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新石器时代人骨遗骸中古代DNA的提取及X-Y染色体同源基因片段的PCR扩增

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摘 要

从中国新石器时代人骨遗骸中提取出古代DNA。通过聚合酶链式反应（PCR）技术扩增得到X-Y染色体上的单拷贝同源基因片段（Amelogenin Gene）。由于扩增的基因片段长度具有性别多态性，从而为古代人骨和牙齿提供了分子生物学的性别鉴定。

关键词 古代DNA，Amelogenin基因，聚合酶链式反应，性别鉴定

近几年来，随着分子生物学方法技术的突飞猛进，它已被广泛地应用于相关的生命科学研究领域。已经证实在古代生物机体遗骸中可以保存有遗传物质DNA（Herrmann and Hummel，1993），学术界不时地出现古代DNA的研究报道。最初的工作尝试用分子克隆技术以研究古代DNA（Higuchi *et al.*, 1984; Pääbo, 1984; Arnemann *et al.*, 1986），但由于生物机体死后的变质降解，克隆技术很难成功，从而使进一步的DNA分析工作无法进行。聚合酶链式反应（PCR）体外扩增DNA技术的建立，为微量古代DNA的扩增提供了理想的工具（Pääbo *et al.*, 1989; Hagelberg *et al.*, 1989; Hummel and Herrmann, 1991）。目前多数古代DNA研究工作集中在线粒体DNA或具有重复序列的细胞核DNA，因为靶序列的高拷贝性有利于DNA的获取。然而单拷贝或低拷贝的古代DNA片段已被证实能够通过PCR扩增得到（Salo *et al.*, 1994）。我们的工作即是从中国新石器时代人骨遗骸中扩增细胞核单拷贝的Amelogenin基因片段。

Amelogenin是一种蛋白质，与牙齿发育相关。编码该蛋白质的基因是位于X和Y性染色体短臂上的一对同源序列（Nakahori *et al.*, 1991; Bailey *et al.*, 1992）。采用不同扩增反应体系，已从现代人标本中扩增出不同长度的该基因片段序列（Nakahori *et al.*, 1991; Akane *et al.*, 1991,1992; Mannucci *et al.*, 1994）。由于古代DNA的降解，其分子长度一般不超过几百个碱基对。因此我们采用Mannuci等人的引物序列，扩增较短的基因片段

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(106/ 112bp)。更重要的是，该扩增体系的产物具有性别多态性，可进行性别鉴定，它在人类学和考古学研究中具有重要意义。

1 材　　料

3 个人骨碎块和一枚牙齿采自中国新石器时代遗址（表 1）。现代对照标本为一男性骨头标本和女性唾液。

表 1　用于 DNA 提取的古代材料

编　号	解剖部位	出土地点	距　今　年　代
XWG2	股骨远端	河南下王岗	5 000—7 000 年 (张振标等，1984)
XWG3	胫骨远端	河南下王岗	5 000—7 000 年 (张振标等，1984)
DDW3	股骨近端	甘肃大地湾	5 600—5 900 年 (林钟雨，1994)
CGP2	下第三臼齿	吉林查干泡	7 800—9 800 年 (尤玉柱等，1984)

在 DNA 提取之前，对骨头材料的保存状况做了组织切片显微观察（图 1）。骨组织结构保存状况良好，仅有轻微程度的微生物侵损痕迹。骨结构单元哈氏系统保存较完整，可看到骨细胞所在处的结构（图 1 箭头所示）。牙齿拔自一下颌骨，牙根牙冠完整无损。由于骨和牙齿是人体最坚硬的组织，内部积有较少量的体液和酶，与其它软组织比较，存在于其中的细胞在机体死后的自降解过程相对轻缓（Herrmann and Hummel，1993; Lassen *et al.*, 1994）。骨头 DNA 主要来自三种细胞：骨细胞，成骨细胞和破骨细胞；牙齿 DNA 来源于牙髓腔细胞和牙骨质成骨细胞。

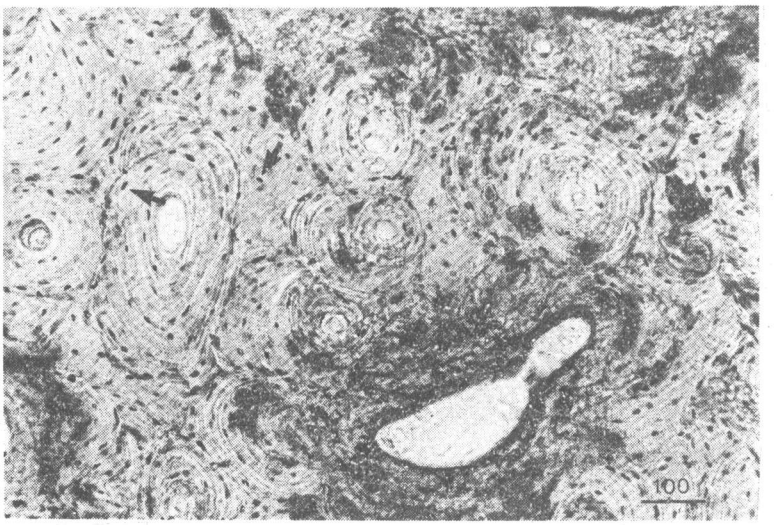

图 1　标本 XWG3 骨组织切片显微照相 (μm)

Micrograph (XWG3) of bone thin section. The microstructure is mainly well preserved exhibiting osteons with the Haversian canals and osteocytes (single arrow)

2 方 法

2.1 备样

手术刀刮净骨块和牙齿表面垢物，紫外光（254nm）照射1.5小时。这一步骤旨在排除发掘、收集、转运过程中带来的现代DNA污染（Cone and Fairfax, 1993）。然后将标本研磨成粉末，称取300毫克，溶于1.5ml 0.5M EDTA（pH8.3）溶液，48小时室温下机械摇动，保持均匀混合状态，充分溶解吸收骨粉中的无机盐，使包围在其中的有机成分释放出来。离心取上清液，转至核酸自动提取仪（GenePure Type 341，Applied Biosystems）。

2.2 DNA提取

提取过程主要通过程序控制，机器自动操作完成，尽可能消除人工操作带来的污染问题。主要步骤：首先进行蛋白酶K消化；然后采用酚和氯仿DNA提取法分离提纯DNA（Sambrook *et al.*, 1989）；在乙戊醇和乙酸钠存在下，沉淀反应产生的DNA分子吸附于小玻璃珠上（glass milk, Dianova），过滤，用乙醇洗脱滤膜上的DNA玻璃珠，将其溶于50μl无菌去离子水（Ampuwa, Fresenius），温浴震荡，使DNA脱离玻璃珠完全溶解，离心得DNA提取液。

2.3 聚合酶链式反应（PCR）

以上得到的DNA溶液中的DNA含量甚微。采用聚合酶链式反应技术，以扩增位于两段已知序列之间的DNA片段。本实验所用的引物序列为Amel A（5′-CCC TGG GCT CTG TAA AGA ATA GTG-3′）和Amel B（5′-ATC AGA GCT TAA ACT GGG AAG CTG-3′）。这对引物可同时扩增XY染色体上一同源序列，即Amelogenin基因的第一内含子多态片段106bp（X染色体）和112bp（Y染色体）（Mannucci *et al.*, 1994）。扩增反应采用两个阶段PCR扩增法（Ruano *et al.*, 1989），在50μl反应混合液中进行。第一阶段的PCR反应液组成如下：10mM Tris-HCL（pH8.0），50mM KCL（Gene Amp 10× PCR buffer，Perkin Elmer Cetus），1.5mM $MgCl_2$（Perkin Elmer Cetus），175mM每种dNTP（Boehringer），0.1μM每种引物（Applied Biosystems）和2μl古代DNA提取液或0.5μl现代DNA提取液，最后加入去离子水（Ampuwa）至50μl。反应开始为5分钟30秒94℃的热启动（Ruano *et al.*, 1991），期间加入1单位的Taq DNA聚合酶（AmpliTaq，Perkin Elmer Cetus）。扩增反应在热循环器（TCL，Perkin Elmer Cetus）中进行：1分钟94℃热变性，30秒57℃退火，1分钟72℃延伸反应，35个热循环。第二阶段的扩增反应是在第一阶段基础上进行，其反应液组成基本相同，引物浓度增至0.14μM，Taq DNA聚合酶1.5个单位，待扩增的5μlDNA溶液是第一阶段PCR产物，在反应开始的热启动中最后加入。35个热循环：1分钟94℃热变性，30秒56℃退火，1分钟72℃延伸反应。反应产物冷却于4℃保存。

2.4 DNA分析

对PCR产物进行14%的非变性聚丙烯酰胺凝胶电泳分离分析。电泳在16×20×0.1cm的垂直装置（Biometra）中进行，所加电压130V，电泳时间17小时。凝胶在

0.1%的 SYBR Greenl 核酸染料溶液（Mo Bi Tec）中染色 30 分钟后，254nm 紫外光透射照相做记录。

3 结果和讨论

4 个古代标本的 DNA 提取液都能成功地进行 PCR 扩增反应，尽管反应的成功率明显低于现代标本，但在不同程度上均具有重复性（表 2）。现代标本总是达到预期的结果（图 2），女性（F）显示单一的 106bp 条带，男性（M）显示 106bp 和 112bp 两个条带；空白对照（X）则没有 PCR 产物，表明在 PCR 实验操作中没有带来污染问题。由于扩增反应引物的特异性，非人类 DNA 污染不会影响反应结果。由此证实新石器时代人骨遗骸中古代 DNA 的存在。

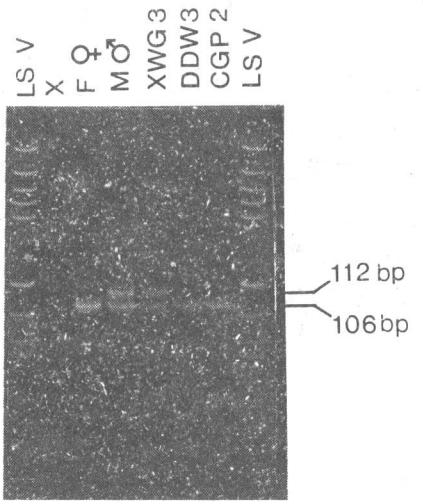

图 2　标本 XWG2，DDW3 和 CGP2 的 PCR 产物凝胶电泳检测

Electrophoretic separation of PCR products. 7μl of the amplification products were loaded on a non-denaturating 14% Polyacrylamide gel, run at 130V for 17 hours, and stained with a 0.1% SYBR GREEN I. solution. The X-Y homologous amelogenin gene sequences were amplified from the ancient samples XWG3 (106/ 112bp), DDW3 (106bp), CGP2 (106bp) and modern samples M (106/ 112bp) and F (112bp). No products were detectable in the no template control(X). The LS V DNA ladder (Boehringer) was used as molecular weight standard

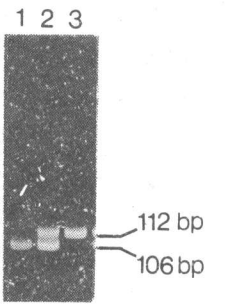

图 3　标本 XWG2 的 3 种不同 PCR 产物的凝胶电泳检测

Electrorephoretic separation of amplification products (8μl) of the sample XWG2 from different PCR reactions. Lane 1 shows a single 106bp band of the X chromosome, while lane 2 shows a 106/ 112bp bands doublet of the X and Y chromosome. Lane 3 shows the 112bp band of the Y chromosome only

表 2　PCR 扩增反应结果

标　本	PCR 总次数	PCR 成功次数
现代男性	14	14
现代女性	14	14
XWG2	13	3
XWG3	6	4
DDW3	6	2
CGP2	6	2

标本 CGP2 和 DDW3 均显示单一的 106bp 条带；标本 XWG3 显示 106bp 和 112bp 两个条带（图 2）；标本 XWG2 则出现 3 种不同的 PCR 结果：单一的 106bp；单一的 112bp；和 106/ 112bp 两个条带（图 3）。

由于 DNA 的降解变性，古代标本仅能提供很少量的完整模板 DNA；另一方面，这些标本由于长时期受埋藏环境的影响，可能含有阻止聚合酶链式反应的抑制因子，如土壤化合物等，从而使单一的或两个等位基因扩增反应出现随即失败的现象，产生不同的 PCR 结果。标本 XWG2 即可能属于这种情况。不过，样品或实验用品污染也可能产生类似结果（Schmidt *et al.*, 1995），因此有必要严格控制污染，多次重复实验结果。同时可对扩增反应产物进行 DNA 序列测定加以确认。

根据以上结果，对 4 个古代破碎标本进行性别判断还是可能的。由于 Y 染色体特征序列（112bp）分别重复出现于标本 XWG2 和 XWG3，从而判断它们为男性个体；单一的 X 染色体特征序列（106bp）分别重复出现于标本 CGP2 和 DDW3，显示二者为女性个体。

在古人类学研究中，研究材料多为破碎的骨块和牙齿，用传统的形态学方法进行性别判断和系统分析比较困难，相比之下，分子生物学方法具有独特的优越性，尤其是古代 DNA 的存在被证实，相信分子人类学将为现代人类起源和演化问题的解决开拓新的前景。

本研究工作得到德国科学技术部和中国科学院资助，于格廷根大学人类学研究所古代 DNA 实验室完成。实验标本由张振标、林钟雨二位先生提供，在此谨致谢意。

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ANCIENT DNA EXTRACTION FROM NEOLITHIC HUMAN SKELETAL REMAINS AND PCR BASED AMPLIFICATION OF THE X-Y HOMOLOGOUS AMELOGENIN GENE

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Ancient DNA was extracted from Neolithic human skeletal remains excavated in China. Single copy sequences of the X-Y homologous Amelogenin gene were amplified from the DNA extracts by PCR technique. Since the amplified sequences exhibit a sex specific length dimorphism they enable a sex identification on a molecular basis for ancient human bone and teeth.

Introduction

Ancient DNA (aDNA) has been extracted from a various number of ancient organic remains (Herrmann and Hummel, 1993). In the beginning of aDNA research there were attempts to study aDNA by molecular cloning (Higuchi *et al.*, 1984; Pääbo, 1984, Arnemann *et al.*, 1986). But due to postmortem degradation, it was the polymerase chain reaction (PCR) (Mullis and Faloona, 1987) which proved to be the ideal tool to amplify minimum amounts of intact aDNA (Pääbo *et al.*, 1989; Hagelberg *et al.*, 1989; Hummel and Herrmann, 1991). Initially, most of the aDNA studies concentrated on mitochondrial DNA or repetitive nuclear DNA, because the high copy numbers of target DNA facilitates its retrieval. However, the amplification of single and low copy DNA fragments had already been demonstrated (Salo *et al.*, 1994).

In the present study, we demonstrate the amplification of the nuclear single copy Amelogenin gene from Neolithic human skeletal material excavated in China.

The Amelogenin gene is a X-Y homologous gene related to tooth development (Nakahori *et al.*, 1991; Bailey *et al.*, 1992). From contemporary forensic samples amelogenin sequences can be amplified with different primer systems (Nakahori *et al.*, 1991; Akane *et al.*, 1991 and 1992; Mannucci *et al.*, 1994). Due to degradation, aDNA fragments usually exhibits not more than a few hundred basepairs. Therefore, the primer system which amplifies short segments of 106 basepairs (bp) and 112 bp (Mannucci *et al.*, 1994) had been employed for aDNA studies and already proved to reveal reproducible results. The sex chromosome specific length polymorphism of 6 bp of the amplification products provides a sex identification, which is a basic feature for physical anthropology and historic demography.

Materials

Three bone fragments and one tooth were chosen from three Neolithic sites in China (Table 1). Contemporary human bone from a male individual (M) and human saliva from a female individual (F) were included as control samples.

Table 1 Ancient materials for DNA extraction

No.	Anatomical Position	Site	Age
XWG2	distal femur	Xiawanggang, Henan	5000~7000 ys BP
XWG3	distal tibia	Xiawanggang, Henan	5000~7000 ys BP
DDW3	proximal femur	Dadiwang, Gansu	5600~5900 ys BP
CGP2	lower M3	Chaganpao, Jilin	7800~9800 ys BP

Prior to DNA extraction, histological thin sections were prepared from the bone samples in order to observe the state of preservation. The micrograph (Fig.1) exhibits a mainly intact bone structure. The major sources of DNA extracted from bone are three types of cells: osteocytes, osteoblasts and osteoclasts, the latter two occuring when bone is remodelled.

From the macromorphological aspect, also the tooth, which was pulled from an intact mandible is well preserved. In the case of teeth mainly the cells of the pulpa tissue and cementoblasts are the sources of DNA.

Since bone and tooth are hard tissues lacking bulk amounts of liquids and enzymes, cells can be expected to suffer less from autolytic processes than soft tissues or fluid tissues. Therefore they are more likely to be better protected against decomposition than soft tissue cells (Herrmann and Hummel, 1993; Lassen *et al.*, 1994).

Methods

Preparation of samples for DNA extraction

After scraping the outer surfaces of the bone and tooth samples with a scalpel the samples were exposed to UV light (254 nm) for 1.5 hours. This procedure removes and inactivates possible contaminating modern DNA (Cone and Fairfax, 1993), which may persist on the surfaces of the samples due to excavation, collection, and transportation. The samples were powdered in a mixer mill (Retsch Typ MM2), aliquots of 300 mg bone powder were mixed thoroughly with 1.5 ml 0.5 M EDTA (pH 8.3), and constantly shaken for 48 hours at room temperature. After a centrifugation for 5 min. in a bench top centrifuge (Eppendorf 5415C) at 5000 rpm the supernatants were transferred to an automated nucleic acid extractor (GenePure Type 341A, Applied Biosystems), which reduces the risk of contamination due to laboratory handling to a minimum.

DNA extraction

The automated extraction started with a proteinase K digestion (0.5 ml, 20 mg/ ml). The incubation was carried out at 56℃ for 1 hour. The following phenol and chloroform extractions

were carried out with standard parameters (Sambrook *et al.*, 1989). The DNA was concentrated by binding to a silica powder (glass milk, Dianova) (Poinar, 1994) in presence of isopropanol and sodium acetate (2.0 M, pH 4.5). The DNA/ silica complexes were collected on filtration membranes (Applied Biosystems), and washed with 80% ethanol. Finally, the DNA samples were eluted manually from the silica beads with 50 μl sterile water (Ampuwa, Fresenius).

Polymerase Chain Reaction

Primers used for amplification are Amel A (5′–CCC TGG GCT CTG TAA AGA ATA GTG–3′) and Amel B (5′–ATC AGA GCT TAA ACT GGG AAG CTG–3′). These primers flank a homologue, length polymorphic sequence on intron 1 of the sex chromosomes, resulting in 106 bp and 112 bp PCR products from the X and Y chromosomes, respectively (Mannucci *et al.*, 1994). Biphasic booster PCR amplifications (Ruano *et al.*, 1989) were carried out in 50 μl reaction mixes. The stage I PCR mixes consisted of 10 mM Tris–HCl (pH 8.0) (Gene Amp 10× PCR buffer, Perkin Elmer Cetus), 50 mM KCl (Gene Amp 10× PCR buffer, Perkin Elmer Cetus), 1.5 mM $MgCl_2$ (Perkin Elmer Cetus), 175 mM each dNTP (Boehringer), 0.1 μM each primer (Synthesis by Applied Biosystems), and 2 μl aDNA. For the modern control samples 0.5 μl DNA was employed, while the no template controls contained no DNA. The reaction mixes were filled to 50 μl with Ampuwa (Fresenius). The PCR reactions started with an initial denaturation (5 min. 30 sec. at 94℃), during this time 1 U Taq DNA polymerase (Ampli*Taq*, Perkin Elmer Cetus) was added (Hot start, Ruano *et al.*, 1991). The amplification was carried out in a Thermal Cycler (TC1, Perkin Elmer Cetus). The 35 amplification cycles consisted of 1 min. at 94℃, 30 sec. at 57℃ and 1 min. at 72℃.

Suppliers of the reaction reagents of stage Ⅱ PCR were the same as for stage Ⅰ PCR. The stage Ⅱ PCR mixes consisted of 10 mM Tris–HCl (pH 8.0), 50mM KCl, 1.5 mM $MgCl_2$, 175 mM each dNTP, 0.14 μM each primer, 1.5 U Taq DNA Polymarase. The reaction mixes were filled with Ampuwa (Fresenius) to the 50 μl reaction volume. During the initial denaturation time 5 μl of stage Ⅰ PCR products were added. The 35 amplification cycles of stage Ⅱ PCR were as followed: 1 min. at 94℃, 30 sec. at 56℃, and 1 min. at 72℃.

DNA Analysis

PCR products were separated by non–denaturing 14% polyacrylamide gel (PAA) electrophoresis (29:1 acrylamide/ bisacrylamide, Biometra). The electrophoresis were performed at 130 V for 17 hours in 16× 20× 0.1 cm vertical electrophoresis chambers (Maxigel, Biometra). The gels were stained with 0.1% aquous SYBR Green 1 nucleic acid gel staining solution (Mo Bi Tec) for 30 min. Documentation of the PAA–gels was done with Polaroid film 667 on a 254 mn UV transilluminator (Bachofer) using an orange filter.

Results and Discussion

After the automated DNA extraction all ancient samples could be amplified successfully although exhibiting a lower frequency of successful PCR amplification than modern samples. The reliability of the results was tested by repeating the amplification procedure several times (cf.

Table 2). The control samples always exhibited the expected results. The female sample revealed a single 106 bp band, the male sample revealed 106 and 112 bp bands. The no template control samples regularly contained no PCR products.

Reproducibly, the ancient samples CGP2 and DDW3 showed single 106 bp bands only. Also reproducibly, the ancient sample XWG3 always exhibited the 106/ 112 bp bands doublet (Fig. 2). In contrast, XWG2 revealed three different PCR products: a single 106 bp band, a single 112 bp band and a 106/ 112 bp band doublet (Fig.3).

Table 2 Frequency of successful PCR amplifications

	No. of PCRs	No. of successful PCRs
modern male	14	14
modern female	14	14
XWG2	13	3
XWG3	6	4
DDW3	6	2
CGP2	6	2

Due to aDNA decomposition and modification, ancient samples provide only small amounts of intact template DNA. Moreover, aDNA samples may contain some PCR inhibitors from soil compounds. Due to this and in correspondence with amplification results of other bone and teeth series, an amplification failure may occur for one of the allels as found in XWG2. Finally, one has to take into account the possibility of occasional contaminations in laboratory disposables which can also lead to artifact amplification results (Schmidt *et al.*, 1995). Therefore, it is neccessary to prove results by repeating the PCR amplification as demonstrated.

On this basis a sex identification is possible for all samples which are more than 5000 years old. Because the Y-chromosomal sequence (112 bp) was present in XWG2 and XWG3, they could be determined as male individuals. The occurance of only 106 bp bands in CGP2 and DDW3 indicate for female individuals. To achieve a sound sex determination for these individuals too, it would however be neccessary to obtain at least one or two further confirming amplification results.

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Key words Ancient DNA, Amelogenin gene, PCR, Sex identification