.*

Amplification for HVR1 fragments

Among the eight samples included in our study, aDNA was extracted successfully from M05t, M42t, M45t, M76t and M90t. In order to eliminate general exogenous contamination, we examined the HVR1 fragment of mtDNA from M05t, M42t, M45t, M76t and M90t. Samples M05t, M42t, M45t and M90t—but not sample M76—were positive for HVR1 fragments in L16055-H16139, L16131-H16218, L16209-H16356, L16055-H16410 and L16313-H16410, respectively, as shown in Figure 4. The failure of amplification of HVR1 fragments in sample M76 might be due to serious degradation of the aDNA or the presence of PCR inhibitors such as humic acid, tannic acid, collagen or fulvic acid.

Table 4 *mtDNA haplotypes (between positions 16055 and 16410) of all persons involved in processing samples*

Sample	*Polymorphic positions*												
	16173	*16185*	*16209*	*16223*	*16260*	*16290*	*16295*	*16298*	*16303*	*16311*	*16319*	*16327*	*16362*
CRS	C	C	T	C	C	C	C	T	G	T	G	C	T
T1	C	C	**C**	**T**	C	**T**	C	T	G	**C**	**A**	C	**C**
T2	C	C	T	**T**	C	C	C	T	G	T	G	C	T
T3	C	C	T	**T**	C	C	C	**C**	**A**	T	G	**T**	T
A1	C	C	T	**T**	C	**T**	C	T	G	T	**A**	C	**C**
A2	**T**	C	T	**T**	C	C	**T**	T	G	T	G	C	**C**
M5	C	**T**	T	**T**	**T**	C	C	**C**	G	T	G	C	T
M42	C	C	T	**T**	C	**T**	C	T	G	T	G	C	**C**
M45	C	C	T	**T**	C	**T**	C	T	G	T	G	C	T
M90	C	**T**	T	**T**	C	C	C	**C**	G	T	G	C	**C**

Sequence analysis of HVR1 fragments of mtDNA

A 356 bp fragment of the mtDNA HVR1 control region was obtained for tooth samples M5, M42, M45 and M90, and compared with the Cambridge Reference Sequence (CRS) (Anderson *et al*. 1981). There were a total of six variable nucleotide positions, all corresponding to C ↔ T transitions. The four mtDNA sequences differ unambiguously from each other, since six different nucleotide positions were observed between them, as indicated in Table 4. The results obtained with the HVR1 region for the four subjects were compared with mitotypes of the technicians (T1, T2 and T3) and archaeologists (A1 and A2), and all were different. All of these findings are in favour of the authenticity of the amplified products.

Results of amplification for the amelogenin gene

After PCR amplification and PAGE electrophoresis analysis, specific positive bands were present in the tooth samples M90t, M76t, M42t, M45t and M05t. Two bands of 106 bp and 112 bp were positive in the tooth samples M90t, M76t and M42t, while one band of 106 bp was positive in the tooth samples M45t and M05t. The target bands were not observed in the tooth samples M96t, M43t and M10t, as shown in Figure 5.

Results of sex identification

Of the selected eight typical tombs, no definite sex identification result was obtained in the three tombs of M10, M43 and M96 using aDNA molecular sex identification methods. The aDNA analytical results revealed that M42, M90 and M76 were males and that M05 and M45 were females.

None of the skeletons of slave victims were identified by osteologists in terms of osteological sex identifications prior to the aDNA sex test. After genetic sex identification of the slave sacrifice victims was finalized for samples M05, M42, M45 and M90, morphometric sex identification was carried out on their skeletons by osteologists. However, no morphometric sex result was obtained for M76 due to an incomplete skeleton. The results obtained from sex identification with PCR methods were consistent with those of traditional osteological sex identification (Table 5).

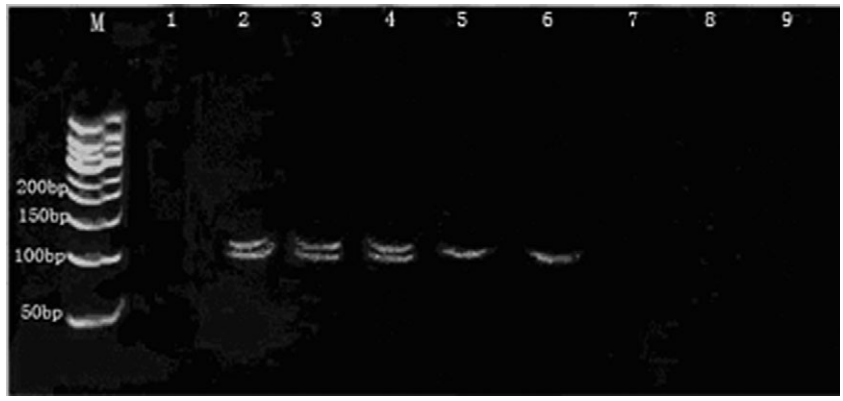

Figure 5 *Electrophoresis results of the PCR products from the tooth samples. M, 50 bp ladder; lane 1, negative control; lanes 2–9, samples M90t, M76t, M42t, M45t, M05t, M96t, M43t and M10t, respectively.*

Table 5 *The results for the selected archaeological samples identified by the molecular sex identification method and the morphometric sex method*

Sample number	*aDNA molecular identification*	*Morphological identification using whole skeletons*
M05	F	F
M42	M	M
M45	F	F
M76	M	No result
M90	M	M

Results of duplicate experiments

In order to further verify the reliability of the experimental results, duplicate experiments were performed by other technicians in the molecular biology laboratory of the Fourth Military Medicine University (Xi'an, China), using the same extraction method, PCR parameters and electrophoresis conditions.

The duplicate experiments were carried out in another laboratory and the results showed that two bands of 106 bp and 112 bp were positive in the tooth samples M90t, M76t and M42t, while one band of 106 bp was positive in the tooth samples M45t and M05t, which was consistent with the results in our laboratory, as shown in Figure 6.

DISCUSSION AND CONCLUSIONS

Discussion

According to previous investigation results, many specific genes have been adopted to analyse sex (Nakahori *et al*. 1991a,b; Andreasson and Allen 2003). The amelogenin gene, which is

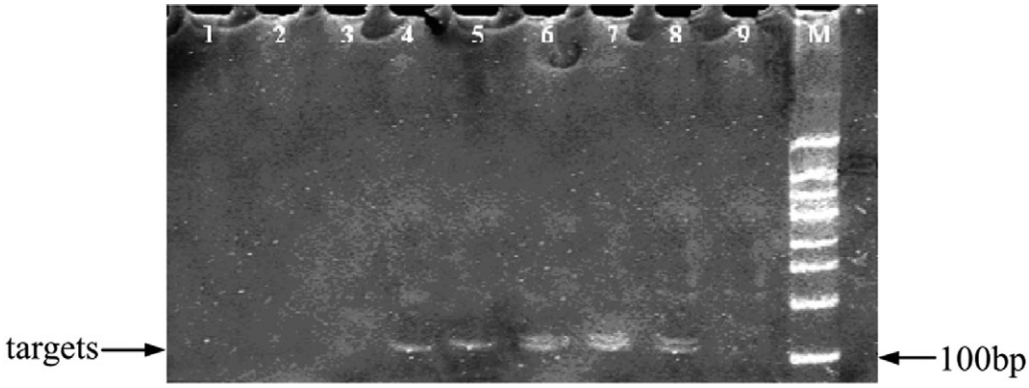

Figure 6 *Duplicate experiments in another laboratory: electrophoresis results of the PCR products from the tooth samples. M, 50 bp ladder; lanes 1–8, samples M43t, M96t, M10t, M45t, M05t, M42t, M76t and M90t, respectively; lane 9, negative control.*

present on both the X and Y chromosomes, is used as a target for PCR to identify sex in modern DNA. After amplification of the amelogenin gene with appropriately designed primers and separation by PAGE electrophoresis, the PCR products of males and females, with different lengths, are visible: the male shows two bands of 112 bp and 106 bp, while only one band of 106 bp appears in females.

PCR detection for the amelogenin gene from ancient DNA was developed after 1993 (Sullivan *et al*. 1993; Mannucci *et al*. 1994; Faerman *et al*. 1995; Andreasson and Allen 2003). Many successful results of archaeological investigations have been reported (Lassen and Hummel 1996; Stone *et al*. 1996; Faerman *et al*. 1997, 1998; Gotherstrom *et al*. 1997; Brown 2001; Matheson and Loy 2001; Mays and Faerman 2001). However, some technical obstacles introduce challenges into the archaeological research, because of the shorter ancient DNA fragments and lower successful extractive efficiency. In particular, the following key problems should be taken into account (Yang *et al*. 2006): (1) contamination prevention against exogenous DNA fragments; (2) improvement of the aDNA extraction efficiency; (3) optimization of PCR technical parameters and removal of PCR inhibitors; and (4) verification of results and conclusions by control and reproducibility experiments.

The methods adopted in our study have the following features. (1) The protocols are independent of the integrity and the age of the skeletons compared using the traditional morphological sex investigation method. However, these protocols have a strong reliance on the preservation quality of the samples. (2) Only a small quantity (0.5 g) of samples is needed and the sampling does not seriously destroy the integrity of the skeleton. (3) The designed primers have good stability and are accepted by many investigators: in addition, the sex analysis results are definite. (4) The shorter amplification products are very suitable for ancient DNA sex identification.

In general, our findings showed that sex information of slave sacrifice victims has been authenticated with aDNA molecular sex identification. Slave sacrifice victims in a master's tomb might be a main marker of Chinese slave society. The results of molecular sex identification in the present study have proved that slave sacrifice victims could be either men and women in the graves of Qin State during the Spring and Autumn Period of China. The observations of extracted DNA and amelogenin gene analysis revealed that females were also chosen as sacrifice victims in China's slave society. The females played critical roles in population growth and fertility in ancient

society. Therefore, female slaves were rarely sacrifice victims, due to the cultural tradition of adoration of and regard for females. Unfortunately, sex analysis on ancient DNA suggested that females, who might have been bondmaids of the tomb master, could become slave sacrifice victims, which shows that there existed some selection probability for female slaves as sacrifice victims. Females could also become sacrificial victims at the funerals of slave masters, and this would correlate with sex relationships and the social status of the slave master buried in the grave.

Conclusions

The clustering of slave sacrifice victims is a special category of burial, with a symbolic characteristic of slave society in the Spring and Autumn Period of China. The identification of slave sacrifice victims can be accurately determined according to the structure of tombs during archaeological excavation. However, the skeletons of slave sacrifice victims are always incomplete, with the consequence of difficulties in sex identification using morphological methods. The molecular sex identification method provides an additional strategy for determination of the sex of slave sacrifice victims.

Many findings of archaeological excavation indicate that the skull bones and teeth of slave sacrifice victims are always intact, so teeth are the preferred raw materials for the extraction of ancient DNA and sex identification analysis. Further investigation is needed to develop more efficient aDNA extraction methods and to examine the sex information of more ancient samples with different backgrounds in China.

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