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Anne C. Stone and Mark Stoneking

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Analysis of ancient DNA from a prehistoric Amerindian cemetery

Anne C. Stone* **and Mark Stoneking**

Department of Anthropology, The Pennsylvania State University, University Park, PA 16802, USA

The Norris Farms No. 36 cemetery in central Illinois has been the subject of considerable archaeological and genetic research. Both mitochondrial DNA (mtDNA) and nuclear DNA have been examined in this 700-year-old population. DNA preservation at the site was good, with about 70% of the samples producing mtDNA results and approximately 15% yielding nuclear DNA data. All four of the major Amerindian mtDNA haplogroups were found, in addition to a fifth haplogroup. Sequences of the first hypervariable region of the mtDNA control region revealed a high level of diversity in the Norris Farms population and confirmed that the fifth haplogroup associates with Mongolian sequences and hence is probably authentic. Other than a possible reduction in the number of rare mtDNA lineages in many populations, it does not appear as if European contact significantly altered patterns of Amerindian mtDNA variation, despite the large decrease in population size that occurred. For nuclear DNA analysis, a novel method for DNA-based sex identification that uses nucleotide differences between the X and Y copies of the amelogenin gene was developed and applied successfully in approximately 20 individuals. Despite the well-known problems of poor DNA preservation and the ever-present possibility of contamination with modern DNA, genetic analysis of the Norris Farms No. 36 population demonstrates that ancient DNA can be a fruitful source of new insights into prehistoric populations.

Keywords: ancient DNA; mitochondrial DNA; sex identification; Amerindians; amelogenin; genetic diversity

1. INTRODUCTION

The extraction of DNA from ancient human materials is fraught with many difficulties, not the least of which is contamination from modern DNA. However, despite the problems, some successes are apparent at the population level for both mitochondrial and nuclear DNA loci (Hagelberg & Clegg 1993; Fox 1996; Parr *et al*. 1996; Zierdt *et al*. 1996). The Norris Farms No. 36 cemetery population, located in the Illinois River valley in the central United States (figure 1), is perhaps the largest sample to date investigated at both types of loci (Stone & Stoneking 1993, 1998; Stone *et al*. 1996). The cemetery dates to about 1300 AD and was used for only a few generations (Santure 1990). It was completely excavated in 1984 and 1985 by the Illinois State Museum, and approximately 260 well-preserved skeletons were recovered. Most of the graves were located in a low mound in slightly alkaline loess. The age and sex distribution of the skeletons correspond to expected human mortality patterns in traditional societies (Milner *et al*. 1989), which suggests that most, if not all, community members were buried in this cemetery.

The cemetery and adjacent village are variants of the Oneota cultural tradition, which represents an abrupt departure from the previous cultural sequence. It is one of the earliest Oneota sites in the Illinois River Valley and is thought to represent an intrusive population that originated from earlier Oneota groups in the upper Mississippi River Valley (Esarey & Santure 1990). Further support that this was an intrusive population that was in conflict with neighbouring villages comes from the fact that the skeletal remains from the cemetery show an unusually high level of violent death; about one-third of the adult remains have indications of decapitation, scalping, shattered bones from blows, or arrow or spear wounds (Milner *et al*. 1991).

Mitochondrial DNA (mtDNA) was previously examined in 108 individuals from the cemetery (Stone & Stoneking 1993, 1998), while a segment of the amelogenin gene was used for DNA-based sex identification of 20 individuals (Stone *et al*. 1996). In this paper, we discuss the preservation of DNA in the cemetery and summarize the results of the previous genetic analyses, as well as present new data pertaining to DNA-based sex identification of the Norris Farms population.

2. METHODS

DNA was extracted primarily from ribs as described in Stone & Stoneking (1993, 1998). Multiple independent extractions were performed for each sample and standard precautions were taken to prevent contamination from modern DNA. For mtDNA analyses, the PCR, restriction digests and sequencing were performed as indicated in Stone & Stoneking (1998), while DNA-based sex identification using the amelogenin gene was performed as described in Stone *et al*. (1996). Amino-acid

*Address for correspondence: Department of Anthropology, University of New Mexico, Albuquerque, NM 87131–1086, USA (acstone@u.arizona.edu).

Figure 1. Map of the Americas indicating the location of the Norris Farms population (solid circle) and the locations of modern Native American populations (shaded circles) to which they were compared.

racemization analysis was carried out by H. Poinar (University of Munich) as described in Poinar *et al.* (1996).

3. RESULTS AND DISCUSSION

(a) *DNA preservation at Norris Farms*

The circumstances that favour DNA preservation are generally acknowledged to be cold, dry conditions (Höss *et al.* 1996; Poinar *et al.* 1996), or wet, anoxic conditions (Hagelberg & Clegg 1991; Hauswirth *et al.* 1991). DNA preservation at Norris Farms was generally very good with approximately two-thirds of the samples (108 out of 152) yielding mtDNA results. The cemetery was located on the edge of a bluff overlooking the Illinois River in well-drained, slightly alkaline loess soil. A silty, clay layer capped the eastern and northern portions of the mound and traces of this cap were found in the other parts of the mound (Santure 1990). The presence or absence of the clay cap over a burial did not significantly affect the preservation of DNA (data not shown), even though the clay is likely to have been more acidic than the loess and hence might be expected to increase DNA degradation. The depth of the grave in the mound, however, does seem to have influenced DNA preservation. Graves were categorized by 50 cm levels; the shallowest grave was 6 cm below the surface, the deepest was 216 cm below the surface, and the mean depth was 115 cm. Skeletons from deeper graves were more likely to have recoverable mtDNA (Spearman's rank correlation test, $R=0.227$, $p<0.05$). The relationship between the presence of DNA and the sex of the individual was also investigated to determine if some other variable related to difference in sex, such as different burial preparation, may have affected DNA preservation; however, no significant difference was found. Successful amplification of mtDNA loci was also independent of the age of the individual, despite the overall less robust nature of juvenile remains.

A useful proxy measure for DNA preservation is amino-acid racemization analysis (Poinar *et al.* 1996). During life, protein biosynthesis uses only the L-enantiomers of amino acids, while after death, racemization takes place, converting the L-form into the D-enantiomer until they occur in equal amounts. Aspartic acid racemizes most quickly and is the most useful indicator of DNA degradation; D–L ratios for aspartic acid less than 0.1 are generally compatible with DNA survival, while D–L ratios greater than 0.1 are typically not compatible with DNA survival (Poinar *et al.* 1996). Bone samples from five individuals were sent to H. Poinar at the University of Munich for amino-acid racemization analysis. These samples ranged from those that appeared to contain little DNA to those for which mtDNA and nuclear DNA loci could be readily amplified. In all five samples the D–L ratio for aspartic acid was 0.05–0.06, indicating the probable survival of DNA in all of the samples. In addition, the D–L ratio for aspartic acid was higher than that for alanine (0.0–0.02) and leucine (0.0–0.02) in these samples. A higher D–L ratio for alanine and leucine, in comparison to aspartic acid, would suggest contamination of the sample from a modern source (Poinar *et al.* 1996); thus, this analysis does not indicate contamination of any of the samples. Although the ability to amplify DNA was different for each sample, no clear trend between DNA recovery and the D–L ratio for aspartic acid is apparent with these five samples. Nonetheless, the use of a D–L ratio for aspartic acid of 0.10 as a 'cut-off' value beyond which DNA survival is unlikely (Poinar *et al.* 1996) is not contradicted by these results.

(b) *MtDNA analysis*

Amerindian mtDNA types cluster into four primary haplogroups that are defined by particular restriction site markers and by particular mutations in the first hypervariable region (HVI) of the mtDNA control region (Schurr *et al.* 1990; Torroni *et al.* 1993). A fifth group has been defined based on mutations at positions 16 223 and 16 278 (Anderson *et al.* 1981) in HVI (Bailliet *et al.* 1994; Forster *et al.* 1996). Most of these haplogroups are found in Asian populations but they are typically rare. In the Norris Farms sample, 108 individuals (out of 152) could be typed for the four markers that define haplogroups A through D. All four lineage clusters were found in the Norris Farms population, while six individuals did not possess any of the four characteristic markers (table 1). The fact that 94.4% of the mtDNAs in this pre-Columbian population belong to one of the four haplogroups that characterize contemporary Amerindian populations indicates that the population decrease that occurred following European contact (Thornton 1987; Ubelaker

Table 1. *Amerindian mtDNA haplogroups in the Norris Farms population and the characteristic markers for each haplogroup*

haplogroup	*n*	Hae III site nt 663	9 bp deletion	Hinc II site nt 13 259	Alu I site nt 5176	number sequenced	characteristic HVI mutations (+16 000)
A	34	+	–	+	+	11	111, 223, 290, 319, 362
B	13	–	+	+	+	7	189, 217
C	46	–	–	–	+	25	223, 298, 325, 327
D	9	–	–	+	–	5	223, 325, 362
other	6	–	–	+	+	2	278 and ?
total	108					50[a]	

[a]Two contaminating sequences were also found, one in haplogroup A and one in 'other'.

Table 2. *The sample size (n), haplotype diversity (h), sequence diversity (π) and the percentage of single lineages for the prehistoric Oneota and 14 contemporary Amerindian groups*

population	*n*	*h*	π	% single	reference
Norris Farms	50	0.91	0.015	73.9	Stone & Stoneking (1998)
Nuu Chah Nulth	63	0.95	0.016	46.4	Ward *et al.* (1991)
Bella Coola	40	0.9	0.015	9.1	Ward *et al.* (1993)
Yakima	42	0.89	0.015	80	Sheilds *et al.* (1993)
Emberá	44	0.94	0.017	25	Kolman & Bermingham (1997)
Huetar	27	0.71	0.011	54.5	Santos *et al.* (1993)
Kuna	63	0.59	0.01	42.9	Batista *et al.* (1995)
Ngobe	46	0.77	0.013	53.3	Kolman *et al.* (1995)
Wounan	31	0.9	0.019	50	Kolman & Bermingham (1997)
Yanomami	50	0.83	0.011	75	Easton *et al.* (1996)
Xavante	25	0.68	0.009	25	Ward *et al.* (1996)
Gaviao	27	0.87	0.013	14.3	Ward *et al.* (1996)
Zoro	30	0.77	0.012	44.4	Ward *et al.* (1996)
Mapuche	39	0.92	0.018	47.4	Ginther *et al.* (1993)
Haida	40	0.68	0.007	55.6	Ward *et al.* (1993)

1988, 1992) did not appreciably alter the composition of the Amerindian mtDNA gene pool.

Of 108 individuals for whom the mtDNA haplogroup was successfully defined, 52 were selected for HVI sequencing. These individuals included members of the four Amerindian haplogroups as well as those who did not appear to fall into one of these haplogroups, in numbers roughly proportional to their presence in the cemetery. HVI sequences from two individuals indicated the probable presence of extraneous DNA contamination; 23 different sequences occurred in the remaining 50 individuals (Stone & Stoneking 1998), for which the characteristic HVI mutations were found that agreed with the haplogroup designation based on the restriction sites (table 1).

The mtDNA haplogroup and sequence diversity at Norris Farms are similar to those found in other Amerind populations (table 2), as would be expected from the geographical location (figure 1) and the archaeological features of this prehistoric site. Despite the probable inclusion of related individuals in the sample, the Norris Farms population has a high level of mtDNA diversity, equivalent to that found in such modern groups as the Nuu Chah Nulth, Emberá and Mapuche. Other populations, such as the Haida (the sole Na-Dene speaking population analysed), Kuna and Xavante, have less diversity, probably due to founder effect and drift (Batista *et al.* 1995; Ward *et al.* 1993, 1996).

The Norris Farms population does have a high percentage of singleton mtDNA types (73.9%) compared to the other modern Amerindian populations (average=45.4%). This could reflect some loss of diversity (i.e. loss of rare mtDNA lineages) in modern populations due to the large decrease in population size associated with European contact (Thornton 1987; Ubelaker 1988, 1992). However, this could also reflect other aspects of population history, as some modern populations do have similar frequencies of singleton types. Additional pre-Columbian populations need to be analysed to see if the high percentage of singleton mtDNA types is characteristic of such populations, or is unique to the Norris Farms population.

Phylogenetic analyses of the mtDNA lineages from the Norris Farms population, modern Amerindians and Mongolians showed that the lineages within each haplogroup cluster together (figure 2), although the bootstrap support is low (Stone & Stoneking 1998). The lineage that falls outside of the four primary haplogroups found in Amerindians typically groups with Nuu-Chah-Nulth and Mongolian lineages that also have mutations at 16 223 and 16 278, or with Mongolian lineages (Kolman *et al.* 1996) that have mutations at 16 093, 16 223 and 16 357. Whether this lineage falls into haplogroup 'X', which is defined by mutations at 16 223 and 16 278 (Bailliet *et al.* 1994; Forster *et al.* 1996), or in another haplogroup is difficult to determine. Brown *et al.* (1997) suggest that a *Dde*I restriction site loss at 1715 is diagnostic

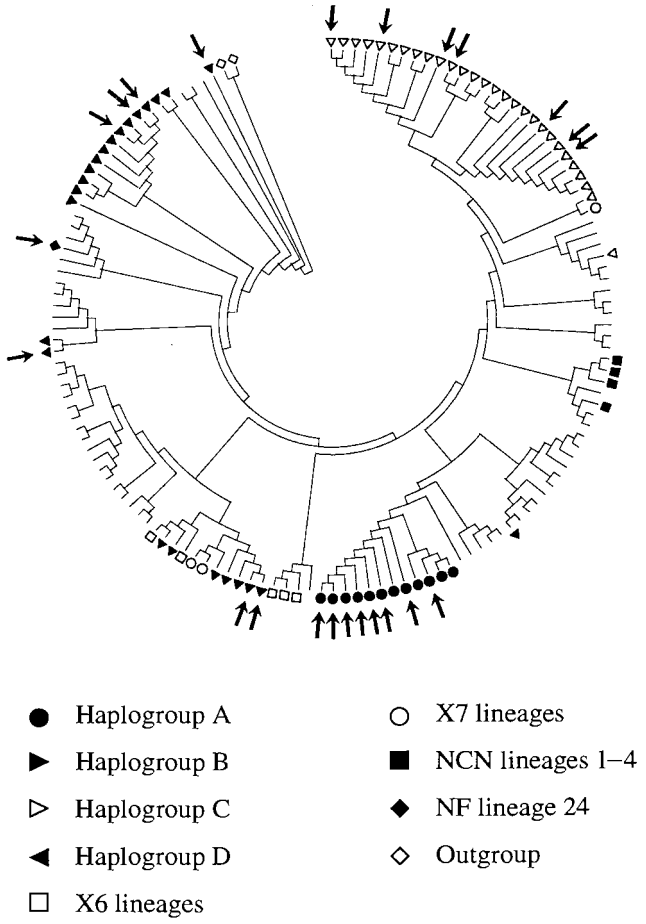

Figure 2. Neighbour-joining tree topology of Norris Farm, modern Amerindian and Mongolian HVI sequences (from Stone & Stoneking 1998). The haplogroup designation is indicated by symbols while unlabelled branches are Mongolian sequences that do not fall into haplogroups A–D. The Norris Farms sequences are indicated by arrows, while NCN sequences 1–4 are from the Nuu-Chah-Nulth (Ward *et al.* 1991) and the X6 and X7 sequences are from the Yanomami (Easton *et al.* 1996).

for this haplogroup; however, this site was not examined in the Norris Farms population.

(c) *DNA-based sex identification*

Stone *et al.* (1996) developed a new method of identifying the sex of skeletal remains using DNA, which is expected to be particularly useful for skeletons for which the morphological indicators of sex are absent (such as juvenile skeletons or fragmentary remains) or uninformative. In this method, a small fragment from the amelogenin gene (for which there are two copies, one on the X chromosome and the other on the non-recombining portion of the Y chromosome) is amplified and bound to a membrane; hybridization with sequence-specific oligonucleotide (SSO) probes is then used to detect the presence of the X and Y copies of the amelogenin gene.

This method was tested in a study of 20 skeletons of adults with unambiguous morphological indicators of sex from the Norris Farms cemetery (Stone *et al.* 1996). A concern arose that if the amplification reaction began with one or a few copies of template DNA, then only the X-chromosome copy of the amelogenin gene might amplify from male individuals, resulting in a false classification of those individuals as female. To minimize this possibility, a strategy was adopted whereby two independent amplification products were obtained from each of two independent DNA extractions from each skeleton; with a total of four amplification products analysed from each individual, the chance that a male would be misclassified if the amplification reaction began from a single DNA copy in each reaction is about 6%.

Table 3. *List of burials examined, the sex of each skeleton as determined by morphology, results from typing four PCR products and the average number of amplification reactions (N) required to obtain each product*

(ND, not determined.)

		PCR 1		PCR 2		PCR 3		PCR 4		
burial	sex	X	Y	X	Y	X	Y	X	Y	*N*
19	M	+	+		+	+		+	+	1
21	F	+		+		+		+		2.25
22	F	+		+		+		+		1.25
26	M	+	+	+	+		+	+	+	1.75
28	F	+		+		+		+		1.75
31	F	+		+		+		+		1.75
35	F	+		+		+		+		3.75
36	F	+		+		+		+		1.25
38	F	+		+		+		+		1.25
47	F	+		+		+		+		1.5
49	M	+		+		+		+		4.5
50	M	+			+	+		+	+	3
51	F	+		+		+		+		1.25
71	M	+			+	+	+	ND	ND	3.5
108	M	+	+	+	+	+	+	+		2.25
194	M	+			+	+			+	2.75
210	F	+		+		+		+		1.25
225	M	+			+	+	+		+	5.75
245	M		+	+		+	+		+	4.25
254	M	+	+	+	+	+	+	+		1

The amplification product from the ten females hybridized only with the X-specific SSO probe, as expected, while DNA from nine of the males hybridized with both the X- and the Y-specific SSO probes (table 3). In several males (such as burial 225) 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 amplification products hybridized only with the X-specific SSO probe. Although mtDNA was successfully amplified, it was difficult to obtain amplification products in this individual, and it is likely that there is insufficient DNA to confidently determine the sex of these remains. An average of 4.5 amplification reactions was required to obtain a single product for sex identification in this individual, whereas the other 19 individuals required an average of only 2.2 amplification reactions.

Assuming that the morphological indicators of sex are correct for this individual, this value of 4.5 amplification reactions per usable product can be used as a lower

Table 4. *List of burials, the estimated age and sex of each skeleton from morphology and DNA-based sex-typing results* (*as in table 3*)

(A indicates an artefact described in the text.)

			PCR 1		PCR 2		PCR 3		PCR 4		
burial	age	sex	X	Y	X	Y	X	Y	X	Y	*N*
56	15–17	M?		+		+	+	+		+	5.25
66	18–21	F?	+	+		+	+	+		A	5.3
95	13–17	1	+	+	+		+	+	+	+	2
181	2–3	1	+	+		A		+	+	+	4.3
183	3–4	1		A		+		A		+	3.5
204	1.3–2.75	1	+	+	+		+	+	+	+	2.75
241	adult	1		+	+		+		+		2
276	5–7	1		A		A					17/none
278	6–7	1	+		+		+	+	+	+	4.75
293	25–30	M?	+			+	+			+	4

bound to indicate the absence of sufficient DNA for sex identification. Burial 225 from this study also required more than 4.5 PCR amplifications on average to obtain a PCR product. Although the DNA data for this individual matched the results of morphological sex determination, it would be prudent to disregard these DNA data as they may instead reflect contamination. The results from one additional adult (burial 245) should also be viewed with some caution, as an average of 4.25 amplifications was required to obtain each PCR product from this individual (however, mtDNA sequencing for this individual did work relatively well). When these three problematic samples are omitted, an average of 2.0 amplifications was sufficient to obtain each PCR product.

This DNA-based sex identification method was then applied to 26 individuals of unknown or uncertain sex from Norris Farms (Stone 1996). At least one PCR product was obtained from ten of these individuals (table 4). Although it was possible to amplify the markers for the mtDNA haplogroups from all of these samples, no PCR products were obtained from 16 individuals after three to seven attempts to amplify DNA using the amelogenin primers. From the remaining ten samples, at least two independent extractions from each served as the DNA source for subsequent amplifications, and two PCR products were generated from each extract, as before (Stone *et al*. 1996). For six out of the ten individuals, an average of less than 4.5 PCR amplifications was required to obtain a product for dot blot analysis. Five were male according to products from both extracts, while burial 241 was male according to the PCR products from one extract and female according to the products of another; thus, additional products from this individual should be examined. Four samples had insufficient DNA to accurately identify sex, requiring an average of more than 4.5 PCR amplifications to obtain a single product.

PCR products slightly larger than the expected size were obtained from four individuals (table 4). For burial 276, two artefact bands were the only PCR products obtained in 17 attempts. In two amplification reactions, both the expected band and the artefact band were obtained from extractions from burial 66. In such cases only the smaller, expected product was cut out of the gel and used for reamplification and SSO-probe analysis. The SSO-probe results for the artefact products gave a signal with the Y probe only. Two of these artefact bands, from burials 183 and 276, were cloned, and five clones were sequenced. The sequences were 151 bp in length and identical with the exception of single point mutations in two sequences that could reflect *Taq* polymerase or cloning errors. The primer sequences in the clones were in the correct orientation, but the sequence did not match the published amelogenin sequence or any other sequence in GenBank. In addition, these sequences did not contain a binding site for the Y-specific probe. Because of the sensitivity of dot blot detection, the positive signal from the Y-specific probe may be the result of hybridization with authentic amelogenin gene fragments that were present in low copy number and thus not visible on the agarose gel. Because of the uncertainty of the source of these positive Y results, these data were disregarded and the sex of burials 181 and 183 were considered not determined by this analysis. Thus, out of 26 individuals of unknown or indeterminate sex for which DNA-based sex-typing was attempted, only three appeared to give reliable results, even though PCR products for mtDNA haplogroup analysis were obtained from all 26.

Presumably, nuclear DNA is not preserved in many of the specimens because it is present initially in smaller amounts than mtDNA. It is worth noting that the bone samples taken from most skeletons for DNA analysis were ribs (usually the 11th or 12th rib). This may account for the apparent discrepancy between the previous study (Stone *et al*. 1996) in which 17 out of 20 samples gave reliable results for DNA-based sex identification, and the present study in which only 3 out of 26 remains (primarily from juvenile or fragmentary skeletons of indeterminate sex) gave apparently reliable results for this nuclear DNA assay. Because ribs are thin, especially in juveniles, nuclear DNA analysis could be more successful at Norris Farms using teeth or more substantial long bones.

4. CONCLUSIONS

The total burial population at Norris Farms consists of over 260 individuals and almost 60% were the subject of genetic analyses. Mitochondrial DNA analyses provide information about the peopling of the New World and the genetic structure of pre-Columbian populations. DNA sex identification is useful for juvenile and fragmentary remains where standard osteological indicators of sex are ambiguous. The genetic results from Norris Farms will also be combined with archaeological data to investigate the social organization of this prehistoric society (in preparation).

The preservation of DNA at this site is relatively good with two-thirds of the individuals sampled yielding mtDNA and approximately one-fifth of those also yielding nuclear DNA. Amino-acid racemization data indicate that all of the samples (even those that do not appear to contain DNA) had aspartic acid D–L ratios in the range of samples that should contain DNA. New extraction techniques, use of more robust samples, such as long bones and teeth, and more sensitive methods of PCR

may yet recover DNA from those samples where it was not successfully obtained.

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Discussion

A. J. Davidson (*Oaklands College, Hertfordshire, UK*). You said that a gene carried on both the X and Y chromosomes could be used to determine gender. Could you give more detail?

M. Stoneking. Yes, the amelogenin gene is present on both the X chromosome and the non-recombining portion of the Y chromosome. There are diagnostic nucleotide substitutions that differentiate the X and Y chromosome copies of the amelogenin gene, and our assay is based on these differences, so we can tell if the DNA came from a female, with only the X-chromosome copy, or a male, with both the X- and Y-chromosome copies of the gene.

A. Millard (*University of Durham, UK*). You showed that ancient genetic variation and modern genetic variation were similar, implying that the bottleneck in population size was at the time of colonization. The ancient data seemed to be a larger sample size, compared to many of the modern populations. Are the population sizes taken into account in making the comparison?

M. Stoneking. Yes, most of the comparisons of mtDNA diversity between ancient and modern populations were adjusted for sample size differences. However, the estimate of the number of rare or unique mtDNA types in a population would certainly be sample-size dependent.

J. Bada (*University of California, USA*). This isn't really a question to the speaker, but rather one to one of the organic geochemists in the audience. Couldn't you sex these skeletons using steroids?

R. Evershed. As you probably know, we have recently shown that cholesterol and various of its diagenetic products survive in archaeological bone. In view of this we have considered the possibility that steroid hormones may also persist and be of some value in sexing skeletons. The major problem I foresee is that of their detection, since these compounds will be present at very low concentrations; a combination of GC/MS and radioimmunological techniques maybe be a fruitful approach. Also of importance is the question of survival of such compounds on archaeological time-scales and the relative rates of diagenetic loss of oestrogens versus androgens, since they are of course both produced by the testes and ovaries with only the balance governing sexual differentiation in adults.

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