Ancient DNA in Anthropology: Methods, Applications, and Ethics

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ABSTRACT Anthropologists were quick to recognize the potential of new techniques in molecular biology to provide additional lines of evidence on questions long investigated in anthropology, as well as those questions that, while always of interest, could not have been addressed by more traditional techniques. The earliest ancient DNA studies, both within anthropology and in other fields, lacked rigorous hypothesis testing. However, more recently the true value of ancient DNA studies is being realized, and methods are being applied to a wide variety of anthropological questions. We review the most common methods and applications to date, and describe promising avenues of future research. We find that ancient DNA analyses have a valuable place in the array of anthropological techniques, but argue that such studies must not be undertaken merely to demonstrate that surviving DNA is present in organic remains, and that no such work should be performed before a careful consideration of the possible ethical ramifications of the research is undertaken. Yrbk Phys Anthropol 45:92–130, 2002.

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INTRODUCTION

The development of new techniques in molecular biology in the late 1980s (Mullis and Faloona, 1987) rendered possible the analysis of the genetic material of deceased organisms. Physical anthropologists had long used molecular characters of modern populations to elucidate human variability and human prehistory (e.g., Wilson and Sarich, 1969). The application of the techniques of ancient DNA (aDNA) allowed, for the first time, a *direct* incorporation of a temporal component in molecular analyses. Anthropologists were quick to adopt these new techniques for the production of previously unobtainable data, which they have applied to the traditional suite of anthropological research problems.

In the past decade, there have been several reviews of aDNA methods and results, some quite general (Pääbo, 1989, 1993; Pääbo et al., 1989; Rogan and Salvo, 1990; Eglinton and Logan, 1991; Macko and Engel, 1991; DeSalle, 1994; Handt et al., 1994; Lister, 1994; Tuross, 1994; Poinar et al., 1996; Audic and BeraudColomb, 1997; Austin et al., 1997; Yang et al., 1997; Cooper and Wayne, 1998; Kelman and Kelman, 1999; Wayne et al., 1999; Hofreiter et al., 2001; Marota and Rollo, 2002), and a few specific to anthropological applications (Sykes, 1993; Brown and Brown, 1994; O’Rourke et al., 2000a; Brown and Pluciennik, 2001). This review presents an updated discussion of recent research, organized by the level of the research question, from individual to species. In addition, we present an extended examination of aDNA methods, including data analysis. Finally, we include a substantial discussion of the ethical, legal, and social (ELSI) issues involved.

Application of aDNA techniques within anthropology permits analyses of patterns of molecular variability in both human and nonhuman organisms, to test hypotheses about human origins and behavior. Population movement is often inferred as an explanation for rapid changes in material culture. This explanation can be rigorously tested by the application of molecular techniques to the human remains recovered from both before and after the inferred population replacement. Additionally, such data can be used to reconstruct ancestor-descendant relationships between populations, and to discern patterns of interrelatedness between ancient groups with various levels of shared material culture. Further, molecular data obtained from ancient human re-

mains can elucidate patterns of social structure. High-resolution analyses allow the sexing of human remains (particularly useful with fragmentary or subadult remains), as well as the development of an understanding of the spatial patterning of maternal and paternal lineages across burial grounds. From such data, light can be shed on issues of social status, marriage patterns, burial customs, and differential patterns of disease and mortality by sex.

Nonhuman remains can also be subjected to aDNA analysis to illuminate several aspects of human prehistory. The patterns of molecular diversity in nonhuman species can assist in the understanding of hunting and dietary behavior, to track the domestication of various species, and to trace the histories of ancient diseases. Furthermore, nonhuman remains can be employed in environmental reconstruction, in addition to being used as proxies for human movement, having been transported as commensal species.

Background

The ability to extract and analyze DNA from ancient remains has a relatively short history. The earliest reported aDNA sequence came from the quagga, an extinct member of the horse/zebra family (Higuchi et al., 1984), followed the next year with the first ancient human sequence (Pääbo, 1985a; but see Wang and Lu, 1981). The next few years saw several reports of additional ancient human DNA recovery (Doran et al., 1986; Pääbo et al., 1988; Hagelberg et al., 1989), accompanied by minimal hypothesis testing. Initial studies were severely limited by the degraded and fragmented nature of aDNA, mostly a result of hydrolytic and oxidative forces acting postmortem. However, the field was revolutionized (as was molecular genetics in general) by the invention and development of the polymerase chain reaction (PCR) procedure for amplifying millions of copies of short fragments of DNA in vitro (Mullis and Faloona, 1987; Saiki et al., 1988; Pääbo, 1989; Pääbo et al., 1989; for details, see Methods, below). As discussed in Pääbo (1993), PCR greatly increased our ability to reliably and reproducibly type ancient genetic markers.

Early aDNA studies concentrated on the biochemistry of DNA degradation, and simply confirmed the endogenous nature of the recovered DNA (Johnson et al., 1985; Pääbo, 1985a,b, 1989; Doran et al., 1986; Higuchi et al., 1987; Rogan and Salvo, 1990; Thuesen and Engberg, 1990; Lawlor et al., 1991). We now have a greater understanding of degradative processes and potential complications (Lindahl, 1993, 1997; Hedges et al., 1995; Hoss et al., 1996; Poinar et al., 1996; Bada et al., 1999; Arroyo-Pardo et al., 2002; Rollo et al., 2002), but there is still much work to be done.

Although early studies suggested that DNA was recoverable from remains more than a million years old (Golenberg et al., 1990; Soltis et al., 1992; Cano et al., 1992; DeSalle et al., 1992; Woodward et al., 1994), recent studies have shown that these initial results were due to contamination from modern sources (Pääbo and Wilson, 1991; Young et al., 1995; Zischler et al., 1995; Wang et al., 1997; Yousten and Rippere, 1997). It does not appear that DNA can survive significantly longer than 130,000 years, even under the best circumstances (Stankiewicz et al., 1998; Loreille et al., 2001), a figure which exceeds some earlier estimates (Pääbo, 1989; Pääbo and Wilson, 1991; Lindahl, 1993, 1997; Poinar et al., 1996; Austin et al., 1997; Wayne et al., 1999; Hofreiter et al., 2001). Nevertheless, studies of aDNA from remains within this time frame have the potential to add greatly to our understanding of human/primate evolution and history, as discussed below.

Within the context of aDNA studies, there are two main DNA sources: organellar and nuclear. Although the vast majority of genomic DNA is present in the nucleus of a cell, each cell only contains two copies of nuclear DNA (one paternal and one maternal). On the other hand, although the mitochondrial and chloroplast organelles only contain a small minority of the total genomic DNA per cell, because there are hundreds of each organelle within the cell, each containing multiple copies of organellar DNA, there are often thousands of copies of mitochondrial or chloroplast DNA per cell. This higher copy number per cell results in a higher likelihood of recovery of intact segments of DNA from these organelles, compared with nuclear DNA, and most aDNA studies to date have concentrated on this type of DNA. However, with improved extraction and amplification techniques, some researchers are beginning to have success in accessing ancient nuclear DNA sequences (Bacher et al., 1990; Hummel and Hermann, 1996; Zierdt et al., 1996; Gerstenberger et al., 1999; Hummel et al., 1999; Schmerer et al., 1999; Schultes et al., 1999; Cunha et al., 2000). This broadens significantly the horizon of potential hypotheses that can be tested using aDNA, although such studies involve substantially higher failure rates at present.

Nuclear markers on the sex chromosomes are most often used to genetically sex ancient individuals (especially juveniles or partial remains; Bacher et al., 1990; Faerman et al., 1998; Schultes et al., 1999; Cunha et al., 2000), but can also be used to identify maternal (X chromosome) or paternal (Y chromosome) lineages, and to detect genetic diseases. Autosomal nuclear markers (found on the nonsex chromosomes) can be utilized for paternity and maternity testing (especially using microsatellite markers), and to detect the presence/absence of particular genetic diseases or geographically specific variation. Examining mitochondrial DNA (mtDNA) allows us to trace maternal lineages through time (Spuhler, 1988; Wilson et al., 1985), as the mitochondrial DNA is passed only from mother to child. Due to a relatively high mutation rate (Brown et al., 1979; Harrison, 1989), mitochondrial markers are

TABLE 1. Anthropological uses of ancient DNA

Application	Implications	Markers
Genetic sexing	Understand marriage and burial patterns, differential mortality rates between sexes, and differential patterns by sex of disease, diet, status, and material possessions	Sex chromosome markers
Nonhuman aDNA	Understand hunting and dietary patterns, domestication of animals and plants, environmental reconstruction, commensal animals as proxies for human populations, and trace history and patterns of prehistoric and historic diseases	Mitochondrial, chloroplast, and autosomal DNA
Maternal and paternal kinship	Understand social structure, status, marriage patterns, burial customs, and migration	Mitochondrial and sex chromosome DNA, and autosomal microsatellites
Population continuity and replacement	Trace prehistoric population movement, ancestor-descendant relationships between groups, and relationships among ancient groups with similar/different morphology or cultural remains	Mitochondrial, sex chromosome, and autosomal DNA
Phylogenetic reconstruction	Patterns of species evolution, and origin of modern humans	Mitochondrial, sex chromosome, and autosomal DNA

also often geographically specific, and in some cases are limited in distribution to a single tribe (private polymorphisms) (Schurr et al., 1990; Merriwether et al., unpublished findings). When analyzing multiple samples from a population, the methods of population genetics, including construction of phylogenetic trees or networks, estimates of genetic diversity, genetic distance between populations, and estimates of gene flow between populations, can be applied. When analyzing nonhuman animal species, mitochondrial and nuclear DNA can be used in the same ways described above. However, to this point, nonhuman aDNA has been used (anthropologically) mostly to identify the genus and/or species of a morphologically indeterminate sample, or to inform phylogenetic analyses. Chloroplasts, found in plants, have varied inheritance patterns (Birkey, 2001). In some cases, chloroplast DNA could be used to trace maternal or paternal lineages of plants, but in general it is more anthropologically useful for the identification of plant genus, and in some cases species (Brown et al., 1994; Rollo et al., 1994). DNA from infectious organisms such as bacteria and viruses can also often be detected in ancient remains (Salo et al., 1994; Taubenberger et al., 1997; Braun et al., 1998; Donoghue et al., 1998; Guhl et al., 1999; Raoult et al., 2000; Taylor et al., 2000). The possible applications of these techniques for anthropology are limited only by the imagination. The most common applications to date are discussed below (and see Table 1).

METHODS

The following is only a brief summary of the methods and considerations involved in aDNA analysis. A detailed description of these methods is given in the Appendix. Ancient DNA has now been successfully extracted from a wide variety of organic remains, including teeth, bone, and preserved soft tissues. Ancient DNA has also been extracted from other resources, that while not "ancient" in the strictest sense, necessitate the use of techniques developed for the challenges associated with aDNA, such as skins held in museums (Higuchi et al., 1984; Horsburgh et al., 2002), hair (Morin et al., 1992, 1994), and feces (Gerloff et al., 1995; Launhardt et al., 1998). Like truly ancient samples, these resources suffer from a fragmented genome and the presence of PCR inhibitors, which are coextracted with the DNA, frequently due to the preservatives that have been applied (Nicholson et al., 2002).

The quality of the DNA that survives in ancient samples is highly dependent on the conditions of the archaeological site from which they were excavated, much less than on the absolute age of the sample (Pääbo, 1989; Rogan and Salvo, 1990; Tuross, 1994; Hoss et al., 1996; Austin et al., 1997; O'Rourke et al., 2000a; Kaestle and Smith, 2001a, b; Robins et al., 2001). The likelihood of success can be predicted, to a degree, from the gross morphology of the sample, as it is affected by many of the same factors as is DNA preservation. Except when the sample has been mineralized, it has been our experience that the harder a bone or tooth sample is, the greater the probability of intact DNA being present in the sample.

Before DNA extraction can begin, the surface of the sample must be treated to remove contaminating (exogenous) DNA. This can be achieved by physically removing the surface of the sample, treating it with bleach, irradiating it with ultraviolet (UV) light, or a combination thereof. Following decontamination, the sample is usually broken to expose internal surfaces. Often, the sample is then treated with a proteinase and a detergent. The digested sample is subjected to one of two protocols. A phenol/chloroform extraction involves incubation with an organic phase (phenol and chloroform) into which many of the cell components migrate, leaving the DNA in the aqueous phase. The alternative approach introduces silica powder to the digested sample, to which DNA binds under the influence of guanidinium thiocyanate, allowing the remainder of the contents of the digest to be washed away. Recently,

several methods combining phenol/chloroform and silica were developed.

The DNA extract is then concentrated, and the section of interest is copied (amplified) using the polymerase chain reaction (PCR). The section of DNA most frequently targeted is the hypervariable region of the mitochondrial genome. This particular stretch of DNA is chosen because the mitochondrial genome is present in multiple copies in most cells, increasing the likelihood that at least a few copies will survive for substantial periods of time. Once amplified, the DNA of interest can be examined by direct sequencing, by using restriction enzymes that cleave the DNA at specific sequences, or by other standard methods, to discern sequence differences between individuals.

The resulting DNA data can by analyzed in myriad ways, but they all seek to recognize meaningful patterns in variability between individuals and groups. These methods include genetic distance statistics, phylogenetic trees and networks, cluster analyses, and simulation analyses.

Proper extraction and analysis of aDNA are quite complicated, and methods continue to evolve (see the Appendix for an in-depth description of methods of extraction and analysis). Despite this, there is general agreement on standard protocols to prevent and detect contamination (see Appendix), which are especially important to follow in analyses of unique or extraordinary samples. Unfortunately, this has not always been done (e.g., Woodward et al., 1994; Adcock et al, 2001a). We also note that aDNA researchers regularly experience failure rates of over 50% (e.g., Malhi, 2001), often discard weeks' (or months') worth of data due to contamination problems (e.g., see Kaestle et al., 1999), and speak irreverently of "PCR gods." This is a task for neither the impatient nor the ill-trained. However, this is also not magic. There is sufficient evidence today to be confident that aDNA can be recovered from a multitude of sources, dating as far-back as tens of millennia in the past.

APPLICATIONS

Human sources

Individual level. At the simplest level, that of the individual, aDNA studies allows us to determine the sex of an individual using markers on the X and Y chromosomes. Ancient DNA can also identify individuals uniquely, using methods similar to those employed by forensic scientists. In this way, mixed remains can be sorted into a minimum number of individuals, and disarticulated remains can be reassociated (whether they became disarticulated at time of burial or after recovery). If morphological attributes suggest that an individual suffered from a genetic or infectious disease, aDNA could be used to confirm the presence of the disease-causing allele or infectious agent. Finally, individuals with known living descendants could be individually identified through comparisons of their aDNA with that of their putative descendants.

Genetic sexing is particularly useful in cases of recovery of fragmentary remains or of juveniles and infants, who are extremely difficult to sex using standard morphological methods (Schutkowski, 1993). Two interesting examples of genetic sexing of stillborn/neonate individuals are those performed on remains from an Ashkelon bathhouse sewer (Israel, late Roman era; Faerman et al., 1998), and those performed on remains from the Aegerten burial site (Bern, Switzerland, 12–19th centuries; Lassen et al., 2000).

Excavations at Ashkelon discovered the remains of approximately 100 neonates in a sewer beneath a Roman bathhouse, presumed to have been a brothel, dating between the 4–6th centuries CE (Smith and Kahila, 1992; Faerman et al., 1998). These remains, found along with animal bones and other refuse, are presumed to have been the result of infanticide, especially when compared with the careful burial of an infant uncovered at the same site (Smith and Kahila, 1992; Faerman et al., 1998). Genetic sex analyses on 43 left femurs from these individuals were able to identify the sex of 19, with 14 being male and 5 female (Faerman et al., 1998). This high frequency of male infanticide ($\sim$74%) is surprising, given that daughters were generally the less valued sex in this society (Pomeroy, 1983; Wiedemann, 1989). The authors suggest that these neonates were the offspring of prostitutes/courtesans working in the bathhouse who preferentially reared females to follow in their professional footsteps (Faerman et al., 1998). However, it is important to note that, with such a small sample size, these results are not quite statistically significantly different from the observed natural neonatal sex ratio of 1.05:1 (Cowgill and Hutchinson, 1963) (chi-square = 3.8377, $P = 0.0501$; our calculations). In addition, the DNA fragments amplified from these remains are relatively long for ancient nuclear DNA, which leaves us with questions regarding the possibility of contamination from modern sources. The publication of mitochondrial sequences from these ancient neonates would help alleviate our concerns.

The Aegerton site is a cemetery site associated with a church, including 263 graves dating between the 12–19th centuries CE, with an additional 132 stillborn or neonate individuals ("Traufkinder") buried near the church walls (Lassen et al., 2000). Morphological sex determination of the infants showed a skewed sex ratio, with 60% of individuals assigned as females (Bacher et al., 1990). This was a surprising result, as the context of burial suggested that these infants had been natural stillbirths or neonatal deaths buried without baptism (Bacher et al., 1990; Lassen et al., 2000), and thus should conform to the slightly higher mortality rate of males during late gestation and shortly after birth (Cowgill and Hutchinson, 1963; Shapiro et al., 1968). The excess in female individuals is statistically significantly different from the expected natural 1.05:1 ratio (chi-

square = 4.002, $P = 0.045$; our calculations). This excess in female individuals suggested, therefore, that some type of sex-biased neglect, or even infanticide, might be taking place. However, genetic sex determinations on 121 of the stillborn/neonate individuals found that many individuals had been assigned to the incorrect sex, and showed instead a slightly male-biased sex ratio (Lassen et al., 2000), as expected for natural stillbirth/neonatal mortality rates. Examination of the morphometric vs. genetic sex of infants between ages 0–6 months from this site also showed an underdetection of male infants using morphometric methods (Lassen et al., 2000).

A high error rate in morphological sex determination of infant and juvenile remains, as seen in the example above, is not terribly surprising (Mays and Cox, 2000). However, evaluation of morphological sexing of adults using genetic techniques has also shown a relatively high error rate (Hummel et al., 2000). Even when limited to remains of fully adult individuals with skulls and, in most cases, the os coxa preserved, from a sample with strong dimorphism of cranial traits, morphological sexing error rates (determined by genetic sexing) were approximately 12% (Hummel et al., 2000). Those subsets of individuals who were less confidently assigned to sex (deemed "ambiguous") using morphological methods were, in fact, incorrectly assigned in 33% of cases (Hummel et al., 2000). These results confirm those based on morphological studies of individuals of known sex (Weiss, 1972; St. Hoyme and Iscan, 1989).

Identifying the sex of ancient remains, especially those difficult to sex morphologically, can help us test hypotheses of differential mortality rates (either natural or through human action), as shown above. In addition, genetic sex identification can allow us to explore differential patterns by sex of disease, diet, status, and material possessions (at least those represented as grave goods), all of which have been hypothesized to be important factors in prehistoric and historic human societies (Domasnes, 1991; Larsen, 1997; Grauer and Stuart-Macadam, 1998; Pearson, 1999; Arnold and Wicker, 2001). However, when interpreting our results, we must remind ourselves that the dichotomous nature of genetic sex does not always map one-to-one onto societies' gender roles (whether dichotomous or more nuanced) (Rubin, 1975; Taylor, 1996; Pearson, 1999). In addition, it is important to temper this "functionalist" viewpoint with the knowledge that "the arena of mortuary rites [forms] a nexus of conflict and power struggle" among living agents (Pearson, 1999, p. 23). As such, mortuary practices are a reflection of the place in society (in terms of age, sex, gender, kinship, status, etc.) of not only the deceased, but also of those still living (Giddens, 1984; Wylie, 1989; Metcalf and Huntington, 1991; Pearson, 1999).

Beyond sexing, individual variation on autosomal chromosomes could be utilized for several purposes. First, in cases where morphological (and/or historical) evidence suggests that an individual suffered from a genetic disease, the region of the gene associated with that disease could be amplified from aDNA, and mutations associated with that disease could be detected. For example, it has been suggested that we could determine if Abraham Lincoln suffered from Marfan syndrome (McKusick, 1991; Reilly, 2000), which is caused by one of several mutations in the fibrillin gene, located on chromosome 15 (Ramirez et al., 1993). Lincoln appeared to suffer from several of the symptoms of Marfan syndrome (e.g., extremely tall, with very long arms and hands), and descendants of his great-great-grandfather (Mordecai Lincoln II) have been diagnosed with the disease (Reilly, 2000). It has been suggested that, had Booth not assassinated him, he might have died at a relatively young age due to the rupture of his aorta, a common cause of death in those with Marfan syndrome. Although Lincoln's preserved tissue is held by the National Museum of Health and Medicine in Washington, DC, and several people have proposed this research, it has not yet been authorized (Reilly, 2000). It has also been suggested that Lincoln suffered from depression, described by contemporaries as "melancholia." Although current research has suggested that there is some genetic component to many depressive disorders (Berrettini et al., 1994; Ewald et al., 1995; Blackwood et al., 1996; Ginns et al., 1996; Reus and Freimer, 1997; Baron, 2001), thus far candidate genes have not stood up to rigorous scientific testing (Baron et al., 1993: Gomez-Casero et al., 1996; Smyth et al., 1997). This does not mean that genes increasing risks of depression will not be identified in the near future, and it has been suggested that Lincoln's "melancholia" could be explored using aDNA as well (Reilly, 2000). The ethics of studying diseases, especially psychological diseases, in deceased individuals of known identity, both for the impact on their reputations and those of their descendants, is problematic (Holm, 2001), and is discussed in more detail in Ethics of Ancient DNA Research, below.

Second, persons with known living descendants could be individually identified through comparisons of their aDNA to that of their putative descendants. If children of the individual can be identified, a "reverse" paternity or maternity test can be performed using microsatellite markers, as was done to identify the remains of Josef Mengele exhumed in Brazil (Jeffreys et al., 1992). If direct maternal or paternal relatives can be identified, mtDNA or Y-chromosome haplotypes can be used to test hypotheses of identity, as was done with much publicity in the case of the Romanov family remains (Gill et al., 1994), and also to show that the remains buried in Delft as "Louis XVII" cannot be the son of Louis XVI and Marie-Antoinette (Jehaes et al., 1998).

Although recently deceased individuals with living descendants or known familial relationships can be identified using aDNA, it is much more difficult to identify the descendants of a single ancient individ-

ual of unknown identity from hundreds or thousands of years ago. If we take mitochondrial DNA as an example, it could be argued that modern individuals possessing hypervariable sequences identical to an ancient individual are likely to be direct maternal descendants, while those without identical sequences are not. However, there are several difficulties with this argument. First, most people alive thousands of years ago have either no direct maternal descendants or a great number of maternal descendants (scattered throughout many living populations, most likely). Avise (1987) showed that if the number of daughters produced by females follows a Poisson distribution with a mean (and variance) of one surviving daughter per female, the probability that any individual mother's mtDNA will survive even 100 generations (approximately 2,000 years for humans) is lower than 2%. Thus, most females alive 2,000 years ago will have no direct maternal descendants. Conversely, the vast majority of people alive today can trace their mitochondrial lineage to a very small number of women living 2,000 years ago. However, since at least some of those "lucky" women were matrilineally related to those whose lineages do not survive, matches (or near-matches) between modern lineages and those derived from prehistoric remains thousands of years old can be expected to occur at a much higher frequency.

For example, if we examine mitochondrial sequences from two ancient individuals from western Nevada (NSM 10, 1,620 ± 50 BP and NSM 11, 1,490 ± 50 BP; Kaestle, 1998; Kaestle and Smith, 2001a), and compare them with sequences publicly available on the GenBank database (maintained by the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/, Benson et al., 2000), we find two very different stories. For the first ancient individual (NSM 10, nucleotide positions (nps) 16090–16330 = 241 base pairs (bp)), there are no identical sequences in the database. Thus, we find no individuals (sequenced to date) alive today who can trace their maternal lineage directly back to this ancient individual. On the other hand, for the same region of sequence for the second individual (NSM 11), there are seven identical sequences found in the database. These matches include three modern Native American individuals: two from the Brazil/Paraguay/Uruguay region of South America (GenBank accession number AF243628, Alves-Silva et al., 2000; and GenBank accession number AF346984, Ingman et al., 2000), and one from the Northwest Coast of North America (GenBank accession number M76011, Ward et al., 1991). The remainder of the matches are individuals from Asia. These include three modern Ainu from Japan (Genbank accession numbers D84762, D84769, and D84773, Horai et al., 1996), and a Kazak individual from far western China (Genbank accession number AF273575, Yao et al., 2000). Although it is possible that an ancient Native American woman alive approximately 1,500 years ago is the ancestress to living peoples in both South and North America, it is unlikely. It is extremely unlikely that she is also the ancestress of people living today in northern Japan and western China. A more likely explanation is that all of these people are descendants of some earlier ancestress, probably living in Asia thousands of years ago, before the peopling of the Americas. Although they are ultimately related to each other in some distant way, it is unlikely that any are direct descendants of the ancient individual in question.

Another difficulty in tying living people to a single ancient individual is that it is impossible to *rule out* a direct relationship using maternal and/or paternal lineages. Because mtDNA is inherited through the maternal line, but nuclear DNA is inherited from both parents equally, while one inherits 100% of one's mtDNA from only 1 of 16 great-great-grandparents, that particular ancestress contributed only 1/16 or about 6% of one's nuclear DNA. Thus, mitochondrial DNA is inherited from only one (female) of many ancestors, and only traces that one relationship of many. If an ancestral connection to an ancient female individual includes just one male (e.g., a great-great-great-grandfather), then the mitochondrial signal of that connection will be lost. The same is true of Y-chromosome DNA, except that it is inherited exclusively through the paternal line. Thus, although specific modern individuals can be included as possible direct descendants of a particular prehistoric individual (as above), no modern individual can be definitely excluded as a descendant of any given prehistoric individual using aDNA data.

Family level. The use of pedigrees has a long history within the field of anthropology, and aDNA analyses now allow us to extend these applications into the past. At a basic level, maternal and paternal lineages (but not necessarily maternity and paternity) can be identified using mtDNA and Y-chromosome DNA, respectively. If long, highly variable DNA regions are examined, people from the same archaeological site or region who share identical, relatively rare mutations are likely to be closely related, because they are not separated by enough generations for a mutational event to have occurred. However, this method does not allow us to identify maternity or paternity with a high degree of confidence. An individual, barring a meiotic mutational event, will share identical mtDNA mutations not only with his/her mother, but also with siblings, maternal grandmother, maternal aunts and uncles, maternal cousins, etc. Furthermore, males, again barring a meiotic mutational event, will share identical Y-chromosome mutations not only with their fathers, but also with their brothers, paternal grandfather, paternal uncles, paternal male cousins, etc.

To actually identify maternity and paternity among ancient individuals, we must examine the variation in a relatively large number of highly vari-

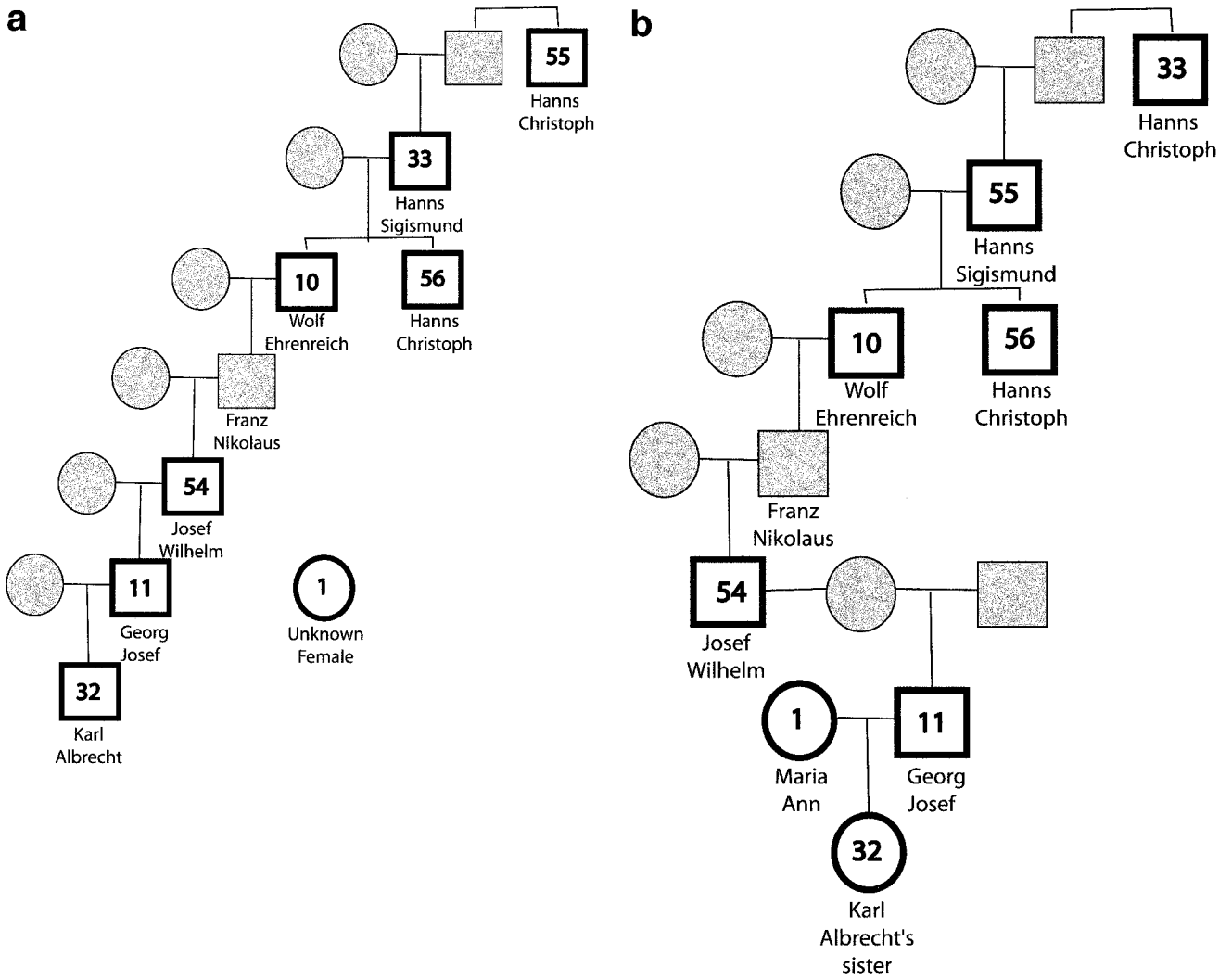

Fig. 1. **a:** Historical reconstruction of Königsfeld family pedigree. Shaded individuals were not buried in the family sepulcher at St. Margareth. Individuals in bold represent recovered skeletal remains, with skeletal ID number associated with each individual indicated. Data from Hummel et al. (1999, Fig. 2, p. 1718). **b:** Genetic reconstruction of Königsfeld family pedigree. Shaded individuals were not buried in the family sepulcher at St. Margareth. Individuals in bold represent recovered skeletal remains, with skeletal ID number associated with each individual indicated. Data from Hummel et al. (1999, Fig. 2, p. 1718).

able autosomal markers. Because children inherit one copy of each chromosome from each parent, half of their autosomal mutations should match each parent. Using these same markers can also help identify other familial relationships, such as siblings (Gill et al., 1994).

Identifying maternal and paternal lineages, or even more specific familial relationships, within the archaeological record represents a huge leap forward in testing hypotheses of social structure, marriage patterns, and burial customs of prehistoric societies. Although kinship relationships can be hypothesized based on burial patterns or morphological similarity (Larsen, 1997), they cannot be directly tested without the use of DNA (Stoneking, 1995). An interesting example of the elucidation of familial relationships in an archaeological context can be seen in the excavation of St. Margareth's Church (Reichersdorf, Germany) (Gerstenberger et al., 1999; Hummel et al., 1999). Inscriptions on memorial stones at the church indicate that eight male members (from seven generations) of the Earl of Königsfeld family were buried there between 1546–1749. Genetic tests on seven of these individuals (the eighth skeleton having been destroyed by grave robbers) showed significant disagreement with the genealogy as reconstructed from historical sources (see Fig. 1). Two of the 7 individuals were genetically female, and thus were not Earls. An analysis of the autosomal and Y-chromosomal microsatellites suggested further anomalies. The pattern of these markers showed that skeletons of the two most senior Earls (Hanns Christoph and Hanns Sigmund) had been exchanged, probably during excavation, and that the most recent male interred (Georg Josef) could not have been the biological son of the previous Earl (Josef Wilhelm). Thus this individual is either not a member of the Königsfeld family or is the

product of a "nonpaternity event." The autosomal haplotype of one of the female individuals, previously identified as Karl Albrecht (Georg Josef's son) based on historical evidence, was consistent with her having been the daughter of Georg Josef, and thus she has been identified as one of Karl Albrecht's sisters. The autosomal haplotype of the second female individual is consistent with her being the mother of the first female (i.e., she was Georg Josef's wife Maria Anna). The authors propose that the presence of a complete family group (Georg Josef, Maria Anna, and their daughter) in the Königsfeld sepulcher suggests that Georg Josef was indeed the product of a nonpaternity event, as it is "rather unlikely that a family not belonging to the Königsfeld genealogy was laid to rest" there (Gerstenberger et al., 1999, p. 475). On the other hand, the remainder of the historically reconstructed relationships for this family are consistent with the genetic analysis. It is important to note that, when dealing with identifiable deceased individuals, one should consider the implications of potential results for the reputation and/or feelings of any living descendants (Holm, 2001), especially if nonpaternity events may be detected (see ethics section below).

Local level. The distinction between family and local levels is somewhat arbitrary, in that some groups are composed of only one or a few families, while many of our questions about larger groups concern how they defined kinship, patterns of marriage, and so on, which are ultimately questions about families. Although it has been suggested that archaeological groups, especially those of hunter/gatherer societies, are likely to have low levels of diversity because they had small population sizes, leading to both inbreeding and high levels of genetic drift (Cavalli-Sforza and Bodmer, 1999), studies of mitochondrial hypervariable region sequence diversity in ancient groups with relatively large samples do not show reduced diversity compared to modern groups (Kaestle, 1998; Stone and Stoneking, 1998; Shinoda and Kanai, 1999; Wang et al., 2000; Malhi, 2001). In addition, a recent study, designed to mimic the sampling possible in aDNA studies, showed relatively high microsatellite heterozygosities, with observed genotypic frequencies approximating those expected under Hardy-Weinberg equilibrium, among the Shamatari (Williams et al., 2002), a cluster of approximately 12 Yanomamö villages (Chagnon, 1997). These reasonably high levels of diversity suggest that it may be possible to distinguish common inheritance/residence patterns (either general patterns of endogamy vs. exogamy, or specific patterns of patrilineal/patrilocal vs. matrilineal/matrilocal), either by direct examination of lineage patterning (Goldstein, 1981; Hummel and Herrmann, 1996) or through comparisons of estimates of levels of variation within maternally, paternally, and biparentally inherited genetic markers within and between groups or classes (Lane and Sublett, 1972; Spence, 1974). A simulation model was recently developed to determine our ability to detect inheritance/residence patterns, using aDNA evidence (Usher et al., 2002). Preliminary results suggest that this should be possible, given large enough sample sizes.

Previously, these methods relied on morphological evidence of kinship (Konigsberg, 1988; Buikstra et al., 1990; Johnson and Lovell, 1994; Larsen, 1997), which assumes morphological similarities are the result of genetic relatedness. However, morphological traits are the phenotypic result of a complex interaction of multiple genes and the environment, along with activity-induced remodeling, and thus can be problematic proxies for genes (Larsen, 1997). For example, craniometric studies of Native American individuals find that populations from Tierra del Fuego and the Arctic cluster with each other, to the exclusion of other Native American groups (Hernández et al., 1997). Obviously, these two groups do not share a more recent common ancestry with each other than with the other Native American groups living between them. It has been suggested that adaptations to similar environments, such as cold stress or heavy mastication, have resulted in similar cranial morphologies for these two groups (Hylander, 1977; Lahr, 1995; Hernández et al., 1997; Larsen, 1997).

With aDNA we can now test hypotheses of inheritance/residence patterns directly. For example, Shinoda and Kanai (1999) are examining the mitochondrial DNA recovered from individuals buried in a Jomon shell midden (4500 BP), located north of Tokyo Bay, Japan, to test the hypothesis that these individuals belong to a single (or small number of) family group(s). Their preliminary results from 29 individuals show that more than 75% of these individuals fall into just two mitochondrial haplotypes, suggesting that this population may, indeed, have consisted of two major families, defined maternally. However, complicating this interpretation is the fact that the remaining seven individuals fall into seven distinct mitochondrial haplotypes. Without information on the sex of these individuals, one can only speculate that these might be male "immigrants" into the family. In general, patrilocal/patrilineal groups will have lower levels of diversity in paternally inherited markers, while matrilocal/matrilineal groups will have lower levels in maternally inherited markers (Usher et al., 2002). In addition, it might be possible to identify individuals as migrants using statistical analyses to identify "atypical" individuals (Waser and Strobeck, 1998; Cornuet et al., 1999; Davies et al., 1999; Rannala and Mountain, 1997; Vasquez-Dominguez et al., 2001). However, it must be remembered that the application of population genetic methods to ancient samples is complicated by the temporal distribution of samples, creating large margins of error in estimated variables (e.g., Hunley, 2002; Hunley and Merriwether, 2002).

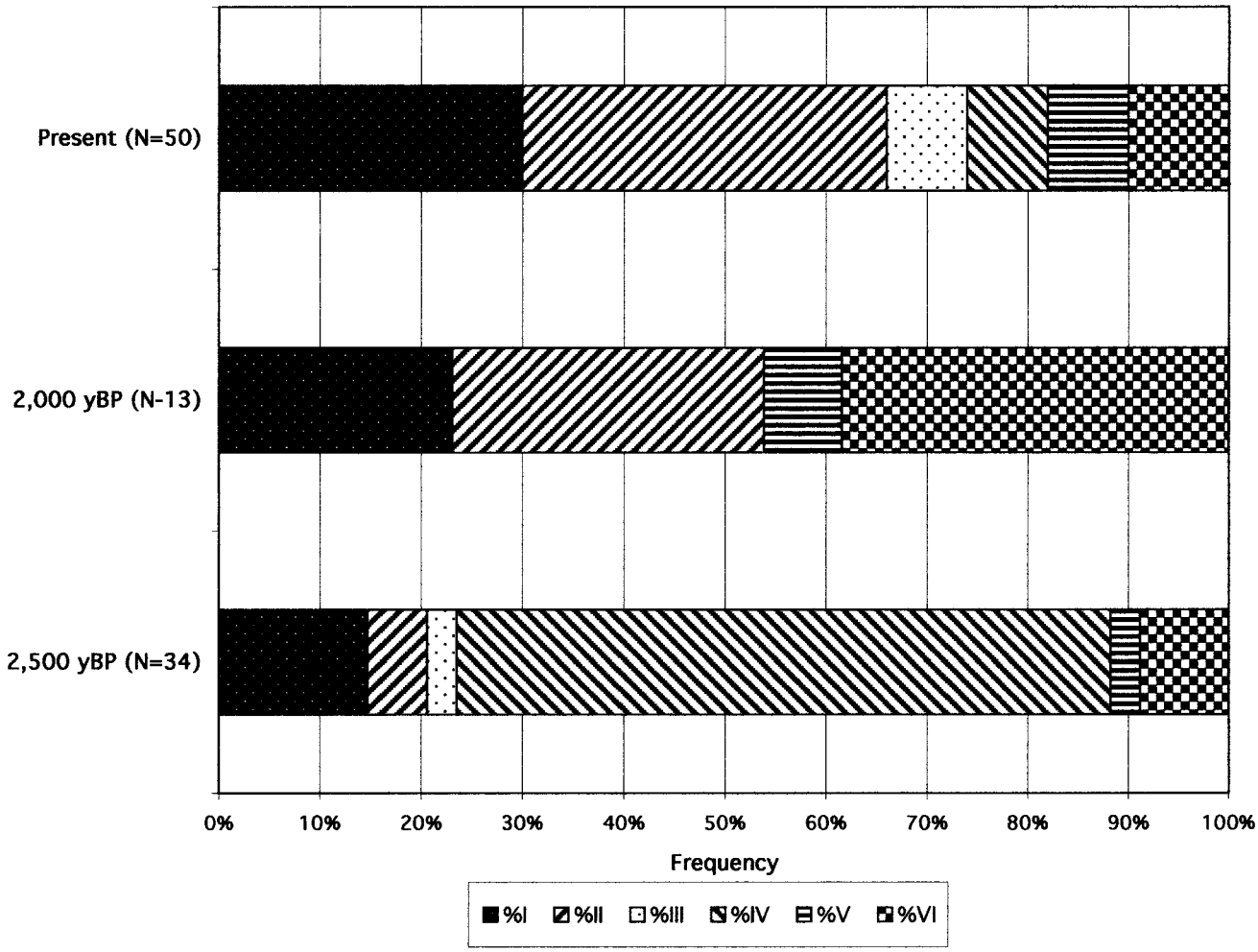

Fig. 2. Frequencies of mitochondrial haplogroups in ancient and modern Linzi groups (data from Wang et al., 2000).

Population level. Events of prehistoric population movement and issues of population continuity or replacement, especially when a large number of ancient individuals are available for testing, can be explored using aDNA. Because genetic variation is inherited from a group's ancestors, modern groups are expected to have similar frequencies of genetic markers to their ancestors, while ancient and modern groups with very different frequencies are not likely to be closely related (except in cases of extreme genetic drift or selective forces). In addition, certain genetic markers have limited distributions and can be used as indicators of relationship. In this way, we can approach questions of ancestor-descendant relationships at many scales.

At a fine scale, aDNA data can be used to test hypotheses of population continuity at a single site. For example, Wang et al. (2000) used mitochondrial sequence data from ancient individuals recovered from the Linzi archaeological site, China. The authors compared data from remains at both the 2,500 and 2,000 BP horizons of Linzi to those of modern groups living both at Linzi and throughout central and eastern Asia, identifying six major mitochondrial haplogroups. The frequencies of these haplogroups have changed drastically in this region (see Fig. 2; the differences in frequencies are statistically significant for comparisons of the 2,500 BP sample to both the 2,000 BP sample and the present-day sample, but the 2,000 BP sample and the present-day sample are not statistically significantly different from each other in haplogroup frequencies, using a chi-square test for homogeneity; $p < 0.05$, our calculations), suggesting to the authors that there was significant movement within this region during this time (consistent with historical records). However, it is important to note that sample sizes, particularly for the 2,000 BP sample, are not large, and this might have contributed to the differences detected (or not detected) among these groups. In addition, when compared with sequence data from around Eurasia, the 2,500 BP sample clusters with modern European rather than Asian populations, a pattern that the authors interpret as evidence of "a genetic shift in the Linzi area from a European-like population to a population more like those found in present-day east Asia, probably caused by migration" (Wang et al., 2000, p. 1,399). However, this

could also be an indicator of contamination of highly degraded DNA. It is possible that laboratory reagents or disposables were contaminated at the manufacturer, resulting in European-type sequences. In addition, very weak signals and/or contamination from multiple sources may be recorded as a signal of the reference sequence, against which most sequences are "corrected" and aligned, which is of European origin (Andersen et al., 1981).

At a slightly broader scale, the exploration of hypotheses of larger population movements has also benefited from aDNA data. For example, linguistic and archaeological evidence has suggested several prehistoric population movements in the Americas that are currently being explored using aDNA (Parr et al., 1996; Hayes, 2001; Kaestle and Smith, 2001a; Malhi, 2001, 2002; Carlyle, 2002; Eshleman, 2002a,b; Kemp et al., 2002). Although O'Rourke et al. (2000b) suggested that aDNA results from North America show geographic variation similar to that found among modern Native North Americans, analyses at finer geographic levels (e.g., Kaestle and Smith, 2001a), analyses using simulations (e.g., Cabana, 2002), and new aDNA studies (e.g., Malhi, 2001; Eshleman, 2002b) all provide evidence of prehistoric population movement and genetic discontinuity in some regions of North America. An example of such a project is that exploring prehistoric population movement in the Great Basin (Kaestle, 1997, 1998; Kaestle et al., 1999; Kaestle and Smith, 2001a). Both linguistic and archaeological evidence has been used to suggest that the current inhabitants of the Great Basin, speakers of Numic languages, are recent immigrants into the area (within the last 1,000 years) who replaced the previous inhabitants (Madsen and Rhode, 1994). However, others have interpreted this same evidence as a sign of local adaptation to a changing environment and increasing population density (Madsen and Rhode, 1994). As part of a larger project to study the prehistory of the western Great Basin, begun by the Nevada State Museum with permission from local Native American tribal groups, mtDNA from approximately 30 ancient individuals from western Nevada was analyzed. Modern Native Americans possess genetic markers in their mtDNA that divide them into at least five maternal lineages, or haplogroups, called A, B, C, D, and X (Schurr et al., 1990; Brown et al., 1998; Smith et al., 1999). These maternal haplogroups represent a subset of those currently found in Eurasia. Recent studies of ancient mtDNA from the prehistoric inhabitants of the Americas confirmed that the majority of ancient Native Americans also fall into these five haplogroups (Stone and Stoneking, 1993; Parr et al., 1996; O'Rourke et al., 1996; Kaestle, 1998; Kaestle and Smith, 2001a; Malhi and Smith, 2002). However, the frequencies of these haplogroups vary significantly among both modern and ancient Native Americans groups, often following linguistic or geographic boundaries (Merriwether et al., 1994; Lorenz and Smith, 1996; Kaestle, 1998; Kaestle and Smith, 2001a). Studies show that the frequencies of these haplogroups in the ancient western Nevadans were statistically different from those of modern inhabitants, and in fact from all modern Native Americans from the western US studied, except for some groups in California (Kaestle, 1997, 1998; Kaestle et al., 1999; Kaestle and Smith, 2001a). This dissimilarity in mtDNA haplogroup frequencies supports the hypothesis that the Numic presence in the Great Basin is quite recent, and suggests that the previous inhabitants are most closely related to the modern central California Native Americans (with whom they appear to have had cultural ties; see Hattori, 1982; Moratto, 1984). However, phylogenetic analysis of these data also suggests that there was a limited amount of admixture between the expanding Numic group and the previous inhabitants of the western Great Basin (Kaestle, 1998; Kaestle et al., 1999; Kaestle and Smith, 2001a). Again, it is important to note that these results may have been significantly affected by small sample size, and sampling across temporal boundaries. However, initial results of simulations to model these effects continue to suggest that genetic drift alone cannot account for the difference in mitochondrial haplogroup frequencies between the ancient and modern inhabitants of the western Great Basin (Cabana, 2002; Cabana et al., 2002). Interestingly, Parr et al. (1996; and see Carlyle et al., 2000) showed that the ancient Fremont inhabitants of the eastern Great Basin also had significantly different mitochondrial haplogroups frequencies from those of the modern inhabitants of the Great Basin. However, these frequencies are also significantly different from those of the ancient inhabitants of the western Great Basin (Kaestle and Smith, 2001a), with the ancient western Great Basin sample possessing haplogroup A but not haplogroup C, and intermediate frequencies of haplogroup B and D, while the ancient eastern Great Basin sample shows the presence of haplogroup C but not A, and extremely high frequencies of haplogroup B but very low frequencies of haplogroup D (for summary statistics, see O'Rourke et al., 2000b). Although both of these regions are currently inhabited by speakers of Numic languages, and are generally considered to be within the Great Basin cultural zone (Driver, 1961), these results suggest that biologically distinguishable populations were inhabiting this region in prehistory.

Ancient DNA data has also entered the debate on large-scale population movements, such as the peopling of whole continents or the Pacific Islands (Horai et al., 1991; Hagelberg, 1997; Stone and Stoneking, 1998; Smith et al., 2000a; Adcock et al., 2001a; Hayes, 2001; Kaestle and Smith, 2001b, 2002). For example, it was shown that the ancient Paleoindians (the first inhabitants of the Americas) are morphologically distinct from living Native Americans (e.g., Steele and Powell, 1992; Neves et al., 1999), leading some to suggest that the initial colonizers of the

Americas were not the direct ancestors of the living Native Americans (Munford et al., 1995; Morell, 1998). However, preliminary analyses of the mitochondrial DNA from these ancient individuals has been able to confirm the presence of mitochondrial haplogroups found among living Native Americans (Smith et al., 2000a; Kaestle and Smith, 2001b, 2002) in the majority of Paleoindians studied to date. This suggests that there is at least some measure of continuity between these earliest inhabitants and modern Native Americans.

Species level. The clarification of relationships between modern humans and other hominids was recently approached with aDNA techniques. The position of Neandertals in our evolutionary history has been debated ever since they were recognized as similar, but not identical, to modern humans. Krings et al. (1997) published a portion of a mitochondrial sequence from a Neandertal type specimen. When replicating their results in an independent laboratory, the replicating laboratory (A. Stone, Pennsylvania State University) amplified only contaminating modern human DNA before amplification was attempted with Neandertal-specific primers that had been designed from the sequence already obtained in the original laboratory (University of Munich). Adcock et al. (2001c) criticized the study for this, arguing that such an approach does not comprise an independent verification. However, the use of species-specific primers when possible is well within the bounds of standard protocols.

Since the original publication of a Neandertal DNA sequence, mitochondrial sequences of two further Neandertal individuals have been published (Krings et al., 2000; Ovchinnikov et al., 2000), and while thus far represented by a sample size of only three, the Neandertal mitochondrial DNA sequences do appear to consistently differ from those of modern humans. Precisely what such sequence divergences mean remains unclear. The nature of the relationship between species or subspecies status, and genetic, specifically mitochondrial, sequence divergence is not well-understood (e.g., Morin et al., 1992), but there does appear to be a relatively high degree of divergence between these populations, when compared with divergences between living human populations. Naturally a sample size of three leaves a great deal unknown, but this is a worthwhile line of research.

Recently, mitochondrial sequence data from 10 ancient Australians, including both robust and gracile types, and most controversially of LM3, a gracile individual dating to about 60 kya (Adcock et al., 2001a), were added to the debate. Adcock et al. (2001a) argued that the sequence of LM3 diverged from those represented by modern humans before the most recent common ancestor of all extant humans. The results of their phylogenetic analysis places the sequence of LM3 outside the clade containing all modern humans sequenced thus far, and sister to the sequence of a nuclear pseudogene. Adcock et al. (2001a,b) argued that the presence of the LM3 sequence outside modern human variation is inconsistent with an "Out of Africa" model of the origins of modern humans. This study, however, has attracted substantial criticisms for the laboratory methods employed, the analytical techniques, and their interpretation of the results of those analyses (Cooper et al., 2001; Colgan, 2001; Groves, 2001; Trueman, 2001). If the sequences produced in this study (Adcock et al., 2001a) are indeed endogenous to the sample, further analysis is certainly required to determine what it is they really mean about the origins of our species (Relethford, 2001).

Nonhuman sources

Environmental reconstruction. Prehistorians are frequently interested in reconstructing the environment in which ancient peoples lived. An understanding of the ecosystem in which prehistoric peoples existed can provide insights into the cultural adaptations required by the environment, such as food acquisition behaviors, and into patterns of seasonal movement. It may also inform our understanding of the domestication process. Environmental reconstruction is typically undertaken by identifying the floral and faunal remains at a site, and inferring the local environment from the preferred habitats of those species. Unfortunately, species identification of archaeological remains is frequently inaccurate (Gobalet, 2001; Matisoo-Smith and Allen, 2001). In addition, closely related but morphologically indistinct species routinely prefer widely varying habitats. Thus, the accurate identification of species can be critical to the reconstruction of local environments. The techniques of aDNA can be employed in this instance to accurately identify the species present in an archaeological site. Barnes et al. (2000), for example, made use of the different environments inhabited by various species of geese (as discussed below) to draw inferences about the local environment.

Insight into cultural practices

Seasonal population movement. While the identification of a species in the archaeological record can assist in reconstructing the local environment during the period of prehistoric occupation, it can also be used to discern a pattern of seasonal site utilization. If organic remains are present that are either only available for harvesting during particular seasons, or are those of a migratory species with a seasonal pattern of utilization, a nonpermanent use of the site can be inferred.

Diet. While the proximate goal of many aDNA studies is the identification of archaeological species, the ultimate goal is frequently an elucidation of dietary patterns. Barnes et al. (2000) undertook a study to ascertain the species of geese at a rural Anglo-Saxon settlement that was occupied from the

7–12th centuries. There were, at the time and in the region, six species of wild goose, which varied widely in their habitat preference, as well as possibly one species of domesticated goose. In an effort to determine if the geese at the site had been hunted, or were domesticated and being bred at the site, Barnes et al. (2000) amplified sections of the mitochondrial genome of the archaeological goose remains, and determined from those species identifications that the resident human population had been engaged in both wildfowling and husbandry.

Such studies have also been undertaken to identify species of hunted animals (Butler and Bowers, 1998), to study the process of animal domestication (Bailey et al., 1996), to distinguish domesticated sheep from domesticated goats (Loreille et al., 1997), and to study plant domestication (Brown et al., 1994).

Stone tools have also been identified as a potential source of target DNA to study the diets of prehistoric peoples. In an effort to investigate the feasibility of studying biological residues on archaeological stone tools, Kimura et al. (2001) undertook an ethno-experimental archaeological study of the lithics manufactured by a modern group in Ethiopia that routinely uses stone tools. They collected stone tools in a variety of stages from manufacture to discard, and successfully amplified DNA extracted from them. They cautioned, however, that although they did amplify DNA from both the manufacturer of the tool and the species upon which it had been used, they also amplified DNA unrelated to the use of the tool. Additionally, Shanks et al. (2001) undertook an experiment on newly manufactured obsidian blades to determine whether cells are preserved on them after washing. They soaked the newly made blades in cell-sized fluorescently labeled latex beads, fluorescently labeled white blood cells, or whole blood. They washed the stone tools and then, after drying, examined them microscopically for fluorescence. They determined that vigorous washing did not remove cells from microfractures in the surface of the obsidian, suggesting that stone tools may indeed be a viable source of aDNA. The study, however, was unable to ascertain the longevity of biological remains in microfractures.

Studying coprolites has also been used to study the diets of prehistoric peoples. By amplifying sections of mitochondrial and chloroplast genomes from three coprolites dating to greater than 2,000 BP (found at Hinds Cave, TX), Poinar et al. (2001) identified several species of both plants and animals, including antelope, rabbit, packrat, squirrel, hackberry, oak, and legumes. Analyses of the human mitochondrial DNA present in the feces revealed one member of haplogroup B, one member of haplogroup C, and a third individual that could not be conclusively assigned to a haplogroup. These haplogroups are typical of both ancient and living Native Americans (Lorenz and Smith, 1996; Kaestle and Smith, 2001a). An earlier study (Sutton et al., 1996) successfully determined the sex of 3 out of 4 coprolites investigated, and suggested that such methodologies could be profitably applied to the study of sex-based dietary differences.

Other biological remains. Ancient DNA has also been used to identify biological remains of cultural significance. Burger et al. (2000) successfully identified plant remains in an Aztec vessel as *Martinella obovata,* a woody vine species used pharmacologically by modern Native Americans as an eye salve. They additionally extracted the DNA of *Salvia,* a species of sage, from an ancient Celtic animal skin container (Burger et al., 2000). Ancient DNA techniques have also been applied to the identification of biological components of remains of material culture. For example, Rollo et al. (1994) have been investigating the clothing of the "Ice Man" found in the Tyrolean Alps, dating to 5,300 BP. The Tyrolean Ice Man had a "cloak" and footwear comprised, at least in part, of plant remains. Analyses revealed DNA from both grasses and microorganisms, thought to have been associated with the grass since it was harvested. Additionally these techniques have been used to identify biological components of prehistoric art, such as in a study by Reese et al. (1996), in which the fat used as a pigment binder in ancient Texan pictographs was identified as being of artiodactyl origin (but see Mawk et al., 2002).

Animals as proxies for human population movement. DNA extracted from faunal remains excavated from archaeological sites has proven to be a valuable resource in tracing prehistoric population movement. Human populations have frequently manipulated animal species, by domestication, and by moving them beyond their endemic range. Matisoo-Smith et al. (1997, 2001; see also Matisoo-Smith and Allen, 2001) have studied one of the species, the Pacific rat (*Rattus exulans*), transported east across the Pacific Ocean by the first colonizers of the region. By examining the patterns of molecular diversity in this commensal species across the islands of the Pacific Ocean, they have been able to clarify some of the paths of colonization and patterns of interaction between prehistoric Polynesians.

Infectious disease. Ancient DNA techniques have also been applied to questions of patterns of prehistoric disease. Various infectious diseases can leave similar skeletal pathologies on human remains, and indeed infectious diseases can manifest in patterns indistinguishable from each other or from inherited diseases (Ortner, 1994; D.C. Cook, pers. comm.). *Mycobacterium tuberculosis,* the pathogen causing tuberculosis (TB), has been successfully amplified from a 1,000-year-old Peruvian mummy (Salo et al., 1994), two fused vertebrae from an Iroquoian ossuary near Toronto from 1400 CE, and a vertebra from a Middle Mississippian ceme-

tery dating to 1020 CE (Braun et al., 1998). The amplification of *M. tuberculosis* from precontact Native Americans proves that TB was not introduced to the New World through contact with European explorers or colonizers. TB has also been identified in a 1,400-year-old Byzantine fragment of calcified lung tissue (Donoghue et al., 1998), confirming its presence in the Old World before European contact with the New World.

Taubenberger et al. (1997) took advantage of pathology specimens collected during the 1918 influenza epidemic that killed 20 million people worldwide. They extracted the virus' RNA and, using RT-PCR (an amplification method that uses a reverse transcriptase enzyme to copy RNA into DNA), amplified and sequenced fragments of nine influenza genes. Contrary to previous speculations that the 1918 flu had been so deadly because it was an avian virus, the sequenced genes were most closely related to known swine flu.

Just as the precise identity of the deadly influenza virus of the 1918 epidemic was unknown, the identity of the pathogen that caused the 17–28 million deaths during the 14th century Medieval Black Death epidemic was unknown. Various pathogens proposed have been anthrax, typhus, TB, hemorrhagic fever, or the plague (Raoult et al., 2000). Teeth were obtained from three individuals in a multiple burial in which an adult female, an adult male, and a child were buried in a 14th century French grave (Raoult et al., 2000). One of the child's teeth, and 100% (n = 19) of the adult teeth, yielded DNA from which could be amplified the *pla* gene of *Yersinia pestis,* the plague pathogen. Primers specific to the other proposed pathogens were unable to amplify aDNA from any of the samples. More recently, Drancourt and Raoult (2002) also found *Y. pestis* DNA in tooth samples from individuals suspected to have died in the European plague epidemics of 1590 and 1722.

These techniques have also been applied to confirming the identification of leprosy in human remains from a Norse cemetery in Orkney, Scotland (Taylor et al., 2000). The remains of two individuals were investigated, one of whom showed skeletal pathology consistent with leprosy, while the other showed no discernible skeletal pathology. Amplification yielded *Mycobacterium laprae,* the leprosy-causing bacterium, in the skeleton with the pathology indicative of leprosy, but not from the nonpathological skeleton. Neither skeleton yielded DNA amplifiable by primers specific to *M. tuberculosis,* the pathogen responsible for TB.

Nonhuman primates. The phylogenetic relationships of many of the recently extinct Malagasy lemurs have also been studied using aDNA techniques. Because many of the now extinct lemur species of Madagascar survived until as recently as 500 years ago, they can be considered the evolutionary contemporaries of extant species. However, their phylogenetic relationships remain unclear due to the high levels of homoplasy present in the morphological characters of Malagasy primates (Eaglen, 1980). While early aDNA data have presented conflicting phylogenetic pictures (Yoder et al., 1999; Montagnon et al., 2001), these lines of research show promise for further clarifying the relationships among these species.

The techniques of aDNA analysis can also be profitably applied to the study of living primate populations. Frequently, traditional molecular techniques that demand tissue samples are unsuitable for wild primate populations, because tranquilizing individuals for sampling is hazardous to both the subjects and the researcher. Further, the trauma inherent in the darting and tranquilizing may have substantial effects on the natural behavior of a wild population (or the habitualization process). The application of aDNA techniques, using biological materials originating from the study organisms that can be collected without disturbing them, allows the molecular study of wild populations without endangering individuals or perturbing natural behavior. These techniques have been successfully applied to the study of chimpanzees, using hair left behind in night nests (Morin et al., 1992, 1994), the feces of bonobos (Gerloff et al., 1995) and Hanuman langurs (Launhardt et al., 1998), as well as wadges and urine from bonobos and chimpanzees (Sugiyama et al., 1993; Hashimoto et al., 1996).

Applications summary

We have described several exciting applications of aDNA studies of humans, applicable at the level of the individual, the family, the population, and the species. Although initial hypothesis testing using aDNA data has concentrated on questions of large population movements (e.g., Hagelberg, 1997; Kaestle and Smith 2001a), there is an increasing interest in smaller-scale questions such as local and kinship relationships. For example, at a recent conference on biomolecular archaeology (Biomolecular Archaeology: Genetic Approaches to the Past, the 19th Visiting Scholar Conference organized by Dr. David Reed at Southern Illinois University, Carbondale, IL, April 19–20, 2002), of 11 formal papers presented, 2 discussed continental-scale population movements (and the African Diaspora), 5 discussed regional-scale movements, 4 discussed local population movements, 3 discussed kinship relationships within sites, and 1 discussed simulation modeling of evolutionary processes (obviously, several papers addressed issues at multiple levels). In addition, the use of aDNA methods on nonhuman samples is increasing (including a paper on plant domestication at the above-mentioned conference). Although the methods are new, they hypotheses addressed using aDNA studies are clearly traditional anthropology questions.

ETHICS OF ANCIENT DNA RESEARCH

The need to understand the host of information often necessary for responsible and ethical decision-making may often demand that . . . subdisciplinary boundaries be crossed. In that very crossing, the discipline's folk categories are at once acknowledged and reshaped, thereby reaffirming the need for the discipline's holistic four-field approach. —Cantwell et al., 2000, p. ix

The application of aDNA methods to anthropological questions holds great promise, as described above. However, as with any study of humans, it is important to consider the ethical, legal, and social (ELSI) implications of research efforts. Dealing as it does with the physical remains of deceased humans and their material culture, anthropological aDNA studies straddle the ever-fluid boundary between physical anthropology and archaeology. Consequently, many of the ethical issues being faced by researchers in the relatively new field of anthropological aDNA have been considered for decades by skeletal anthropologists, and more particularly, by archaeologists. However, increased awareness of the potential ELSI implications of archaeological and physical anthropology research for living people and communities has reinforced the importance of cultural anthropology for our research. We therefore find that the literature of all these fields may be profitability plumbed for insights relevant to our own research.

Ancient DNA and destructive analysis

Although it is possible to extract DNA from long bones with minimal damage, and to glue teeth back together after extraction of the dentin, in general, aDNA methods are destructive. Thus, it is important to bear in mind our obligation to proper stewardship of anthropological material (Lynott and Wylie, 1995a; Monge and Mann, 2001; Turner, 2001; AAPA, 2002). Because these resources are irreplaceable (Lynott and Wylie, 1995a; but see Zimmerman, 1995), destructive analysis should only be undertaken in cases where the results are likely to inform important debates or provide data to test interesting hypotheses, and/or when their destruction does not imperil other research avenues. In many cases, aDNA may not be the most productive approach to hypothesis testing. For example, establishing cultural affiliation between a single very ancient individual and a living group is very difficult using genetic evidence, although perhaps not impossible, depending on what is meant by "cultural affiliation" (Kaestle and Smith, 2002, and see Applications, above).

Another significant concern is the likelihood that DNA is sufficiently preserved in the sample in question for profitable analysis. Destructive analysis should not take place when it is unlikely to yield results. Different approaches to assessing this likelihood are discussed in the Appendix. In addition, some of the sample should be reserved for possible testing in the future, whether to confirm results or to apply new techniques not available at the time of initial study.

Human subjects

A much more complex issue, or set of issues, surrounds the idea of accountability, sensu Watkins et al. (1995), including the responsibility of the researcher to consult with groups that may be affected by the research, and the idea of beneficence (NCPHS, 1979; Turner, 2001), central to studies involving human subjects, i.e., that research should strive to avoid harm to subjects. A large body of literature discusses these issues as applied to archaeology and physical anthropology in general (e.g., Green, 1984; McBryde, 1985; Fluehr-Lobban, 1991; Lynott and Wylie, 1995b; Vitelli, 1996; Greely, 1997; Cunningham, 1998; Foster et al., 1998; Foster and Freeman, 1998; Juengst, 1998; Cantwell et al., 2000), the discussion of which is beyond the scope of this paper. However, there are several concerns specific, or particularly relevant, to aDNA studies that should be considered.

Ancient DNA and individual consent. Today, biological studies of living humans generally involve varying levels of informed consent from the study participants, in compliance with both federal and institutional regulations. It is, however, impossible to obtain informed consent from deceased individuals, and anthropological research on them has not generally been subject to federal or institutional human subject regulations (except when the project also involves the participation of living people). Philosophical debate regarding the rights of the dead has a long history (e.g., Aristotle, translated by Rackham, 1962; Bellioti, 1979; Partridge, 1981; Marquis, 1985; Callahan, 1987; Grover, 1989; Fisher, 2001; Scarre, 2001), and most discussions focus on the rights to privacy and preservation of the reputation or respect of the wishes of the dead (except for those who presume some form of personal immortality).

Holm (2001) discussed these issues productively with respect to aDNA research. He first dismissed interests ascribed to deceased individuals based on their beliefs if we do not know what these beliefs were (which will generally be true except for cases of recently deceased individuals). Even in cases where we have some knowledge of the common practices of the society of the individuals in question, the interpretation of this knowledge is difficult. Given the well-established difficulties of extrapolating other beliefs from mortuary/archaeological data (e.g., Pader, 1982; Giddens, 1984; Wylie, 1989; Metcalf and Huntington, 1991; Pearson, 1999), such as beliefs about kinship and gender, the presumption that we can understand beliefs of prehistoric societies (and more specifically, beliefs of individual members of those societies) regarding their interests in proper treatment of their remains after death is problematic. In a similar manner, it would be diffi-

cult to discern what individuals would consider to be a "slur" on their good name (Holm, 2001, p. 447).

If, on the other hand, the individual is known and his/her beliefs on proper treatment of his/her remains or good name were made explicit during his/her lifetime, this must obviously have a large impact on decisions regarding aDNA research. This situation is expected to be exceedingly rare.

Some form of proxy consent, usually made by the descendants of the deceased, has been suggested as a substitute for the consent of the deceased. However, proxy consent implies that the proxy is making a decision based on the best interests of the deceased. As discussed above (and in Holm, 2001), the interests of the deceased are very difficult to discern. Holm (2001) also points out that multiple descendants may disagree regarding the study of their ancestor. This also presumes that direct descendants can be identified, which is generally unlikely.

Increasingly, living people who are "culturally affiliated" with the deceased are being asked to make these decisions. The presumption is that these people, because they share a common culture with the deceased, are more likely to make decisions regarding the study of deceased's remains with which the deceased would agree. Identifying cultures that are "affiliated" with that of the deceased is, at best, difficult (Haas, 2001; Barker et al., 2000; Killion, 2001; Kemp et al., 2002). In fact, what constitutes evidence of cultural affiliation itself is an arena of great disagreement (Kaestle and Smith, 2002). In the face of these difficulties, there has been a movement to define "cultures" in increasingly general terms (National Parks Service, 2000). Implicit in this suggestion is that any Native American group can serve as a proxy for an unidentifiable culturally affiliated group. Expanding the scope of what is meant by cultural affiliation only increases the likelihood that living groups' decisions will not reflect the beliefs of the deceased (Meighan, 1984; Renfrew and Bahn, 1996; Tsosie, 1997; Goldstein and Kintigh, 2000; Mitchell and Brunson-Hadley, 2001), and that different living groups will disagree regarding the disposition of the remains (for several conflicting/differing Native American and Australian views on the treatment of ancient remains, see Tsosie, 1997; Cantwell, 2000; Bary, 2001a). In addition, in the case of many indigenous groups, members may not belong to a recognized cultural group (e.g., in the case of unenrolled Native Americans, who make up the majority of individuals of Native American descent in the United States; Thornton, 1997).

Assuming that a living group can be identified to consult, and a satisfactory method for that consultation can be established (a matter of great controversy; see Williams and Mununggur, 1989; Pyburn and Wilk, 1995; Pyburn, 1999; Weijer et al., 1999; Cantwell, 2000), we still must deal with the assumption that this living group is likely to make a decision with which the deceased would agree. This may not be a reasonable assumption, even for the recently deceased (Meighan, 1984; Mulvaney, 1991; Hill, 2001; Holm, 2001).

On a side note, most discussions of these issues, including ours, center around consultation with indigenous communities (Weijer et al., 1999). However, much anthropological research is focused on nonindigenous communities, and this trend is increasing (Comitas, 2000; Silverman, 2000). Weijer et al. (1999, p. 279) point out the problems "with applying protections developed for aboriginal populations to other less cohesive communities, especially ones without legitimate political authorities." These difficulties include delineating the community, identifying legitimate political institutions or leaders of these groups (if they exist at all), and identifying community-wide consensus on needs and priorities.

For the reasons enumerated above, in most cases we do not believe that the argument can be upheld that culturally affiliated groups, even if they can be identified, *will protect the interests of the deceased.* Using similar logic, Holm (2001, p. 447) concluded that this type of study can be done "without seeking the consent of the dead person's descendants or his present-day culturally affiliated cultural community" in most cases. We are uncomfortable with this conclusion, and feel that the issue is not quite so simple.

Ancient DNA and living communities. Living groups have an interest in the aDNA research performed on deceased individuals *independent* of the interests of the deceased. The results of aDNA studies may impact the social, political, and legal situation that living groups find themselves in, and may contradict or offend beliefs about their ancestors and origins.

As with studies on living peoples, the results of aDNA studies may have implications for group members, even if they did not participate in the research. Given the genetic essentialism (sensu Nelkin and Lindee, 1995) so prevalent in Western society today, genetic evidence has the potential to take on significant weight in social, political, and legal arenas. Just a few examples should serve to make this point.

Because aDNA studies have the potential to provide evidence of biological ties between living and ancient individuals and groups (ancestor/descendant relationships), this type of evidence could be used to advance land claims (or other Native rights), or to reject them, in countries that recognize such rights (e.g., the USA, Canada, and Australia). For example, the Western Mohegan tribe has undergone genetic testing to support their claims of lineal descent from Mohegan ancestors to gain official state and federal recognition (Lehrman, 2001; Tallbear, 2000), and this claim could be supported by genetic evidence from deceased individuals buried on traditional tribal lands. In fact, several tribal groups have contacted aDNA specialists to explore the possibilities of this type of research.

Another example of the ELSI implications of aDNA research involves repatriation decisions (or identification of cultural affiliation) of extremely ancient Native American individuals. The Native American Graves Protection Act (NAGPRA) accepts both biological evidence in general, and molecular genetic evidence specifically, for cultural affiliation (43 CFR 10.14 (c)(2)(i)–(iii)), stating that "genetic evidence is a kind of biological evidence that may be relevant in determining cultural affiliation" (Department of the Interior, 2000). Ancient DNA studies of both the Kennewick man and Spirit Cave man remains, Paleoindians from Washington and Nevada, respectively, were considered when determining their NAGPRA status (for details, see Dansie, 1997; Jantz and Owsley, 1997; Preston, 1997; Kaestle et al., 1999; Barker et al., 2000; Kaestle, 2000; Merriwether and Cabana, 2000; Smith et al., 2000b; Thomas, 2000; Tuross and Kolman, 2000; Chatters, 2001; Dewar, 2001; Kaestle and Smith, 2001a). It should be noted that, although these examples are of situations in which aDNA did not support a particular cultural affiliation, this will not always be the case, and aDNA results could be used by indigenous groups to bolster requests for the repatriation of ancient remains, and could also be used to help sort mixed or improperly identified remains for proper repatriation (Cantwell, 2000).

The implication that living groups do not, or cannot, know their own history without the intervention of outsiders/experts, can be deeply troubling and offensive to living peoples (Andrews and Nelkin, 1998; Garza and Powell, 2001), and has been interpreted as an infringement on their religious freedoms (White Deer, 1997; Pyburn, 1999; Deloria, 2000; Mihesuah, 2000; Grimes, 2001; Haas, 2001). The control of ancient remains by nonindigenous peoples has also become a focus of the debate on self-determination and colonialism (Pyburn, 1999; Cantwell, 2000; Frichner, 2000; Meskell, 2000; Riding In, 2000; Cash Cash, 2001; Zimmerman, 2001).[1] Thus, it is necessary to have full knowledge of perceived potential hazards and the explicit recognition of many different stakeholders to move ahead with ethically sound, scientifically based historical research. These issues are not limited to studies of aDNA, but apply more generally to the study of ancient peoples and their cultures (Zimmerman, 1989; Echo-Hawk, 1992; Mihesuah, 2000; Grimes, 2001). As such, they are beyond the scope of this paper, but should be important considerations for those pursuing aDNA research.

We have established that living groups have an interest in the use of aDNA techniques in anthropology. However, does this interest overwhelm that of the scientist? How are we, as anthropologists, to deal ethically with these issues? It should be noted that the American Anthropological Association (AAA) code of ethics uses the word "can" rather than "must," when discussing the possibility that our obligations to the people we study may supersede our own goals, and includes as ethical obligations, in this same section, the long-term conservation of archaeological, fossil, and historical records (AAA, 1998). As Silverman (2000, p. 214) noted, the code "also enumerated responsibilities to the public, to the discipline, to students, to sponsors, and to one's own government and to host governments. That these responsibilities were bound to clash, and that it would be up to the individual to make ethical choices, was the necessary condition of anthropological work" (although Silverman (2000) is speaking of the 1971 AAA code, these responsibilities are also included in the current code). Thus, balancing the rights of all the involved parties remains a complex process.

Suggestions for the future

Most suggestions on how to deal ethically with these situations have involved consultation or collaboration with living groups on a voluntary basis (e.g., AAA, 1998; WAC, 1991; Pyburn, 1999; Killion, 2001; Loring, 2001; Spector, 2001). Unfortunately, the history of colonial interaction with indigenous groups can only be described as abysmal. Non-Native anthropologists have a large hurdle to clear in developing trusting relationships with these groups, and are distinctly hampered by our own historical record of complicity with colonial powers (e.g., Bruce, 2000; Killion, 2001). Ancient DNA research has been particularly hampered by negative perceptions of previous interactions with human geneticists (e.g., Tierney, 2000; AAA, 2002). In addition, the process is made more difficult when scientists do not recognize themselves as nonobjective stakeholders. We discuss below several approaches to consultation/collaboration/cooperation that might be helpful to anthropologists negotiating this space.

A "contact perspective" (Bray, 2001b) enables a profitable conceptualization of the interactions between anthropologists and other stakeholders. It allows the meanings of the interactions, as well as the meanings of the biological and cultural remains to be considered both emergent, and contingent on the participants, rather than inherent or essentialized. This approach emphasizes cross-cultural communication, often involving both linguistic and cultural translation (Jacknis, 2000; Bray, 2001b). Bray (2001b) suggested that this endeavor may also be aided by the notion of "embodied objectivity" (Haraway, 1991), which recognizes complete objectivity as an impossible state, but strives for situated knowledge, with the acknowledgment of the importance of individual perspective. It is our belief that embodied objectivity should be made explicit in all anthropological studies.

[1]The assumption that repatriation is desired, and that scientific study is rejected, is also problematic, given that this will not always be the case, and may result in reburials that are unwanted by the community (examples in Cantwell, 2000).

It has also been suggested that anthropologists work with (or for) native peoples in what Garza and Powell (2001) call "covenantal archaeology," Loring (2001) calls "community archaeology," and Spector (2001) calls simply "partnership," in which the goals of indigenous peoples define the problems and research questions, and establish priorities for these studies (Garza and Powell, 2001; Loring, 2001; Watkins, 2001; Zimmerman, 2001). Pyburn (1999) made the point that the inclusion of indigenous viewpoints from *within* the anthropological endeavor is likely to improve our field in a manner similar to that accomplished by the inclusion of women. As she pointed out, women were once considered inappropriate members of our profession, and yet we have made great strides forward as a result of inclusion (see also Farnham, 1987; Haraway, 1989, 1991; del Valle, 1993; Lloyd, 1995; Conkey and Gero, 1997; Arnold and Wicker, 2001; Pyburn, 2002).

There cannot be a single standard when it comes to the ethics of anthropological research, or even of aDNA research in anthropology. Because aDNA research generally falls outside the domain of institutional review boards, we must regulate ourselves, both through adhering to our field's sometimes contradictory ethical standards as best as we can, and through serious case-by-case consideration and discussion among ourselves, our colleagues within and outside of anthropology, and other interested parties (stakeholders). We hope that the points enumerated above provide a starting point for these discussions, both within individual laboratories and for the field as a whole. The issues are complicated, but this should not provide an excuse to ignore them.

As the use of aDNA in anthropological research continues to be mainly self-reviewed, and guided by a range of laws in different nations, we suggest that the following questions be addressed by researchers before they undertake a specific aDNA research program:

1) Does the application of the method address an anthropological question?
2) Are there nondestructive methods that can be used to achieve the result?
3) Do the conditions of the remains or other material suggest aDNA is more likely to be present than not?
4) How will different stakeholders view the destruction of the remains in question?
5) What are the ELSI implications of possible study results, if any, for living groups?
6) Has a reasonable attempt been made to define and receive informed consent from different stakeholders?

CONCLUSIONS

Both within anthropology, and further afield in the biological and paleontological sciences, aDNA studies had a rocky start. Extravagant claims were made, and retracted; studies were published, soundly criticized for their methods, and then had their results revealed to be the product of contamination (Pääbo and Wilson, 1991; Young et al., 1995; Zischler et al., 1995; Wang et al., 1997; Yousten and Rippere, 1997). Such events led to widespread skepticism of the possibility that any aDNA study could produce real, reliable, and reproducible results. Nonetheless, with more careful analyses, and more sober discussion of the possibilities and necessary precautions, aDNA is becoming ever more respectable. With such respectability come further challenges for the field. Demonstrating the endogenous nature of aDNA is no longer newsworthy, and undertaking destructive analysis merely to prove that DNA has survived in particular organic remains is no longer justifiable. Ancient DNA studies must now be undertaken to answer specific research questions, and to test specific hypotheses. Further, we must now strive to protect our hard-won respectability. We should resist the temptation to rush into print with new and exciting results before we have appropriately verified them. Retractions of our results serve to jeopardize our respectability in the wider academic community, and to our respectability is tied both our abilities to effectively disseminate our results and our access to funding with which to further our work.

We have devoted a substantial portion of this paper to a discussion of the ethical concerns involved in analyzing the DNA of deceased organisms. We do this because we believe that such considerations are important in influencing the paths of our research, despite our inability to offer concrete rules about appropriate behavior within our field. It is precisely this lack of hard rules, offered by us or anyone else, which makes the ethical decisions so difficult. However, the complicated nature of the ethical issues raised by our work requires that we think about such issues more, not less.

In general, the tone of this paper has been somber, cautious, and highly concerned with the minutiae of aDNA work. Lest we confuse a concern for scientific rigor with pessimism, let us note that it is precisely because we are so enthusiastic about much of the work that has already been done in the field, and even more so about the potentials of aDNA studies within anthropology, that we treat it with such care. The application of aDNA techniques, explicitly informed and directed by traditional anthropological concerns, is only beginning to exert its full impact on the field.

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APPENDIX: ANCIENT DNA METHODS

The discussion of methods presented here assumes a basic knowledge of both theoretical and practical aspects of molecular biology and genetics. For readers lacking such knowledge, we suggest consulting Witherly et al. (2001), Avise (1994), and/or Lewin (1999) for basic details.

The great proliferation of methodologies for extraction and amplification of aDNA that took place in the 1990s has now been whittled down to two semistandard protocols (Phenol Chloroform and Silica), often with minor modifications to deal with specific situations (see below). The first study to successfully amplify DNA from ancient remains was published in 1984, in which the extinct quagga, a member of the horse/zebra family, was sequenced from a museum sample (Higuchi et al., 1984). Since then, there have been tremendous advances in the techniques of molecular biology.

Potential sources

Ancient DNA can be found in a variety of organic remains. The more obvious sources of such DNA are the soft tissues, teeth, and bones of ancient organisms, but less obvious sources, such as coprolites (Sutton et al., 1996; Poinar et al., 2001), can be equally valuable. Such materials can be housed in a variety of locations. It can be of great advantage if the remains to be studied are earmarked for use in aDNA analyses from the time of their discovery. In such cases, the handling of samples can be controlled by the person who will be performing the molecular analyses, thereby reducing the contamination problems encountered in many aDNA studies (see below). Frequently, however, the collections of organic materials of interest are housed in museums, medical collections, private collections, and art museums.

Will it work?

The likelihood of successfully extracting aDNA is affected by the age of the sample. However, age is far from the most significant factor determining success. Of substantially more importance is the environment to which the sample has been exposed since its death (Pääbo, 1989; Rogan and Salvo, 1990; Tuross, 1994; Hoss et al., 1996; Austin et al., 1997; Kaestle and Smith, 2001a,b; Robins et al., 2001). Depurination is the most important route of decay in aDNA (Lindahl, 1993; O'Rourke et al., 2000a), followed by strand breakage and the destruction of the ribose ring (Austin et al., 1997). The rate of DNA degradation is affected by ambient temperature, humidity (including the relative location of a water table), and the pH of the soil if the sample is buried. In addition, the DNA extraction process (described below) frequently coextracts chemicals that inhibit the PCR amplification reaction, such that any DNA that is present cannot be accessed. While this problem can be addressed somewhat with modifications to extraction techniques (see below), high levels of such inhibitors can prove an insurmountable problem. Thus, the soils in which archaeological samples have been buried are of importance both in that their characteristics can affect the rate of DNA degradation, and in the inhibitors that can be deposited in the samples.

Many of these factors compound to influence the gross morphology of remains, so the potential of many samples can be reasonably predicted by an examination of the samples. In particular, skeletal and tooth remains that are soft or crumble under mild pressure are unlikely to yield amplifiable DNA. Conversely, except when the sample has become mineralized, the harder such remains are, the higher the probability that there is sufficient intact DNA for analysis.

An additional predictor of the presence of amplifiable DNA in ancient samples is the degree of racemization of amino acids. All amino acids in biological organisms have a conformation described as laevorotatory, or left-handed, meaning that they rotate plane-polarized light anticlockwise. After death, the laevorotatory amino acids begin to spontaneously alter conformation, or racemize, to become dextrorotatory, or right-handed, such that they will rotate plane-polarized light clockwise. Many of the same environmental conditions that affect the rate at which DNA is degraded affect the rate at which amino acids racemize (Poinar et al., 1996). Therefore, an assay of the ratio of dextrorotatory to laevorotatory amino acids in a sample can give an indication of the likelihood of there being surviving DNA. While a high ratio of dextrorotatory amino acids to laevorotatory amino acids, indicating extensive racemization, reasonably accurately predicts a lack of intact DNA, conversely, a low ratio of dextrorotatory amino acids to laevorotatory amino acids, indicating limited racemization, does not necessarily imply that there is amplifiable DNA present, because additional factors affect DNA preservation that do not influence the rate of amino-acid racemization. Because amino-acid racemization testing is, like aDNA analysis, a destructive technique, and its determination is not an accurate predictor of the presence of intact DNA, we do not, contrary to the opinions of others (Poinar et al., 1996; Cooper and Poinar, 2000; Hofreiter et al., 2001), advocate its use prior to DNA analysis except in unusual cases, or in cases in which a large amount of material is available. It has also been suggested that bone collagen

content can be used as a rough indicator of biological preservation (and thus likelihood of successful aDNA extraction) (Taylor, 2001). As with amino-acid racemization, we do not advocate destructive analysis solely for the purposes of determining bone collagen content. However, if dating of samples is planned, bone collagen content may be determined in the process, and could provide valuable insight into the preservation of aDNA in the sample. O'Rourke et al. (2000a) advocated direct dating of each sample used in aDNA analyses, although this can be prohibitively costly.

Another potential test for intact human DNA at a site involves testing animal remains from the same site for aDNA. This allows the confirmation of aDNA preservation under the conditions of the site, before destructive analysis of human remains is undertaken.

Controlling for contamination

Due to the sensitivity of the polymerase chain reaction (PCR, described below) and the degraded nature of DNA in ancient samples, the contamination of samples and laboratory preparations by exogenous DNA is a constant concern. Such contamination can derive from a variety of sources, including the DNA of other workers who have handled the samples before they reach the laboratory, such as archaeologists, museum staff, and medical workers. Additionally, some relatively standard procedures for dealing with skeletal remains (such as stablizing with geletin-based glues; Nicholson et al., 2002) can serve to either worsen contamination problems, or degrade the endogenous DNA. For example, washing samples in water can facilitate the infiltration of contaminating DNA deep into the bone matrix, rendering more difficult the decontamination process. Further, x-raying bones can increase the fragmentation of the endogenous DNA (Götherström et al., 1995). Several decontamination procedures (see below) are employed in an attempt to remove contaminating surface DNA from samples before beginning the extraction protocol.

Exogenous DNA can also be introduced into samples from a variety of other sources. A substantial source of contaminating DNA can be the modern DNA extracted in laboratories for other purposes, as well as the DNA that has been PCR-amplified for analysis. Consequently, the laboratories in which aDNA analyses are performed must be physically separated from other laboratories conducting molecular analyses, and must be dedicated solely to the extraction and analysis of DNA from ancient samples. Additionally, workers cannot move from laboratories in which modern and post-PCR work is conducted directly into the aDNA laboratories because of the high probability of transporting DNA on their clothing, hair, and shoes. While transporting modern or amplified DNA is a particularly high risk associated with moving from other laboratories, it remains a risk at all times. Therefore, the use of protective clothing is necessary. A combination of laboratory coats, coveralls with hoods, hairnets, shoe covers, gloves, and facemasks proves effective.

A further source of exogenous DNA can be the plasticware and reagents used in the process of DNA extraction. The most effective strategy to minimize the chances of contamination via this route is to purchase both reagents and disposable plasticware that are guaranteed to be DNA-free by the manufacturer. Additionally, reagents should be aliquoted into small volumes that will be used quickly to avoid the introduction of DNA to stock solutions. Finally, laboratory surfaces need to be maintained to prevent the accumulation of DNA. Regularly wiping surfaces with bleach, and subjecting them to periods of UV-irradiation, can achieve this (Oh et al., 1991).

Even when all the precautions described are followed, contamination is an inevitable reality of working with aDNA. Recognizing that contamination will occur necessitates the ability to identify it when it does. Negative controls are run in parallel with samples throughout the extraction procedure, in which empty tubes are treated in exactly the same manner as the tubes containing samples. If the products of these negative controls yield amplifiable DNA, it is apparent that the extraction has been contaminated. A negative control of the PCR reaction is also run, to assist in determining at which point in the procedure the contamination occurred. A further test for contamination is at the level of analysis. DNA sequences obtained from ancient samples should be phylogenetically sensible.

A further method to assist in determining the veracity of obtained aDNA sequences is to quantify the starting molecules in an extract. Handt et al. (1996) found that when amplification was started with fewer than 40 template molecules, several different sequences were recovered from clones of the amplicons. Consequently, they advocated the quantification of starting molecules to determine whether authentic results are likely to be obtained. The quantification of starting molecules is undertaken by a competitive PCR procedure (Hirano et al., 2002), in which a reaction is spiked with a known quantity of constructed templates with the same primer binding sites as the target sequence, but of slightly shorter or longer length. When the number of introduced competitor templates is approximately equal to the number of endogenous target sequences, the proportions of each amplified fragment (amplicon) should be approximately equal. The relative quantities of amplicons can be determined by visualization on a gel. Note that this method could be confounded by the presence of contaminating exogenous DNA.

The final line of defense against contamination is replication. In all cases, results should be replicated in multiple independent amplifications from at least two independent extractions, preferably separated by at least a month. In addition, external replication, in which a portion of the sample is sent to an independent laboratory for extraction and analysis,

should be performed on at least a subset of samples. Reciprocal arrangements between laboratories can be established for the exchange of samples for the mutual replication of results. While it is logistically and financially impractical to have all the results of one laboratory replicated by another, replicability is a central feature of all good science, and demands that a subset of samples from each study be replicated. Any samples that yield surprising or unusual results must be added to the randomly selected subset of samples sent for replication. This standard has not yet been applied consistently across the discipline (e.g., Adcock et al., 2001a).

Extraction methods

Decontamination. Before extraction can begin, any exogenous DNA contaminating the surface of the sample must be removed. This can be achieved physically, (by removing the surface of the sample), chemically (by wiping with, or soaking in, bleach), or by UV irradiating all surfaces. Each of these methods has advantages and disadvantages. Physically removing the surface with sandpaper or a dremel tool is efficient, and should reliably remove all surface contamination. However, this method generates significant amounts of dust, which can contain the DNA that was removed from the surface of the sample, and thereby provide an additional source of contamination. Wiping with bleach may not allow sufficient penetration of the sample to eliminate exogenous DNA in pores of the sample, but soaking a porous sample in bleach may allow bleach to penetrate to the core of a sample, possibly destroying much of the endogenous DNA along with the contamination. Finally, UV irradiation can prove effective, but can be difficult to undertake systematically if the sample is irregularly shaped. It also will not penetrate the surface of the sample, and therefore cannot destroy exogenous DNA that has infiltrated the samples. Many researchers in the field find a combination of these methods to be the most effective.

Extraction. In most cases, the sample is then reduced to fragments or a powder to expose the surfaces not treated by the decontamination protocols, and to increase the surface area available to chemical manipulation, but see O'Rourke et al. (2000a) for an alternative method. There are two major approaches to extracting DNA from samples. One involves the introduction of an organic phase (phenol and chloroform), into which many of the cell components (but not the DNA) migrate, and which is then removed. The other approach involves binding DNA to a substrate (silica, or glass beads) and washing everything else away.

Phenol-chloroform protocol. This protocol is an adaptation of a standard phenol-chloroform DNA extraction procedure (Sambrook et al., 1989), in which samples are digested with proteinase K, and a detergent such as Triton X-100 or SDS, to break down the proteins in the sample. The digest is rocked with an equal volume of phenol for 15 min, and then centrifuged for 15 min at 13,000 rpm, and the organic (phenol) phase is removed. An equal volume of phenol:chloroform:isoamyl alcohol (25:25:1) is then added, and the samples are rocked for 10 min and centrifuged at 13,000 rpm for 10 min. This is repeated, usually once, until much of the discoloration has been washed away. To remove traces of phenol, 800 μl of chloroform:isoamyl alcohol (24:1) are added, and the samples are rocked for 5 min and centrifuged for 5 min. The aqueous phase, containing the DNA, is then removed to a clean tube, and the DNA is either precipitated with ammonium acetate and cold 100% ethanol, or concentrated into a small volume using a centrifugal filtration system. Although the aqueous phase is usually found above the organic phase during phenol-chloroform extraction, it is important to note that high salt concentrations can cause phase reversals (Sambrook and Russell, 2001). This is more frequently the case when extracting aDNA, because the matrix itself may have a high salt content due to preservation conditions.

Silica-guanidinium thiocyanate (GnSCN) protocol. This protocol is a derivation of that of Hoss and Pääbo (1993), which was adapted from that of Boom et al. (1990). The powdered sample is digested overnight in 500 μl of 0.1 M Tris-HCl (pH 7.4), 0.02 M EDTA (pH 8.0), 1.3% Triton X-100, and 0.01 mg of proteinase K under constant rotation at 37°C. One milliliter of extraction buffer (10 M GuSCN, 0.1 M Tris-HCl, pH 6.4, 0.02 M EDTA, pH 8.0, and 1.3% Triton X-100) is then added, and the digest is further incubated under constant rotation at 55°C for between 1 and several hours. The digest is centrifuged for 5 min at 13,000 rpm, and 500 μl of the supernatant are transferred to a clean tube, to which is added 500 μl of extraction buffer and 40 μl of silica suspension (Boom et al., 1990) or glass milk (Burger et al., 1999). The mixture is incubated at room temperature for 10 min to allow the DNA to bind to the silica under the chaotropic influence of the GuSCN.[2] The silica is then washed twice with a wash buffer (10 M GuSCN, 0.1 M Tris-HCl, pH 6.4) and once with cold 70% ethanol. The pellet is dried, and the DNA is eluted in two aliquots of 50 μl of ddH_2O or TE buffer, pH 8.0, at 56°C. The DNA extract is then frozen for future use. Kits are currently available from biotechnology companies in which the silica or glass milk suspensions are confined to a column through which the digest is passed.

In extracting samples with substantial concentrations of coextracting PCR inhibitors, this silica method can have an advantage over the phenol-chloroform method because everything that does not bind to the silica is washed away. However, probably because the

[2]A chaotropic agent is one that distrupts hydrogen bonds such as those between water molecules and DNA, such that the solubility of DNA in an aqueous solution is reduced. It thereby promotes the precipitation of DNA, in this case, precipitation on silica particles.

aDNA is damaged, researchers may find that aDNA does not bind to the silica as efficiently as modern DNA. Thus, the phenol-chloroform method may extract a larger quantity of aDNA.

Amplification

The polymerase chain reaction (PCR) is an in vitro technique used to synthesize copies of a fragment of DNA under investigation. The total genomic DNA extracted from samples is subjected to a number of cycles of heating and cooling, during which time copies of a specific region of interest are constructed. Upon heating (usually to 92°C), the hydrogen bonds down the center of the DNA molecule (the rungs of the helical ladder of DNA) are broken, and the DNA is then described as single-stranded. Upon cooling, short sections of DNA called primers bind to the target DNA. It is this annealing step that confers the specificity of the reaction. The primers are designed, on the basis of known DNA sequences, such that they are complementary to the ends of the target sequence. The reaction mixture is then heated to 72°C, which is the optimum temperature for the function of the *Taq* DNA polymerase enzyme. The *Taq* extends the complementary strand by binding free nucleotides (dNTPs) to the template strand and to each other. After a period of time, the reaction mixture is reheated to 92°C to separate the original template from the newly synthesized strand, which serves as an additional template in subsequent rounds of synthesis. Employing this technique allows the molecular analysis of samples with very limited or degraded DNA.

PCR inhibition. As mentioned earlier, coextracted PCR inhibitors can be a substantial problem in working with aDNA. Employing the silica GuSCN protocol in preference to the phenol-chloroform protocol can eliminate some inhibitory problems, but inhibitors are frequently found to be in extracts despite using this protocol. Additional strategies include diluting the DNA extract in the hope that the inhibitory elements will be sufficiently diluted for successful amplification, before the target DNA is diluted to such a degree that it is no longer amplifiable. Further, bovine serum albumin (BSA) can be added to the PCR reaction, which can serve to bind to inhibitors, thereby removing them from solution and allowing the reaction to proceed. Other strategies include further digesting the samples with proteinase K or a collagenase, or adding NaOH.

Electrophoresis

Electrophoresis is a technique employed to size-fractionate DNA molecules. The PCR product is placed in wells in a gel matrix, and being negatively charged, is attracted to the positive electrode when an electric current is applied. The rate at which a DNA molecule migrates through the gel matrix is proportional to its length. PCR products are subjected to electrophoresis to determine whether the reaction has been successful, and whether the PCR product is of the expected size. If the amplification is successful, the PCR product can then be tested for the presence of particular mutations, e.g., through the use of restriction enzymes, or further processed to remove unbound primers, dNTPs, and BSA, to allow direct sequencing.

Sequencing

Sequencing reactions are a variant on the theme of PCR. Only one primer is added to the reaction so that all the DNA synthesis moves in one direction, and a portion of free dNTPs are replaced by dye-dideoxynucleotides. DNA is called deoxyribonucleic acid because a portion of the structure is ribose, a type of sugar, which, in DNA, has one fewer OH groups than normal ribose has (i.e., it is deoxygenated). In normal DNA synthesis, the remaining OH group reacts with the phosphate group on the adjacent nucleotide, forming a phosphodiester bond. Dideoxynucleotides are *di*deoxygenated, and so do not have this OH group necessary to form the bond with the next nucleotide. For this reason, they are called chain-terminating nucleotides: they prevent further extension of the DNA chain (Sanger et al., 1977). These chain-terminating nucleotides are synthesized to carry dye molecules, with each color dye specific to the base of the nucleotide.

At some point in the synthesis of a complementary strand, the DNA polymerase will incorporate a dye-labeled chain-terminating nucleotide. Synthesis of that strand will then stop, resulting in a fragment that is color-labeled specific to the final nucleotide in the chain. This reaction then produces a population of DNA fragments terminating at varying points along the sequence that are color-labeled, specific to the final nucleotide incorporated. By running these PCR products on an acrylamide gel, the single-nucleotide length differences in fragments are resolved, and the DNA sequence can be read from the order in which the colors line up. Sequencing is automated, and a computer reads and records the color of the dye, and hence the terminal nucleotide, of each fragment as it passes under a laser.

Protocol modifications

Slight variants of the protocols described above are used in different laboratories. Additionally, modifications are made to improve success rates under particular circumstances.

Decalcifying. The efficacy of the extraction protocol can be enhanced by decalcifying the sample, if it is bone or tooth. The sample is incubated in 0.5 M EDTA (pH 8.0) for up to 72 hr, with a change in EDTA every 24 hr. This can be done before (Malhi, 2001), after (Hagelberg et al., 1989), or if EDTA decalicification sufficiently demineralizes the bone, instead of (O'Rourke et al., 1996, 2000a; Carlyle et al., 2000) reducing the sample to powder. This protocol has been found to both increase the DNA yield

from a sample, and to decrease the level of coextracted inhibitors.

PTB. It is frequently suspected that DNA of sufficient quality is present in samples that fail to yield analyzable DNA. Poinar et al. (1998) reported that one of the potential reasons for this is the extensive cross-linking between macromolecules that occurs postmortem. They suggested that DNA can become trapped within cross-linked products, preventing its successful amplification. To release the DNA from such cross-linked matrices, Poinar et al. (1998) employed N-phenacylthiazolium bromide (PTB) to cleave the cross-links, and reported both an increase in the success rate per sample, and an increase in the strength of signal obtained from samples.

Combined protocol. An additional method to improve the chances of a successful extraction was developed by Burger et al. (1999), in which they combined both the phenol/chloroform and the silica/GuSCN protocols. Having conducted the phenol/chloroform extraction process as described above, they then precipitated the extracted DNA onto 10 μl of glass milk (Bio 101) with 90 μl of sodium acetate and 3.2 ml isopropanol (rather than precipitating with cold ethanol and no glass milk). After washing the glass milk twice with cold 80% ethanol, it is left to dry in an incubator, before eluting the DNA in 100 μl of TE buffer. The glass milk is retained in the extract and homogenized throughout the sample before it is added to a PCR reaction.

DNase. Even when all the precautions against contamination described above are adhered to rigorously, contamination by foreign DNA does occur, and one of the sources of this contamination may be disposable labware or reagents used during the extraction and amplification procedures. Eshleman and Smith (2001) advocate the use of DNase I to digest any potentially contaminating DNA in labware or reagents prior to the addition of primers and template DNA. The PCR master mix, containing PCR buffer, BSA, $MgCl_2$, and *Taq*, was subjected to digestion with 0.4 μl of DNase I with 0.4 μl of DNase I buffer at room temperature for 15 min, after which the DNase I was denatured at 70°C for 10 min. This protocol was found to successfully eliminate DNA added to the master mix prior to DNase I digestion, allowing successful amplification of template DNA added following enzyme denaturation.

Degenerated oligonucleotide-primed PCR (DOP-PCR). The degraded nature of aDNA frequently makes the amplification of segments of interest difficult. To ameliorate the effects of working with such fragmentary DNA, Telenius et al. (1992) developed a protocol to increase the quantity of DNA available for specific amplification. DOP primers are partially degenerated, and when used in conjunction with a low annealing temperature, they anneal throughout the genome and allow a general DNA amplification. This amplified product is then used for specific amplification with primers designed for the region of interest. While this method can prove successful, it substantially increases the opportunities for contamination, and thus must be employed with caution.

Touchdown PCR. In the early cycles of a PCR reaction, primer concentration is extremely high, and particularly when working with highly degraded aDNA samples, there may be very few target sequences for them to bind to. As a consequence, a large proportion of the early amplification products may be primer dimers, in which two primers bind together and the *Taq* amplifies new sequences of the short overhanging primer ends. The vast excess of primers in early amplification cycles can produce a substantial population of products of primer dimer synthesis, which can then outcompete the target sequence in later cycles, resulting in a low yield of target amplicons. Touchdown PCR (Don et al., 1991) is employed to counter this problem, by systematically reducing the annealing temperature, such that a substantial population of desired target molecules has been synthesized by the time the annealing temperature has fallen sufficiently to permit primers to bind to each other.

The annealing temperature in the first two cycles is usually set about 3°C higher than the melting temperature of the most GC-rich primer with its perfect hybrid template. The annealing temperature is then lowered by 1°C for every two amplification cycles. Thus, the onset of nonspecific primer annealing is delayed.

Touchdown PCR can also be usefully employed if there is limited information about the absolute sequence of the target. With limited information about the target, it is impossible to calculate the melting temperature, and thereby the optimum annealing temperature, of the primers for that species. Simply amplifying the target with a low annealing temperature will likely produce multiple PCR products, as the primers anneal in several locations throughout the genome. Employing touchdown PCR reduces the likelihood of this outcome.

Finally, touchdown PCR can be usefully implemented when amplifying DNA from coprolites. Generally, the DNA found in coprolites is analyzed for one of two reasons: to learn about the animal itself, or to learn about the diet of the animal. If the goal is to study the diet of the animal, nonspecific PCR priming can be an effective approach. However, if the goal is the DNA of the animal itself, highly specific priming can be essential. This is particularly true if the organism consumes relatively closely related species. In such an instance, the accurate priming of the target-organism DNA, rather than that of its prey species, can be achieved by using the increased specificity of reaction conferred by the touchdown PCR protocol.

Various DNA polymerases. In the early days of PCR, new DNA polymerase had to be added with each cycle, as the high temperatures required to denature the DNA target also denatured the polymerase enzyme. Since then, there has been a proliferation of recombinant enzymes that ameliorate many of the problems associated with amplifying problematic templates. The stability of DNA polymerases at high temperatures remains an issue, particularly when working with aDNA, because of the high number of PCR cycles generally employed. Even relatively thermostable polymerases can begin to denature after repeated exposure to high temperatures. There are now recombinant DNA polymerases available that have very long half-lives at high temperatures, thereby eliminating this problem (e.g., Deep Vent DNA Polymerase, New England Biolabs).

Additionally, the low temperatures through which a PCR reaction tube must be passed can cause mispriming, and thereby produce nonspecific amplification. Primers bind to random sites while the reaction temperature is low, and the DNA polymerase extends the strand before an increase in temperature can cause the primer to melt from the misprimed target. Mispriming in this fashion can be avoided by employing a hotstart protocol. If the DNA polymerase is either inactive or not present until the reaction mixture is at a high temperature, it cannot extend nonspecifically bound primers. This can be achieved by adding the DNA polymerase only when the reaction has reached a high temperature (although this method has the substantial side effect of creating an additional opportunity for the introduction of contaminating DNA), or by placing a physical barrier of wax between the DNA polymerase and the rest of the reaction mixture, which melts only when the reaction temperature is sufficiently high. DNA polymerases are now available that do not become active until a high temperature is reached (e.g., AmpliTaq Gold, Perkin Elmer; Platinum *Taq*, Life Technologies), thereby avoiding the need to open the reaction tubes or create a physical barrier.

Finally, DNA polymerases in PCR reaction tubes are known to incorporate mismatched bases periodically, just as DNA polymerases in cells do. The product with the misincorporated base is then a template for further rounds of synthesis. If such a misincorporation occurs early in the cycle sequence, then the daughter molecules of that mutated product can represent a substantial proportion of the final population of molecules, potentially resulting in an erroneous sequence. This problem can only ever be fully addressed by direct sequencing of multiple PCR products or clones of PCR products, but it can be ameliorated by the use of a DNA polymerase with a proofreading exonuclease function (e.g., Deep Vent DNA Polymerase, New England Biolabs; Platinum *Pfx* DNA Polymerase, Life Technologies).

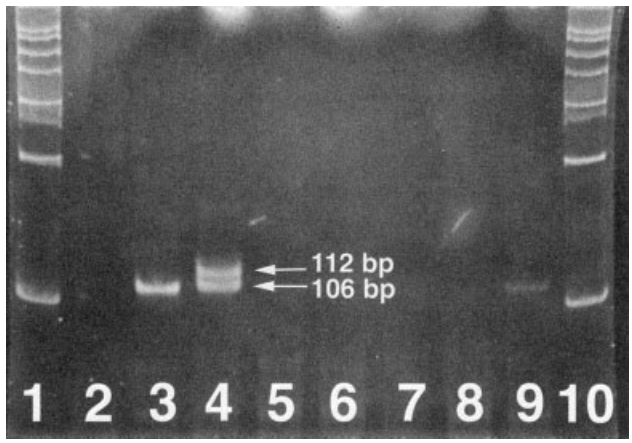

Fig. 3. Electrophoretic gel image for Amelogenin sexing markers (Mannucchi et al., 1994). **Lanes 1** and **10** molecular size marker; **lane 2** amplification negative control; **lane 3,** modern female sample; **lane 4,** modern male sample; **lanes 5–8;** extraction negative controls; **lane 9** ancient sample (morphometrically identified as female).

Important markers

The most common genetic markers used in aDNA analyses are discussed below. Basic descriptions of how they can be applied to anthropological questions are given, and some cautions are noted.

The most common method of genetic sexing takes advantage of differences in the Amelogenin gene, present on both the X and Y chromosome, but with slightly varying sequences. The favored protocol involves amplifying a short segment of the Amelogenin gene that contains a 6-base-pair (bp) deletion in the copy on the X chromosome, when compared with the Y (Mannucci et al., 1994). Thus, the DNA fragment amplified from an X chromosome is only 106 bp long, while that from a Y chromosome is 112 bp. Amplifications from a male individual will therefore contain DNA fragments of two sizes, while those from a female individual will contain DNA fragments of only one size (Fig. 3). Another sexing protocol also utilizes the differences between the copies of the Amelogenin gene on the X and Y chromosomes, but rather than detecting size differences, it probes the amplified DNA with oligonucleotides (short single-stranded fragments of DNA) that are specific to sequence differences (mutational substitutions) between the X and Y versions of the gene (Stone et al., 1996). Amplifications from a male individual will bind both probes, while those from a female individual will bind only the probe specific to the X chromosome sequence. A further method of genetically sexing individuals relies on the presence of microsatellites (areas containing multiple repeats of a few DNA bases, also called short-tandem repeats or STRs) or other genetic markers that are found only on the X or Y chromosome (Santos et al., 1998; Schultes et al., 1999; Cunha et al., 2000; Matheson and Loy, 2002). If Y-chromosome markers are detected, one can conclude that a Y chromosome

is present in the extract (i.e., the individual was male). Genetic sexing should always be replicated with multiple extracts and amplifications, because the low copy number of nuclear DNA (one copy of each sex chromosome per cell for males) results in a high likelihood of allelic dropout, in which the amplified product represents only one of the chromosomes. This can lead to a false negative for the presence of the Y chromosome, and thus the categorization of a male individual as female.

Another application of aDNA analyses is to use genetic markers (usually autosomal microsatellites) to sort mixed remains into a minimum number of individuals. The use of several (6–13) autosomal microsatellite markers in combination has been shown to differentiate between individuals very accurately (the results of such analyses have been deemed admissible evidence in court cases; Lygo et al., 1994; Sparkes et al., 1996a,b; Chakraborty et al., 1999). Because of the increasing demand for this type of analysis for forensic purposes, several proprietary kits, specifically designed for use with degraded (ancient) DNA, are now available that allow streamlined analysis of multiple microsatellite markers (Sparkes et al., 1996a,b). Although this method has been used forensically (Clayton et al., 1995; Corach et al., 1997; Goodwin et al., 1999), this type of analysis is both time- and money-intensive, and no examples of such use have been found in the anthropological literature (but regarding the Dead Sea Scrolls, see Watzman, 1995). As with sex chromosome markers, due to the low copy number of nuclear DNA, there is a high probability of allelic dropout in this type of analysis, leading to apparent homozygosity in individuals who are actually heterozygous for a particular marker (Zierdt et al., 1996; Schmerer et al., 1999). Therefore, multiple extractions should be tested using multiple independent amplifications for each individual.

In the case of mtDNA, the most variable region is found in the noncoding displacement loop (d-loop), where mutation rates are estimated to be between 7% and 12% per million years (Stoneking et al., 1992; Horai et al., 1996). Mitochondria are maternally inherited without recombination (Merriwether and Kaestle, 1999), and as such, people with identical mtDNA sequences (having the same haplotype) for this region belong to the same matriline (i.e., are relatively close maternal relatives) (Gill et al., 1994). Another region of mtDNA often examined for the purposes of species identification is the cytochrome b gene (Newman et al., 2002). In the case of Y-chromosome DNA, the most variation found to date involves microsatellite markers, in which the number of repeat units per locus varies among individuals (Jobling et al., 1999). Multiple microsatellite markers can therefore be combined to define patrilines, and male with identical microsatellite alleles (having the same haplotype) are relatively close paternal relatives (Roewer et al., 1992; Gerstenberger et al., 1999).

To identify parentage with confidence, enough markers must be used to (statistically) eliminate the possibility of a random match with a nonparent. As mentioned above, due to the forensic demand, several kits utilizing highly variable autosomal microsatellite markers have been developed specifically for this purpose, but the same technique can be applied using researcher-designed sets of markers (de Pancorbo et al., 1995; Sparkes et al., 1996a,b; Hummel et al., 1999).

Analysis

Phylogenetic trees. A phylogenetic tree (see Fig. 4a for an example) is an evolutionary hypothesis about the proportional relatedness of individuals, populations or species (Hillis et al., 1996). The central premise of phylogenetic reconstruction is that measures of similarity in some way reflect the recency of a common ancestry.

There are currently a multitude of methods for estimating phylogenies, which essentially break down along two lines, i.e., character methods and distance methods. Raw sequence data are discrete, and they can be analyzed as such, using each nucleotide site during the analysis. Alternatively they can be converted to a distance matrix, in which sequence divergence between each pair of sequences is calculated. While there are several different methods for calculating pairwise sequence divergences, each taking into account observed patterns of molecular evolution, distance methods nonetheless result in a significant loss of information (Hillis et al., 1996). Steel et al. (1988) offered the example of nine taxa with 20 four-state characters. There are at least 10 distinct sets of sequences that will produce the same distance matrix. This loss of information is a definite disadvantage associated with distance methods. However, by reducing the complex patterns present in raw data to a single two-dimensional matrix, the computational power necessary to implement distance methods is limited, making these analyses much faster than character-based methods.

The second dichotomy in phylogenetic analyses is that between clustering methods and search methods. Clustering methods use distance data and implement an algorithm which judges the best edge to which to join the next sequence. Clustering methods are fast and always produce a single tree (Page and Holmes, 1998). In contrast, search methods scan many trees, judging them against an objective criterion, such as parsimony or likelihood. There are several search methods, but they all tend to have the same faults. They are slow, requiring considerable computational power, and while still preferable to a clustering algorithm, they are only as good as the objective criterion chosen.

The objective function against which trees are judged is essentially a model of molecular evolution. It is generally chosen on the basis of previous evidence about the evolutionary behavior of the section of the genome being examined. For example, transi-

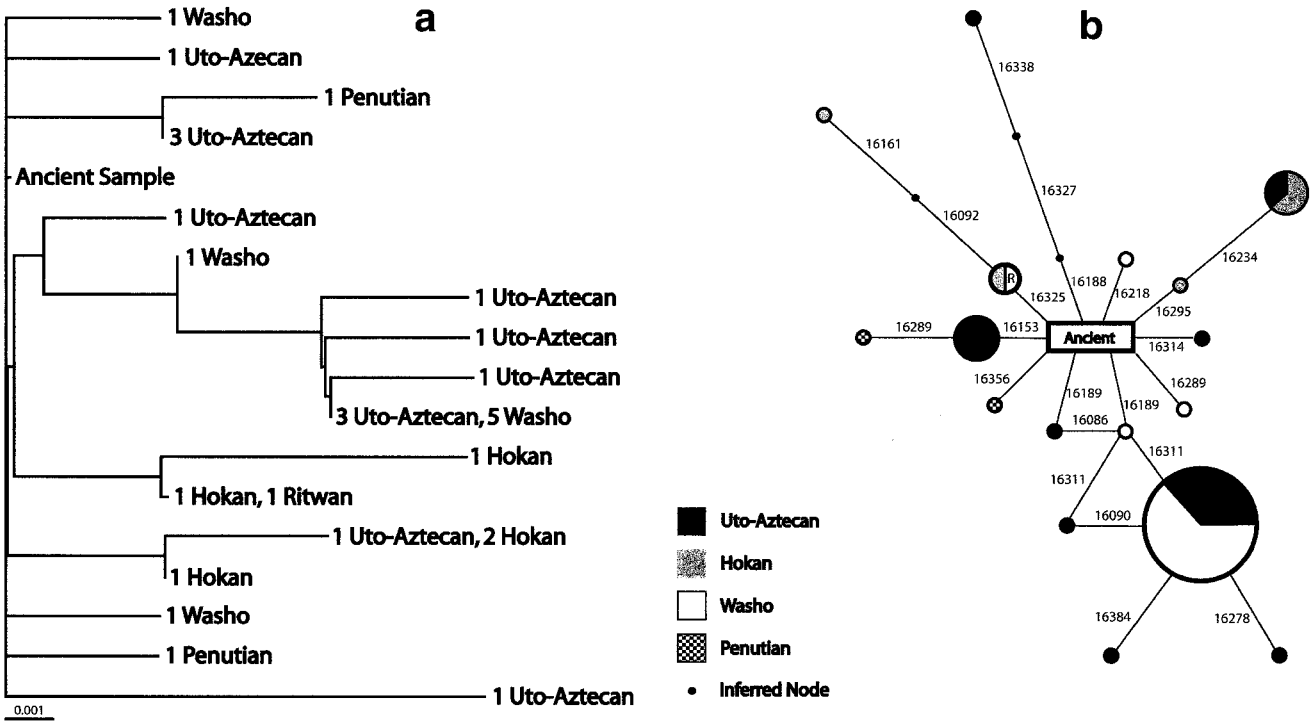

Fig. 4. **a:** Neighbor-joining phylogenetic tree of 29 contemporary Native Americans from the western United States, and one ancient Native American (Wizards Beach, 9,200 BP) from western Nevada. **b:** Statistical parsimony network of 29 contemporary Native Americans from the western United States, and one ancient Native American (Wizards Beach, 9,200 BP) from western Nevada. Size of circle represents number of individuals possessing that haplotype, while pattern/shading represents linguistic affiliation of those individuals. Numbers represent nucleotide position of mutations defining each node.

tional mutations, the change from a purine to a purine, or a pyrimadine to another pyrimadine (such as a C to a T), are more common than transversional mutations, changes between purines and pyrimadines (a C to a G, for example), because stereoscopic differences hinder mutations between classes of nucleotides (Aquadro and Greenberg, 1983). Consequently, a transition:transversion bias is often factored into the model of molecular evolution. In shallow evolutionary events, such as within the African great apes, transitions are slightly more than nine times more common than transversions (Spuhler, 1988). For deeper splits, however, as in the case of primates vs. nonprimates, transitions make up slightly less than half the mutational differences, due to mutational saturation obscuring historical changes (Hillis et al., 1996). It becomes a judgment call, then, to decide at what level to set the ratio. It is also worth noting that mutational changes to the genome do not fall simply into these two probability classes of transitions and transversions (Hillis et al., 1996). There can be as many as six different mutational probabilities if mutations occur symmetrically, such that the probability of an adenine mutating to a cytosine is the same as the probability of a cytosine mutating to an adenine. If this is not the case, there are up to 12 mutational probabilities.

Each of the phylogenetic methods operates on sets of assumptions about the nature of the DNA sequences, as well as the populations from which they were taken. When working with aDNA, one of the central assumptions is violated because the taxa are not contemporary. The taxa at the ends of the branches, in addition to not being contemporary, can represent entities at a variety of levels. The taxon can be a haplotype, representing either an individual or multiple identical individuals. It can be a group, reduced to haplogroup frequencies, or an individual representing a group. Likewise, the taxon can be an individual representing a species. In these last two cases, where individuals represent groups or species, caution must be exercised in the interpretation, because a single individual, however "randomly chosen," cannot possibly encompass the variability and population structure of the group or species it is standing for.

It remains difficult to assess the accuracy of phylogenetic trees, although some methods are employed which are able to give a sense of the reliability of a tree. Bootstrapping (Felsenstein, 1985) is the most popular of these methods. It is a method adopted from statistics to produce pseudoreplicates of the data, in which data columns are randomly selected with replacement to manufacture a varied data set from the information contained among the true data. The bootstrap pseudoreplicate data sets are analyzed to generate a phylogenetic tree for each of them, and then a consensus tree is calculated. Bootstrap trees have numbers associated with each node, representing the proportion of pseudorepli-

cates that generated that split. It is important to appreciate that a bootstrap value for a particular node is no indication of the truth of that split, but simply reflects the likelihood that the split will be retained as longer sequences become available for analysis (Felsenstein, 1985).

Figure 4a shows an example of a tree, based on mitochondrial sequence data from the first hypervariable segment (nps 16075–16394) of several contemporary Native Americans from the Western United States[3] and from one ancient individual from western Nevada (Wizards Beach, dated to approximately 9,200 BP; Dansie, 1997). Genetic distances among haplotypes were generated by the DNADist program (Kimura-2 parameter model, transition: transversion ratio of 15:1; Felsenstein, 1993). A neighbor-joining clustering algorithm was used to construct an unrooted tree from these data (using the Neighbor program, randomized input order; Felsenstein, 1993). Note the basal (underived) position of the ancient sample within this tree.

Networks. Another way to approach evolutionary relationships is a network, which allows reticulation (or cycling; see Fig. 4b). In many cases, this may be a more appropriate representation of our knowledge (or of reality) than a tree, which presumes dendritic (branching) evolution. For example, when working with autosomal DNA, there is the possibility that recombination between homologous chromosomes has led to reticulate evolution for a region of interest (Templeton et al., 1992; Posada and Crandall, 2001). More relevant for aDNA research is the fact that high mutation rates (such as those observed in mitochondrial DNA) can lead to recurrent mutation at particular nucleotides, which can cause ambiguity in the evolutionary pattern we are attempting to detect (Bandelt et al., 1999, 2000; Posada and Crandall, 2001), as can the generally small genetic distances found between individuals within the same species (Bandelt et al., 1999; Posada and Crandall, 2001). A network allows us to depict these ambiguities in connections between nodes in a topology by connecting nodes through multiple pathways. The same types of data can be used to construct networks as are used to construct trees (see above). Network-building algorithms generally begin with raw sequence or haplotype data, rather than distance data, and generate their own genetic distances from these data (Huson, 1998; Clement et al., 2000; Bandelt et al., 2000; Posada and Crandall, 2001). These methods generally fall into two classes: those that begin with a tree and add reticulation, and those that begin with a highly reticulated network and eliminate reticulation. Both types often consider subsets of the data iteratively to generate or eliminate the reticulation. As an example, we will consider the median network approach (Bandelt et al., 1995, 2000). In this method, variant sites are sorted into two classes: those that are compatible (do not require multiple mutational events per site and therefore can be accommodated in a tree-like topology), and those that are incompatible (those characters which cannot all be uniquely derived on a tree without reticulation). The incompatible characters are mapped onto unrooted trees produced from the compatible characters (generally using parsimony, or step-minimizing, methods; Bandelt et al., 2000), with all possible reticulations included. A data set with no incompatible sites will produce a network that is the equivalent of an unrooted tree. Once a network with all of the most likely reticulations is produced ("most likely" being determined by a semi-arbitrary tolerance level chosen by the researcher; Bandelt et al., 1995, 2000), additional rules can be used to reduce the number of reticulations in the network (Bandelt and Dress, 1992; Hendy and Penny, 1992; Templeton et al., 1992; Huson, 1998; Bandelt et al., 1999, 2000; Clement et al., 2000; Posada and Crandall, 2001). These additional rules generally rest on our understanding of molecular genetic evolution (such as rates of transition vs. transversion, described above, or observed variation in mutation rates among nucleotides in the genetic region under study, or the observation that ancestral sequences tend to be more frequent than derived sequences in a population), allowing us to rule out "evolutionary pathways which are extremely unlikely" (Bandelt et al., 2000, p. 15).

As with phylogenetic trees, it is difficult to assess the accuracy of network methods (Posada and Crandall, 2001), although networks are more likely to at least include the true tree, simply because they encode multiple tree topologies at once. Bootstrapping methods can be used to generate pseudoreplicates of the data, and network results can be assessed in a similar manner to tree results (Huson, 1998). A simulation study on data sets of known evolutionary pathways, generated to mimic expected human demographic patterns, was performed to assess the greedy reduced median (GRM) method of generating networks (Bandelt et al., 2000). This preliminary assessment of the method gave mixed results. For incompatible sites produced by a single recurrent mutation, all reductions in the network were correct. However, in cases where recurrent mutations were more common (i.e., a single nucleotide mutated more than twice), the authors found that only 80% of reconstructed networks contained the true tree. Nevertheless, when this method was applied to mitochondrial data sets from the literature (Oota et al.,

[3]Samples comprise only members of mitochondrial haplogroup C and include 8 Washo (members of the Washo language isolate); 6 Northern Paiute, 7 Pima, 1 Vanyume, 4 Luiseño, and 1 Tubatulabal (all members of the Uto-Aztecan language family); 2 Kumiai, 1 Achumawi, 1 Chumash, and 1 Diegeño (all members of the Hokan language family); 1 Coos and 1 Wintu (members of the Penutian language family); and 1 Yurok (a Ritwan speaker) (data from Kaestle, 1998; Malhi, 2001). Note that the inclusion of these languages within larger linguistic families (Ruhlen, 1991) is for convenience only, and does not represent an endorsement of these particular linguistic divisions (which are, in some cases, highly questionable; Campbell, 1997).

1995; Calafell et al., 1996), the GRM networks did contain all of the most parsimonious trees estimated with phylogenetic tree algorithms (Bandelt et al., 2000). Note that these results are applicable only to the GRM method of producing networks, and may not reflect the reliability of network algorithms in general.

Networks have several advantages over trees when working with aDNA. As mentioned above, they are more likely to reflect the ambiguities of our data. In addition, their structure facilitates the depiction of temporally distributed samples (in that haplotypes can occupy internal nodes, whereas trees require haplotypes to occupy terminal branches). Networks also simplify the depiction of sample and mutational data. In general, the size of a node reflects the number of individuals possessing that haplotype, and pie charts or similar methods can be used to depict information on group membership. Networks traditionally also display mutational events on the links between nodes (although this can also be done along tree branches).

Figure 4b depicts a network generated from the same data used to generate the tree in Figure 4a. This network was produced using a statistical parsimony method (Templeton et al., 1992), as implemented in TCS software (with gaps treated as a fifth state, using the default 95% parsimony probability; Clement et al., 2000). Note the central position of the node representing the ancient sample. The reticulation among two different sets of three haplotypes (located in the lower portion of the network) represents the different possible mutational pathways between these haplotypes (in other words, mutations may have occurred twice at nps 16189 and/or 16311).

Population statistics. In addition to representing relationships with trees or networks, many statistical analyses can be done to compare populations with one another, or to derive estimates of important population genetic variables from observed data (such as migration rates, effective population size, and the like). Most of these methods rely on estimating total variation within a population sample, and comparisons of variation within and between samples. The most common statistics used for these studies are F_{IT} and F_{ST}, the total inbreeding coefficient and the coancestry coefficient, respectively (Wright, 1951; also estimated as F and θ, respectively; Cockerham, 1969; Weir and Cockerham, 1986). As two populations become more differentiated from each other, F_{ST} increases. For example, if $F_{ST} = 0.07$, then two alleles or sequences chosen at random from within a population are 7% more likely to be the same than if you pick two alleles or sequences at random from the sample as a whole (the combination of two or more populations you are comparing). These variables can be estimated in a number of ways, such as from allelic (or haplogroup) data, or from sequence data (Nei, 1975; Jorde, 1980; Hartl, 1981; Lynch and Crease, 1990; Excoffier et al., 1992; Gillespie, 1998). These statistics allow a calculation of an estimate of differentiation between populations. Population differentiation is influenced by the elapsed time since common ancestry (with greater times leading to greater differentiation through the action of genetic drift; Slatkin, 1991) and the level of migration between populations (with higher levels of migration leading to reduced differentiation through the action of gene flow; Cockerham and Weir, 1993). Under a simple model of migration, for biparentally inherited loci, the equilibrium value of F_{ST} is estimated as

$$\theta = \frac{1}{1 + 4\mathrm{Nm}}$$

where N is the effective population size, and m is the migration rate between populations (Weir, 1996). Unfortunately, it is difficult to separate the effects of drift and gene flow (dependent on N and m), and migration between populations is almost certainly more complicated than the model suggests. Thus, more complicated analytical and simulation models have been developed in an attempt to separate and estimate these two terms (e.g., Nei, 1975; Jorde, 1980; Slatkin, 1985; Slatkin and Barton, 1989; Hudson et al., 1992; Gillespie, 1998; Hunley, 2002). In addition, most population genetics methods assume that a sample has been drawn from a single (or limited number of) generation(s) from a population. This is generally not the case when dealing with ancient samples, which may span hundreds or thousands of years (tens of human generations). This temporal scatter of the sample introduces an additional source of error when estimating population parameters, which is not accounted for in statistical estimates of error on these variables.

Nonphylogenetic "cluster" analyses. In addition to expressing relationships between populations using trees or networks (as described in the phylogenetic analyses above), genetic similarities among populations can be visualized on two- (or more) dimensional plots. These graphical representations of the relationships between groups have the advantage that they do not presume any branching order, simply that some groups will cluster more closely with each other than with other groups (for whatever reason). In addition, several of the methods utilized to generate these relationships have beneficial statistical attributes when dealing with DNA data.

The most common method of revealing these relationships used with genetic data is principal components analysis (PCA). The aim of PCA is to identify and represent the most important of these relationships between populations (or objects) with a smaller number of variables, allowing them to be displayed graphically along relatively few dimensions. This method discards some of the data features as uninformative, the result of "noise" (Krza-

nowski and Marriott, 1994). This method utilizes variance-covariance matrices to estimate a vector (eigenvector) that maximizes the variance among populations, calling this the first principal component (PC) (Mardia et al., 1979). It then estimates a second vector that maximizes the remaining variance not correlated with the first vector, calling this the second PC. The method continues to estimate vectors, or PCs, until all of the variance in the input data is accounted for. The PCs are then assessed based on the proportion of total variation that they explain (Mardia et al., 1979), and some subsets explaining low amounts of variation are excluded (where this cutoff point is set can vary; Bartlett, 1950; Kaiser, 1958; Cattell, 1966; Mardia et al., 1979; Krzanowski, 1987; Jackson, 1991; Johnson and Wichern, 1998). Plotting the first two or three PCs against each other can reveal structure in the data, and additional pairs of PCs can also be plotted (e.g., the third PC against the fourth, and so on) (Krzanowski and Marriott, 1994). This structure may involve the clustering of a subset of the data, or an ordering of data points along an axis that appears to represent some real-world variable (e.g., geographic or linguistic groups; Kirk, 1982). It is important to remember that PCA can be sensitive to scaling of variables, and input data measured in different units (of noncomparable scale) must be scaled to equalize both unit of measurement and variance (Krzanowski and Marriott, 1994) before PCA is applied. This is usually accomplished by dividing variances by their standard deviations (creating a correlation matrix in place of the variance-covariance matrix) (Krzanowski and Marriott, 1994; Johnson and Wichern, 1998). A PCA of the four most common Native American mitochondrial haplogroup frequencies in four modern Native Americans groups (defined geographically) and one ancient group from western Nevada (discussed in more detail in Applications, above) is presented in Figure 5 (data from Kaestle and Smith, 2001a, Table 3. Note that erroneous haplogroup frequencies for the Baja group were reported in this table. The correct frequencies are: A, 0.02; B, 0.68; C, 0.30; D, 0; and X, 0).

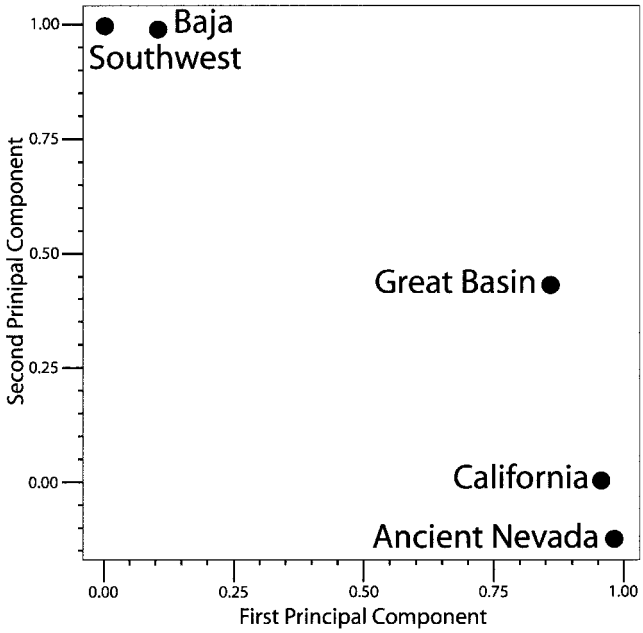

Fig. 5 Principal component analysis of Native American mitochondrial haplogroup frequencies, including data from an ancient western Nevadan group (Kaestle and Smith, 2001a), utilizing a Varimax rotation to simplify the structure (Bryant and Yarnold, 2001). The first principal component explains 58% of the variance, and separates the Southwestern and Baja groups from the rest, while the second principal component explains 39% of the variance, and differentiates the Great Basin group from the California/Ancient Nevada cluster (and also adds to separation of Baja/Southwest cluster from remaining samples).

Another method of describing population relationships based on genetic data of many kinds (haplotype frequencies, SNP frequencies, or sequence data as represented in genetic distance/F_{st} estimates between groups) is multidimensional scaling (MDS). Multidimensional scaling is similar to PCA, in that it detects relationships between objects (in this case, populations), and can depict them in a graphical way using a plot. MDS, however, does not assume linear relationships between these groups, nor that the data are distributed (multivariate) normally, can accommodate asymmetric matrices, and do not require the computation of a correlation matrix (Lalouel, 1980). For these reasons, it is applicable to many more types of data. The plot produced through MDS usually depicts two or three dimensions for ease of display, although the proper number of dimensions to use can be explored using a scree test or other methods (Cattell, 1966; Kruskal and Wish, 1978), and may be significantly more than three, but in these cases the plots are so difficult to interpret that other methods of analysis may be more fruitful. MDS essentially develops a κ-dimensional plot of the populations that minimizes the differences between the (usually Euclidian) distances between the points in this plot and the matrix of input data (usually genetic distances), using a stress function (Kruskal and Wish, 1978). Another way of saying this is that the k-dimensional plot produced seeks to nearly match the original distances/dissimilarities, although in fewer dimensions (Johnson and Wichern, 1998). In many cases, interesting patterns (whether they are clusters that seem to reflect significant differences, or sorting on dimensions that appear to reflect real-life variables such as geographic distance) can be identified simply by visual inspection of these plots, but more objective methods also exist (e.g., Borg and Lingoes, 1987). This process, when performed with metric data (i.e., actual magnitudes of differences or similarities between populations), is also called principal coordinate analysis (Gower, 1966; Johnson and Wichern, 1998) or classical scaling (Krzanowski and Marriott, 1994). Although sometimes also abbreviated PCA, principal coordinate analysis should not be considered interchangeable with principal component analysis (Gower, 1966; Mardia et al., 1979; Lalouel, 1980;

Seber, 1984; Krzanowski and Marriott, 1994). Principle coordinate analysis is only equivalent to principal component analysis when the dissimilarity matrix utilized consists of Euclidean distances between points (Gower, 1966; Krzanowski and Marriott, 1994), and in this case the principal coordinates of the data matrix in "k" dimensions are given by centered scores of the these groups on the first "k" principal components (Mardia et al., 1979). When MDS is performed on the rank orders of distances (i.e., ordinal information), this process is called nonmetric multidimensional scaling (Johnson and Wichern, 1998).

Simulation models. In addition to the abovementioned analytical models, the computing power available on desktop computers now allows sophisticated simulation models to be developed to deal with the complexities of aDNA analysis (and population dynamics in general) (Cabana, 2002; Cabana et al., 2002; Hunley, 2002; Hunley and Merriwether, 2002; Usher et al., 2002). This allows us to begin to explore the special sampling problems inherent in studying ancient groups, and to try to incorporate multiple processes that can affect population genetic variables.

Cabana (2002; and Cabana et al., 2002) developed a model to test hypotheses of population continuity that allows the effects of genetic drift, including issues of migration and population structure and size, to be examined over any number of generations. This model uses aDNA data as a starting point to generate multiple populations of a user-determined size that are allowed to interact through migration (again, at user-determined rates) over any number of generations. The differences between the starting and ending populations are noted, and the results of multiple simulation runs are used to generate a distribution. The actual differences between the two populations (whether they are two ancient groups separated in time, or an ancient group compared with a living population) can then be evaluated in light of this distribution.

Usher et al. (2002) developed a simulation model to evaluate our ability to detect some aspects of social structure using aDNA data. Assuming particular inheritance and residence patterns (patrilineal/patrilocal, matrilineal/matrilocal, or matrilineal/avunculocal), the authors simulated landscapes containing multiple cemeteries, which they filled with simulated individuals possessing mtDNA and Y-chromosome markers, who belonged to simulated families. The spatial distributions of these markers were assessed both within and between cemeteries to determine if it was possible to distinguish between different patterns of inheritance and residence. Preliminary results suggest that patrilineal and matrilineal patterns, at least, are clearly distinguishable.

Hunley (2002; and Hunley and Merriwether, 2002) developed a general simulation model to incorporate several human behaviors that can affect population genetics parameters, and in particular, effective population size. This model allows the user to vary human behavior, such as individual reproductive success or migration rate between subpopulations, while simulating the evolution of groups (composed of individuals possessing mtDNA and/or Y-chromosome markers, which are assigned based on the known frequency of these markers in a living group). For each simulation, population parameters (such as measures of genetic diversity) are calculated, and a distribution is generated. The observed values of genetic diversity in and among living groups are then compared with this distribution, allowing the generation of more informed estimates of population parameters (such as migration rates). Although the data used to test this model were generated from living populations, the model could easily be adapted to incorporate data from both ancient and modern populations.

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