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Sex Determination of Ancient Human Skeletons Using DNA

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ABSTRACT A method for determining the sex of human skeletons was developed using molecular genetic techniques. The amelogenin gene, found on the X and Y chromosomes, was examined using the polymerase chain reaction (PCR) and a nonradioactive dot blot procedure. DNA was analyzed from 20 modern individuals of known sex and 20 skeletons from an archaeological site in central Illinois dating to A.D. 1300. An independent assessment of the sex of each skeleton was made according to standard osteological methods. The sex of 19 ancient and 20 modern individuals was accurately determined using this molecular genetic technique. Molecular sex determination will be especially useful for juvenile and fragmentary remains when it is difficult, or impossible, to establish an individual's sex from morphological features.

Until recently, the size and shape of bones were the sole means of establishing the sex of skeletons from archaeological or forensic contexts. The discovery that DNA can be recovered from old bone (Hagelberg et al., 1989; Horai et al., 1989) provides an opportunity to determine sex using DNA from the X and Y chromosomes. Such an analysis is particularly useful when standard osteological methods for determining sex produce, at best, equivocal results, such as when juvenile or fragmentary remains are examined. The ability to establish the sex of such skeletal remains contributes important information to forensic cases, and allows the expansion of archaeological mortuary analyses.

Several molecular methods of sex determination using the polymerase chain reaction (PCR) have been developed for medical or forensic purposes (Aasen and Medrano, 1990; Akane et al., 1992; Cui et al., 1994; Ebensperger et al., 1989; Handyside et al., 1990; Kogan et al., 1987; Nakahori et al., 1991a; Norby and Eriksen, 1992; Witt and Erickson, 1989). Most of these methods, however, are inappropriate for ancient DNA. Some require the amplification of DNA fragments that are too large to be routinely obtained from ancient specimens. Others rely on the presence or absence of a Y-specific PCR product to indicate sex; for example, PCR amplification of alphoid repeats found on the Y chromosome (Honda et al., 1990; Hummel and Herrmann, 1991, 1993). Although the presence of a product after PCR amplification of the alphoid repeats indicates that an individual is male, the absence of a product does not necessarily indicate that an individual is female. It may simply reflect low quantity or quality DNA for amplification or the inhibition of the PCR by other agents in the sample; both occur frequently with ancient DNA (Cooper, 1993; Hagelberg and Clegg, 1991; Pääbo et al., 1988).

We present a method of determining the sex of skeletons using a small fragment (112 bp) of the amelogenin gene. Fixed sequence differences between the X and Y copies of

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this fragment are then revealed via chemiluminescent detection with sex-specific oligonucleotide probes. This method of sex determination was tested using 20 adult skeletons exhibiting clear male or female morphology from a 700-year-old archaeological site. In addition, DNA from 10 modern males and 10 modern females was also tested. Our results demonstrate that this nonradioactive dot blot procedure allows rapid, DNA-based sex determination from ancient as well as modern samples.

SAMPLES

The skeletal sample used for this analysis consists of 20 adults from the west-central Illinois Norris Farms #36 cemetery dating to A.D. 1300 (Milner and Smith, 1990). The cemetery was completely excavated in the mid-1980s by Illinois State Museum archaeologists, and approximately 260 skeletons were found that belong to an Oneota cultural component. These bones came from a cemetery that is thought to have been used by a community for a short period of time. They were also exceptionally well-preserved, and previous research demonstrated that they contained sufficient DNA for mitochondrial DNA typing (Stone and Stoneking, 1993).

The modern DNA samples were from Indonesian individuals of known sex and were previously extracted from blood (Redd et al., 1995).

METHODS

When choosing the 20 archaeological skeletons for this analysis, an effort was made to select specimens that had morphological characteristics that were readily identifiable as either male or female. In determining the sex of these individuals, the greatest weight was placed on several cranial and pelvic features, including the shapes of pubic bones, the widths of greater sciatic notches, and the sizes of preauricular sulci, supraorbital ridges, mastoid processes, and superior nuchal lines (e.g., Bass, 1971; White, 1991). Such features were consistent with other less precise means of determining the sex of skeletons, including the general robustness of bones.

For sex determination using DNA, primers for PCR were designed to amplify a small fragment (112 bp) in exon 6 of the amelogenin gene. The amelogenin gene, important for enamel development in teeth, is found on both the X and Y chromosomes outside the pseudoautosomal, or recombining, region (Nakahori et al., 1991b). The primers amplify corresponding fragments from both the X and Y copies of the amelogenin gene.

DNA was extracted from rib bones, usually the 11th or 12th ribs, that were free of pathological lesions. These particular specimens were chosen for analysis to minimize the destruction of parts of the skeleton frequently used in standard osteological studies. The outer layer of bone was removed with a sterile razor blade or a rotary tool (Sears Craftsman) to prevent contamination from previous handling. The bones were then ground to a fine powder using a bone mill (B. Braun Biotech) or an electric coffee grinder (Mr. Coffee). The mill and coffee grinder were washed with 1 N HCL or bleach and UV irradiated between uses. DNA was extracted from approximately 0.25 g of bone using the silica and guanidine thiocyanate extraction protocol described in Höss and Pääbo (1993).

The PCR was carried out in a 50 μl volume in which a wax-mediated hot start was performed (Chou et al., 1992). The lower phase of the PCR contained the deoxynucleoside triphosphates (dNTPs) and primers (Fig. 1), while the upper phase contained the DNA, 1 unit of Taq polymerase (Perkin Elmer, Roche, NJ), and BSA. Both phases contained tris buffer and $MgCl_2$. The two phases mixed as the temperature increased to 94°C during the first cycle of PCR. After the two phases mixed, the final concentrations were 100 μM of each dNTP, 20 pmol of each primer, 50 μg of bovine serum albumin (fraction V from Sigma), 67 mM Tris-HCL (pH 8.8), 2.5 mM $MgCl_2$, and 5 μl (approximately 5%) of the extracted DNA. Forty cycles of amplification were carried out, with each cycle consisting of denaturation at 94°C for 1 minute, annealing at 65°C for 1 minute, and elongation at 72°C for 1 minute. The PCR products were visualized with ethidium bromide in a 2.8% NuSieve agarose gel (FMC). For the ancient samples, PCR product bands were excised from the gel and placed in 100 μl of TE or

X: CTGCCGCCACAGCCACCTCTGCCTCCGATGTTCCCCATGCAGCCCC**TGCCTCCCATGCTTCCT**
Y: ....T.....A.....C...G.....**C.....A.....**

X: GATCTGACTCTGGAAGCTTGGCCATCAACAGACAAGACCAAGCGGGAGG
Y: ......CA.....G.....A.....

PRIMERS:		PROBES:	
A1:	CTGCTGCCACAGCCACCTCTG	X:	TGCCTCCCATGCTTCCT
A2:	CCTCCCGCTTGGTCTTGTCTG	Y:	TGCCCCCCATACTTCCT

Fig. 1. Sequence of the X (nucleotides 2270–2382) and Y (nucleotides 2090–2202) amelogenin gene fragment (Nakahori et al., 1991a) with the location of the primer sequences underlined and the probe sequence in bold. Dots signify identity to the X chromosome copy. Primer and probe sequences are listed below.

ddH_2O. The gel plug was melted at 65°C and 3 μl were used in a 15 cycle reamplification, consisting of denaturation at 94°C for 1 minute, annealing at 67°C for 1 minute, and elongation at 72°C for 1 minute. In addition to increasing the amount of DNA present, the reamplification step avoided using up the original PCR product, so that many dot blots could be made if necessary.

DNA was obtained from a minimum of two independent extractions per individual. At least four separate PCR products were used for the sex determination of each individual. To detect contamination by modern DNA, each set of extractions included a negative extraction control that contained all extraction reagents except bone powder. In addition, PCR negative controls containing all reagents except for DNA were routinely performed. PCR reagents were also regularly tested with primers for mitochondrial DNA sequences, which are more sensitive to DNA contamination than primers for nuclear single-copy sequences. To further minimize the potential for contamination, all DNA extracts and PCRs involving the skeletal samples were prepared in a room dedicated to ancient DNA research that is physically separated from the main genetics laboratory. Dedicated reagents and equipment, including gamma-sterilized filter pipet tips (VWR), were also used.

Dot blots of the reamplification products were prepared using uncharged nylon membranes (Biodyne). For each sample, 5 μl of PCR product was added to 50 μl of denaturation solution [0.4 N NaOH, 25 mM EDTA (pH 8.0)]. The denatured DNA was placed on the membranes using a manifold (Bio-Rad) and washed with 100 μl TE. After briefly blotting the membrane, the DNA was fixed to the membranes with UV irradiation (550 mJ) in a Stratalinker (Stratagene).

The membranes were prehybridized in 20–30 ml of 5 × SSPE (0.76 M NaCl, 0.05 M $NaH_2OPO_4H_2O$, and 6.25 mM EDTA), 5 × Denhardt's solution (Sambrook et al., 1989), and 0.5% SDS for 15 minutes at the hybridization temperature of 45°C. After prehybridization, 30 μl of 1 μM X or Y specific biotinylated probe (Fig. 1) was added and hybridization was carried out for at least 15 hours.

After hybridization, the membranes were placed in trays and washed twice for 5 minutes at room temperature in a wash solution of 2 × SSPE, 0.5% SDS. This was followed by a wash for 1 minute at the hybridization temperature and another wash at room temperature for 5 minutes. The membranes were then incubated at 42°C in 30 μl of the wash solution and 90 μl of streptavidin-horseradish peroxidase (Perkin-Elmer) for 10 minutes. Following the incubation step, the membranes were washed twice for 1 minute each using room temperature wash solution. They were subsequently placed in 42°C wash solution and gently agitated for 15 minutes while the wash solution cooled to room temperature.

The membranes were rinsed briefly in 0.1 M sodium citrate to remove the remaining SDS. Each membrane was agitated for 1 minute in 20 ml of chemiluminescence detec-

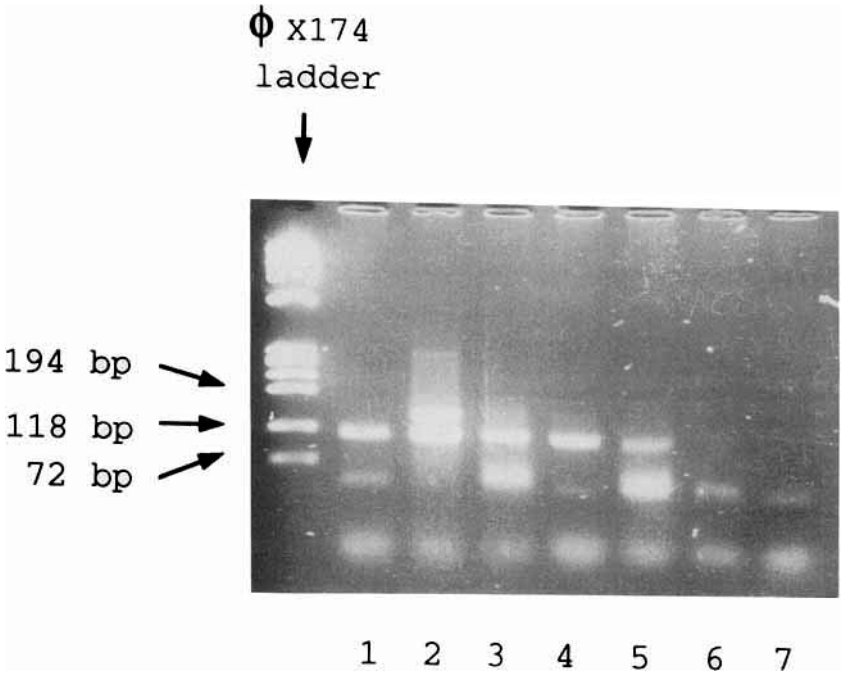

Fig. 2. Results of PCR amplification of the 112 bp amelogenin gene fragment using a dilution series of DNA. **Lane 1,** DNA extracted from a single hair bulb; **lanes 2–6,** 1 ng, 100 pg, 10 pg, 5 pg, and 1 pg DNA; **lane 7,** PCR blank.

tion reagents prepared according to the manufacturer's directions (ECL kit, Amersham), and blotted on 3M Whatman's paper. While the membranes were still damp, they were sealed in plastic wrap and placed on X-ray film for 25 minutes.

RESULTS

This method for DNA-based sex determination utilizes fixed differences between the X and Y copies of the amelogenin gene (Fig. 1). The sensitivity of this method for sex determination of samples containing minute amounts of DNA was established using a dilution series of male DNA (Fig. 2) ranging from 1 ng to 1 pg. In addition, ten separate PCR reactions of both the 5 pg and 1 pg dilutions were performed. PCR products were obtained from all ten of the reactions containing 5 pg of DNA but only from one of the ten reactions containing 1 pg of DNA. Dot blot results indicate that the X copy of the amelogenin gene fragment was detected in all 11 of the amplification products while the Y copy was observed in only five of the ten 5 pg test samples and not in the 1 pg sample. Since one haploid genome contains about 3 pg of DNA, it is likely that PCR is beginning from just one or two copies of the amelogenin gene in these samples.

Twenty modern samples from individuals of known sex were typed blindly; for these samples approximately 50 ng of DNA were used, and the 15 cycle PCR reamplification was unnecessary. The sex of 19 of the 20 modern individuals was correctly ascertained, but one male was incorrectly identified as female. A second DNA-based sex-typing method (Sullivan et al., 1993) also indicated that the individual was female. Therefore, this sample appears to have been simply mislabeled in the field and, hence, the new method correctly identifies this person's sex.

The results from a typical PCR amplification of ancient samples are depicted in Fig. 3. DNA from each ancient sample was extracted at least twice, and a minimum of

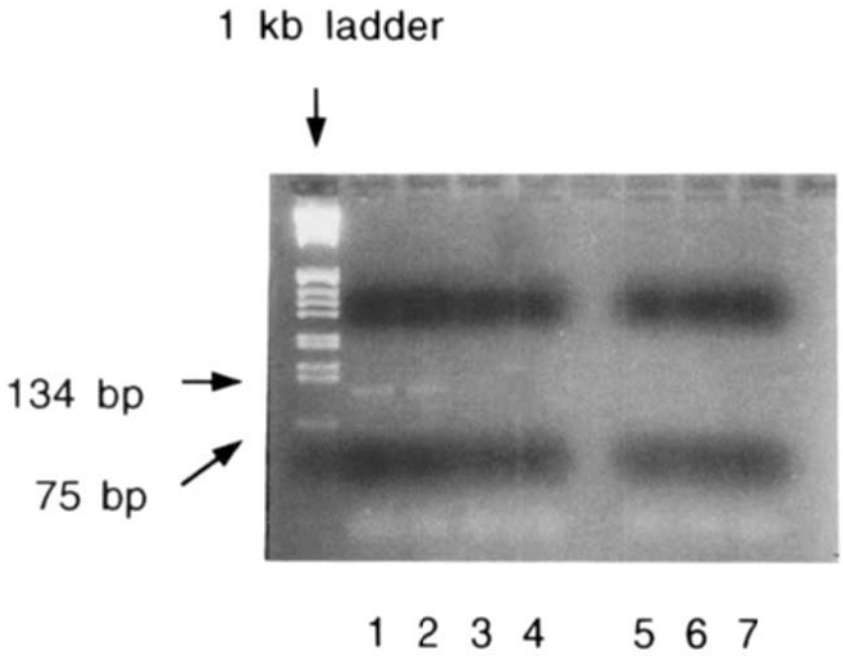

Fig. 3. PCR amplification of the 112 bp amelogenin gene fragment. **Lanes 1–4,** bone samples; **lanes 5** and **6,** extraction blanks; **lane 7,** PCR blank. The amplification of DNA from the bone samples was successful only in lanes 1 and 2.

four PCR products were obtained for dot blot hybridization. A typical dot blot of ancient samples is illustrated in Fig. 4. DNA from the ten ancient females hybridized only with the X specific oligonucleotide, as expected. DNA from nine of the males hybridized with both the X and the Y specific oligonucleotides (Table 1). In several males (such as burial 225 in Figure 4), some amplification products hybridized with only one of the two specific probes, consistent with the assumption that the PCR began from one or a very small number of copies.

One individual (burial 49) was classified as a male based on pelvic morphology and robust skeletal features, but the PCR products hybridized only with the X specific oligonucleotide. Although mtDNA was amplified, it was difficult to obtain amplification products from the amelogenin gene in this individual, and it is likely that there is insufficient DNA to determine sex with confidence. An average of 4.5 PCR amplifications was required to obtain a single product for sex determination in this individual. For the

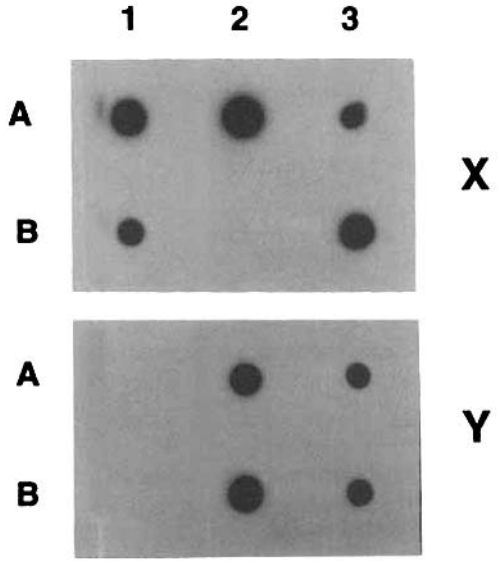

Fig. 4. Dot blots probed with X (top) and Y (bottom) specific oligonucleotides. A1, modern female; A2, modern male; A3, 5 pg male control; B1, burial 21 (female); B2, burial 225 (male); B3, burial 19 (male).

TABLE 1. List of burials examined, the sex of each skeleton determined by morphology, results from four PCR products, number of extractions, and number of PCR amplifications required to obtain four independent products

		PCR 1		PCR 2		PCR 3		PCR 4			
Burial	Sex	X	Y	X	Y	X	Y	X	Y	Extracts	PCRs
19	M	X	X		X	X		X	X	2	4
21	F	X		X		X		X		3	9
22	F	X		X		X		X		2	5
26	M	X	X	X	X		X	X	X	2	7
28	F	X		X		X		X		3	7
31	F	X		X		X		X		3	7
35	F	X		X		X		X		2	15
36	F	X		X		X		X		3	5
38	F	X		X		X		X		3	5
47	F	X		X		X		X		3	6
49	M	X		X		X		X		2	18
50	M	X			X	X		X	X	2	12
51	F	X		X		X		X		4	5
71	M	X			X	X	X	ND	ND	2	14
108	M	X	X	X	X	X	X	X		3	9
194	M	X			X	X			X	2	11
210	F	X		X		X		X		3	5
225	M	X			X	X	X		X	3	23
245	M		X	X		X	X		X	3	17
254	M	X	X	X	X	X	X	X		3	4

other ancient samples, an average of 2.4 PCR amplifications was sufficient to obtain a product.

DISCUSSION

The present method to determine sex employs PCR and a nonradioactive dot blot procedure to examine sequences from the amelogenin gene on the X and Y chromosomes. The sex of 19 ancient and 20 modern individuals was accurately determined giving a success rate of 95% and 100% respectively. This assumes that one modern sample was mislabeled, as indicated by this and other sex determination methods (Sullivan et al., 1993). One ancient individual was classified as a male based on morphology, but the PCR products hybridized only with the X specific oligonucleotide. This may be the result of insufficient DNA, or mutations in the priming or oligonucleotide binding sites on the Y chromosome copy of the amelogenin gene. Since PCR products for mitochondrial DNA were also difficult to obtain, it is likely that an insufficient quantity of DNA for sex determination was present.

Another molecular technique for sex determination that has been applied to skeletal remains was developed by Sullivan et al. (1993). For this method, which also uses the amelogenin gene, a short DNA fragment from intron 1 is amplified that contains a 6-bp deletion in the X chromosome sequence that is not present in the corresponding Y chromosome sequence. Gill et al. (1994) employed this technique to determine the sex of skeletal remains found near Ekaterinburg, Russia, that were reputed to be those of the Romanovs, their doctor, and three servants.

The method described in this paper, like the technique designed by Sullivan et al. (1993), solves the problem encountered when using the alphoid Y repeat sequences for sex determination, because both the X and the Y copies are amplified during PCR. Our method, however, appears to be more sensitive to very low quantities of DNA, such as those likely to be found in ancient bones. A dilution series testing the sensitivity of the primers indicates that they can begin amplification from as few as one or two copies of the gene (Fig. 2). The Sullivan et al. (1993) technique, on the other hand, in our experience requires at least 100 pg of DNA during 40 cycles of PCR in order to obtain a product (data not shown). Attempts to amplify DNA from the bones used in this research were not successful using the Sullivan et al. (1993) primers (data not shown).

Because of damage and degradation, analyzing ancient DNA is difficult, and it requires great care to prevent contamination

from modern sources. In addition to taking the precautions that were mentioned above, all results must be confirmed with independent extracts. For sex determination, samples that do not hybridize with the Y specific oligonucleotide should be examined using at least four separate PCR products in order to have a 94% certainty that no Y copies are present. This is especially true if the amount of nuclear DNA present is low (as indicated by difficulty in amplifying bands), possibly indicating that the PCR begins from only one copy (Fig. 4 and Table 1). In such instances, there is a 50% chance that the Y fragment will be amplified from a male in any one PCR. Even with four PCR amplifications, 6% of samples that start PCR from single molecules are expected to give incorrect results; thus, it is recommended that the DNA from such samples be quantified prior to testing (Handt et al., 1994). If insufficient DNA is present, then the samples should not be tested.

Sex determination using DNA can be valuable for both forensic and archaeological research. Standard osteological methods, however, are less expensive and more rapid when the skeletons of adults are complete and the bones are in good shape. The success rate using morphological techniques to determine the sex of well-preserved adult skeletons is generally said to range from about 80% to over 90% (St. Hoyme and Iscan, 1989). Techniques based on hard tissue morphology, however, are often ineffective for determining the sex of juvenile and fragmentary remains. For archaeological research, the use of DNA to determine the sex of juveniles provides an opportunity to extend traditional mortuary analyses through the inclusion of children of known sex. Molecular analyses can also address questions regarding the sex of adult skeletons that fall in the overlapping range of male and female morphological variation.

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LITERATURE CITED

Aasen E, and Medrano JF (1990) Amplification of the ZFY and ZFX genes for sex identification in humans, cattle, sheep and goats. BioTechnology *8:*1279–1281.

Akane A, Seki S, Shinono H, Nakamura H, Hasegawa M, Kagawa M, Matsubara K, Nakahori Y, Nagafuchi S, and Nakagome Y (1992) Sex determination of forensic samples by dual PCR amplification of an X-Y homologous gene. Forensic Sci. Int. *52:*143–148.

Bass WM (1971) Human Osteology: A Laboratory and Field Manual of the Human Skeleton. Columbia: Missouri Archaeological Society.

Chou Q, Russell M, Birch DE, Raymond J, and Bloch W (1992) Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res. *20(7):*1717–1723.

Cooper A (1993) DNA from museum specimens. In B Herrmann and S Hummel (eds.): Ancient DNA. New York: Springer-Verlag, pp. 149–165.

Cui K-H, Warnes GM, Jeffrey R, and Matthews CD (1994) Sex determination of preimplantation embryos by human testis-determining-gene amplification. Lancet *343:*79–82.

Ebensperger C, Studer R, and Epplen JT (1989) Specific amplification of the ZFY gene to screen sex in man. Hum. Genet. *82:*289–290.

Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, Evett I, Hagelberg E, and Sullivan K (1994) Identification of the remains at the Romanov family by DNA analysis. Nature Genet. *6:*130–135.

Hagelberg E, and Clegg JB (1991) Isolation and characterization of DNA from archaeological bone. Proc. R. Soc. Lond. [series B] *244:*45–50.

Hagelberg E, Sikes B, and Hedges R (1989) Ancient bone DNA amplified. Nature *342:*485.

Handt O, Richards M, Trommsdorff M, Kilger C, Simanainen J, Georgiev O, Bauer K, Stone A, Hedges R, Schaffner W, Utermann G, Sykes B, and Pääbo S (1994) Molecular genetic analyses of the Tyrolean ice man. Science *264:*1775–1778.

Handyside AH, Kontogianni EH, Hardy K, and Winston RML (1990) Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature *344:*768–770.

Honda K, Harihara S, Nakamura T, Hirai M, and Misawa S (1990) Sex identification by analysis of DNA extracted from hard tissues. Jpn. Forensics J. *44(4):*293–301.

Horai S, Hayasaka K, Murayama K, Wate N, Koike H, and Nakai N (1989) DNA amplification from ancient human skeletal remains and their sequence analysis. Proc. Japan Acad. *65(B):*229–233.

Höss M, and Pääbo S (1993) DNA extraction from Pleis-

tocene bones by a silica-based purification method. Nucleic Acids Res. *21(16)*:3913–3914.

Hummel S, and Herrmann B (1991) Y-chromosome-specific DNA amplified in ancient human bone. Naturwissenschaften *78*:266–267.

Hummel S, and Herrmann B (1993) Y-chromosomal DNA from ancient bones. In B Herrmann and S Hummel (eds.): Ancient DNA. New York: Springer-Verlag, pp. 205–210.

Kogan SC, Doherty M, and Gitschier J (1987) An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. N. Engl. J. Med. *317(16)*:985–990.

Milner GR, and Smith VG (1990) Oneota human skeletal remains. In SK Santure, AD Harn, and D Esarey (eds.): Archaeological Investigations at the Morton Village and Norris Farms 36 Cemetery. Illinois State Museum Reports of Investigations, No. 45. Springfield: Illinois State Museum, pp. 111–148.

Nakahori Y, Hamano K, Iwaya M, and Nakagome Y (1991a) Sex identification by polymerase chain reaction using X-Y homologous primer. Am. J. Med. Genet. *39*:472–473.

Nakahori Y, Takenaka O, and Nakagome Y (1991b) A human X-Y homologous region encodes "amelogenin." Genomics *9*:264–269.

Norby S, and Eriksen B (1992) Sex identification of forensic samples using PCR analysis for the presence of Y-chromosome specific DNA sequences. Adv. Forensic Haemogenet. *4*:38–40.

Pääbo S, Gifford JA, and Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Res. *16(20)*:9775–9787.

Redd AJ, Takezaki N, Sherry ST, McGarvey ST, Sofro ASM, and Stoneking M (1995) Evolutionary history of the COII/tRNA^{Lys} intergenic 9 b.p. deletion in human mitochondrial DNAs from the Pacific. Mol. Biol. Evol. *12(4)*:604–615.

St. Hoyme LE, and Iscan MY (1989) Determination of sex and race: accuracy and assumptions. In MY Iscan and KAR Kennedy (eds.): Reconstruction of Life from the Skeleton. New York: Alan R. Liss, pp. 53–93.

Sambrook J, Fritsch EF, and Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Stone AC, and Stoneking M (1993) Ancient DNA from a pre-Columbian Amerindian population. Am. J. Phys. Anthropol. *92*:463–471.

Sullivan KM, Mannucci A, Kimpton CP, and Gill P (1993) A rapid and quantitative DNA sex test: Fluorescence-based PCR analysis of X-Y homologous gene amelogenin. BioTechniques *15(4)*:636–641.

White TD (1991) Human Osteology. San Diego: Academic.

Witt M, and Erickson RP (1989) A rapid method for detection of Y-chromosomal DNA from dried blood specimens by the polymerase chain reaction. Hum. Genet. *82*:271–274.